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Pakistan Academy of Sciences

Editorial

PAS-ANSO Strategic Planning for Epidemic and Pandemic Preparedness

Under the ANSO (Alliance of International Science Organizations) collaborative research grants (ANSO-CR-PP-2021-05), we arranged the first event "MAAP-PAS-ANSO hybrid workshop on ecosystem restoration: one-health and pandemics" (June 05, 2022; on the occasion of world environment day) at Pakistan Academy of Sciences, Islamabad. The workshop stresses communication and collaboration for sustainable health (human, animal, and environment) and gauged issues related to the health of the planet [1]. Under the same project, the second event "ANSO-PAS-MAAP conference on the epidemic and pandemic preparedness" (December 05-07, 2022) was organized at the Pakistan Academy of Sciences, Islamabad. The three days event emphasizes more scientific efforts and brainstorming to discuss strategies for controlling the current COVID-19 pandemic as well as future epidemics, pandemics, and emerging pathogens. The conference abstract book highlights the resource persons and their thoughts (in the form of abstracts and their biographies) [2]. Selected articles are published as a special issue in the Proceedings of the Pakistan Academy of Sciences: Part B. Life and Environmental Sciences. Moreover, the conference proceedings and recommendations have also been published in the special issue.

The third event which was organized under the project is "ANSO-PAS workshop on biological safety and risk management" (December 23, 2022) at the Department of Biotechnology, Quaid-i-Azam University Islamabad. The workshop discussed bio-risk management in scientific/educational institutions, dual-use research of concern, risk assessments; and practical exercises were performed on different aspects like donning and doffing of PPE, waste/spill management in the lab., using biological safety cabinets in the laboratory, transportation and/or packing of infectious material, and certain scenario-based exercise for laboratory biosecurity.

In addition to the above-mentioned events, wet-lab experiments are being performed under the ANSO project, and bats-associated beta-coronavirus were reported for the first time from Pakistan [3]. Keeping in view, emerging/re-emerging infectious diseases (EIDs) as a major threat to public health, and bats as potential carriers; we are looking forward to more collaborations in further assessing the bat-viromes through high throughput sequencing (HTS), next-generation sequencing (NGS) and/or metagenomics techniques.

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REFERENCES

1. A. Nawaz, N. Khan, H. Ahmad, I. Iqrar, M. Ali, and Z.K. Shinwari. Proceedings of the MAAP-PAS-ANSO Hybrid Workshop on "Ecosystem Restoration: One-Health and Pandemics". Proceedings of the Pakistan Academy of Sciences: Part B 59(2): 79-83 (2022).
2. I. Iqrar, M. Ali, S. Rahman, and S.A. Shahid. Abstract Book "ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness". *Pakistan Academy of Sciences, Islamabad* (2022). ISBN: 978-969-8223-32-8.
3. S. Rahman, S. Ullah, Z.K. Shinwari, and M. Ali. Bats-associated beta-coronavirus detection and characterization: First report from Pakistan. *Infection, Genetics and Evolution* 108:105399 (2023).

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Pakistan Academy of Sciences

Review Article

Biogenic Nanomaterials: A Way Forward in Preventing Bacterial Infections

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Abstract: Antibiotic resistance puts a tremendous strain on the healthcare system. Bacteria such as *Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* that cause diseases like endocarditis, pneumonia, and Urinary tract infections have now become resistant to many previously used antibiotics. Antibiotic overuse must be reduced as it has become a public health threat paving the way to pandemics. Instead of creating new antibiotics, repurposing existing medicines that have faced resistance is one way forward. Plant-based antimicrobials have been explored as antibiotics to boost or augment the capability of existing antibiotics. It has been proposed that conjugates of plant-based products and antibiotics have increased activity and that the conjugated groups could help circumvent the beta-lactam antibiotic resistance mechanisms. Antibiotics have been combined with plant-based substances like Berberine, and a considerable synergy has been reported among them. Nanomaterials also promise a powerful environment-friendly strategy for weaponizing antibiotics with plant compounds. Nanoparticles could attach with many biological molecules such as DNA, enzymes, ribosomes, and lysosomes, further affecting the permeability of the cell membrane. The interaction of nanoparticles with many biological targets makes it hard for bacteria to develop resistance against them. Low molecular weight nanomaterial based on antibiotics could be very effective against multidrug-resistant gram-negative pathogens. Our study aims to analyze the progress done at the front of nanomaterials and nano-antibiotics against infectious diseases.

Keywords: Biogenic nanomaterials, Multi-drug resistant microorganisms, Metallic nanoparticles, Nanoparticles-biomolecule conjugate, Lipid nanoparticles

1. INTRODUCTION

The evolution of bacteria to acquire resistance to antibiotics dates back to the time when humans were trying to produce antibiotics at a large scale [1]. The reason behind the early and continuing evolutionary mechanisms of resistance includes the struggle of bacterial strains for resources, including the natural production of secondary metabolites, which are analogous to antibiotics used today as therapeutic agents [1]. However, most types of currently used antibiotics were revealed in 1940-1960, known as the golden era of antibiotics. At that time, it was believed that it would control the rate of infectious diseases [2]. Unfortunately, worldwide antibiotic resistance increased with time due to selection pressure, overpopulation, increased use of antibiotics in hospitals, wildlife spread, enhanced global migration, and poor sewage removal systems [3]. In the past few decades, many pandemics have occurred; the most recent one among them is COVID-19, which affected millions of lives. It is believed that COVID-19 worsened the global problem of antimicrobial resistance (AMR). The mortality rate is approximated to be 700,000 deaths per year due to unsuccessful antibiotic treatment,

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and it is expected to reach 10 million by 2050 [4]. Due to the rapid increase of multidrug resistance (MDR) bacteria, new therapeutic strategies are required to control the rate of infectious diseases because no new class of effective antibiotics against Gram-negative bacteria have been discovered in more than half a century, and only ~40 new antibiotics are in pre-clinical testing. Now it has become necessary to adopt new treatment strategies to halt the mechanism of resistance [5].

The surge of nanotechnology gives new hope for reducing the problem of antibiotic resistance. It has been reported in various laboratory-based studies that nanoparticles and antibiotics can synergistically act against pathogens. Various strains of bacteria are reported to be succumbed to nanoparticle stress and become susceptible to drugs [6]. Recent reports showed that by making conjugation of nanoparticles and antibiotics alleviates their toxicity to human cells and is also found to be effective at low dosages with enhanced bactericidal properties. Nanoparticles also repair the ability of antibiotics to destroy resistant bacteria. Nanoparticles when bound with antibiotics, enhances their bioavailability and facilitate their interaction with bacteria.

Similarly, nanoparticles combined with biomolecules possessing antimicrobial properties, such as antimicrobial peptides and essential oils are highly effective against resistant bacteria [7]. Nanoparticles previously produced by physical and chemical methods have limited use due to their toxicity to human cells. Now the most suitable method has been adopted, which is known as green synthesis because it is safe, non-toxic, and inexpensive. In nanobiotechnology, several biological systems, such as biomolecules, bacteria, fungi, yeasts, and plants, serve as ideal nano factories [8]. In the synthesis of nanoparticles plant metabolites, for instance, polyphenols have shown significant results because of their therapeutic value [9, 10]. The nanoparticles synthesized from plants are gold, zinc, magnesium, copper, silver, titanium, alginate, etc. Silver-based nanoparticles are more significant and show antimicrobial activity against bacteria, fungi, or protozoan pathogens [11]. A study revealed that silver nanoparticles conjugated with various drugs, such as ampicillin, streptomycin, gentamycin, and tetracycline, increase their stability and functionality and enhance antimicrobial potential against several resistant strains of bacteria *Escherichia coli, Staphylococcus aureus,* and *Staphylococcus pneumonia* [12]. Compared to chemically synthesized nanoparticles, plant-based nanoparticles show a better antibacterial effect, especially against multidrug-resistant organisms (MDROs), both individually and in synergy with current or conventional antibiotics [13]. Thus, this review focuses on applying green synthesized nanoparticles to combat antibiotic resistance to tackle current infections and prevent the emergence of new outbreaks.

2. BURDEN OF ANTIMICROBIAL RESISTANCE

Since the first use of antimicrobials, the burden of bacterial resistance has grown steadily and rapidly during the past ten years. Before the discovery of antibiotics, antibiotic-resistant genes were present in a few numbers. Still, the overuse/misuse of antibiotics and exposure to antibiotics with their companions, humans, animals, food, and environment led to antimicrobial resistance [14]. Antimicrobial-resistant is a global concern because it causes a burden on the healthcare and economic sectors, mostly because it limits the treatment options, increases the risk of failure of available therapies, increases the time of hospitalization, cost of treatment, and unrecognized outcomes such as increased mortality and morbidity [15]. Antimicrobial resistance is increasing rapidly, and AMR is expected to kill 10 million people annually by 2050. Murray *et al*. [16] estimated the resistance of strains of *E. coli* and *Klebsiella pneumoniae*. He concluded that both strains have become resistant to third-generation cephalosporins and carbapenems in almost 193 countries, which is alarming [16].

Similarly, *Salmonella spp*. is estimated to cause 3 billion human infections annually. Ciprofloxacin is the first-line drug to treat patients suffering from typhoidal salmonellae. Still, there is an increase in bacterial strains against ciprofloxacin, which causes a fear of treatment failure and necessitates the need for new antibiotics [17]. Some other pathogens due to their multi-drug resistant properties have shown to escape clinical treatments: *Campylobacter, Salmonella, Enterobacteriacea,* Methicillin-resistant *Staphylococcus aureus* (MRSA),

Vancomycin-resistant Enterococci (VRE), and New Delhi Metallo-β-lactamase (NDM)-1 [18]. To reduce the current burden of AMR, it is important to know the pathogen-drug combinations contributing to the burden of bacterial AMR and its global trend. Development of AMR is a continuous process and if it is not treated, many pathogenic bacteria could become much more lethal in the future than they are now [18].

3. MECHANISM OF ANTIBIOTIC RESISTANCE

AMR is an inescapable evolutionary outcome of all organisms evolving genetic alterations to evade deadly selection pressure. As long as antibacterial medications are employed against bacteria, they will evolve and adapt resistance methods (Figure 1) [19]. The lack of effective antibiotics in development contributes to the rise of resistance to existing antibacterial agents. In addition to acquiring antibiotic resistance by horizontal gene transfer and mutation in a chromosomal gene, bacteria can also possess intrinsic resistance to certain antibiotics [20]. For instance, triclosan has an extraordinary antibacterial effect against the diverse class of bacteria, which is why innate resistance in an individual species occurs when the bacteria are not susceptible to an antibiotic [21]. Although active efflux has initially been thought to explain this, it fails to inhibit the growth of Gram-negative *Pseudomonas* due to the occurrence of the fabl gene, which produces an enzyme named enoyl-ACP reductase which targets triclosan in susceptible strains [22].

Bacteria can acquire or evolve antibiotic resistance in addition to their intrinsic resistance. Four mechanisms are responsible for causing this effect: they either 1) reduce the intracellular concentration of the antibiotic due to inadequate bacterial penetration; 2) activate antibiotic efflux, [23]; 3) modify the target of the antibiotic via genetic mutation or post-translationally, and/or 4) inactivate the antibiotic by hydrolyzing or modifying it [24].

Gram-negative bacteria show impermeability to certain drugs because their external membrane, which contains a lipopolysaccharide layer, generates a permeability barrier [25]. A good example of the effectiveness of the bacterial outer membrane can be seen from the fact that glycopeptide antibiotics, such as vancomycin, remain ineffective against Gram-negative bacteria due to a lack of penetration through the outer membrane [26]. Changes in the outer membrane's permeability significantly impact hydrophilic compounds, such as β-lactams, tetracyclines, and some fluoroquinolones [27]. Another way in which bacteria colonize is through the production of biofilms. Polysaccharides, proteins, and DNA make up the biofilm matrix, which makes it difficult for antimicrobial agents to enter the bacterium and serves as protection [28].

The expression of the efflux pump is one of the intrinsic resistance strategies possessed by Gram-negative bacteria. Most of the drugs expel out of the bacterial cell through these pumps [29]. Most bacteria have a variety of efflux pumps. The five major classes of efflux pumps, grouped as a result of their structure and energy source, are the ATP-binding cassette family, small multidrug resistance family, large facilitator superfamily, and multidrug and toxic compound extrusion resistance-nodulation-cell division [30]. Except for resistance-nodulation-cell division, which has multiple pumps that cause the efflux of substrates over the cell envelope, all others possess single pumps that expel drugs through the cytoplasmic membrane.

Bacteria may produce enzymes capable of attaching different chemical groups to medicines [31]. This inhibits the antibiotic's ability to attach to its target in the bacteria. To inactivate a drug, chemical group transfer is the most effective method that involves the transfer of acetyl, adenyl, and phosphoryl groups [32]. Acetylation is the most used method, and it is thought to be used with chloramphenicol, fluoroquinolones, aminoglycosides, and streptogramins. In contrast, adenylation is thought to be involved in targeting aminoglycosides. Aminoglycoside modifying enzymes covalently change an aminoglycoside molecule's amino groups or hydroxyl, rendering it inactive. It is one of the most reported instances of antibiotic resistance [32].

β-lactam drugs including cephalosporins and penicillin, are extensively used, antibacterial agents. All members of this pharmaceutical category have a β-lactam loop with four sides, which serves as

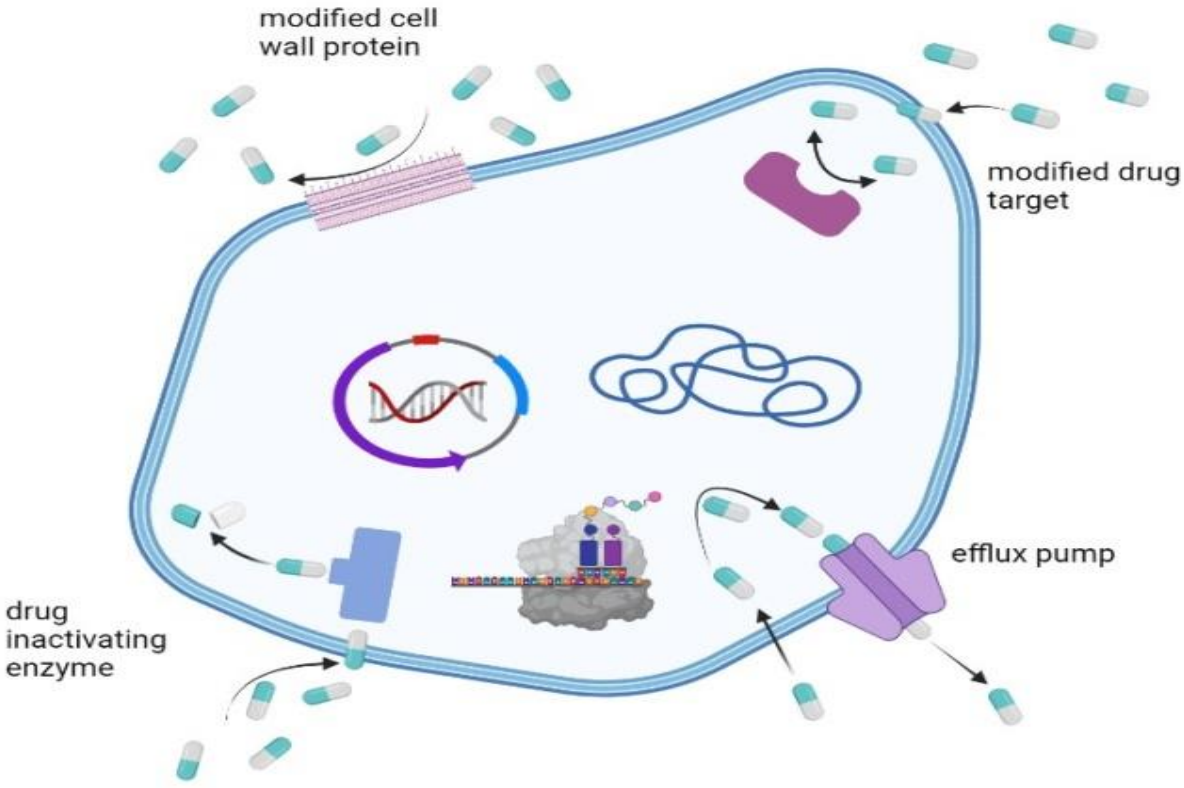

Fig. 1. Diagrammatic representation of antibiotic-resistant mechanisms adopted by bacteria

its primary structural component. The primary mechanism of β-lactam resistance, the β -lactam loop, is destroyed by the action of β -lactamases. The hydrolysis of β-lactam ring formation by the β -lactamases prevents it from attaching to penicillin-binding proteins (PBP) [33].

One typical pathway for the development of antibiotic resistance is the modification of the drug's target [34]. One of the mechanisms of resistance to β-lactam antibiotics is causing changes in the organization and/or quantity of PBPs. Changes in the number of PBPs alter the quantity of medication that can bind to the target [35]. A structural change, such as activating the mecA gene in S. aureus, will limit or prohibit drug binding. In response to drugs that block nucleic acid syntheses, such as fluoroquinolones, DNA gyrase, and topoisomerase IV may become resistant due to changes in their proteins [36]. Gyrase and topoisomerase undergo structural changes due to these alterations, which decreases or eliminates the drug's ability to bind to these proteins.

4. POTENTIAL OF RESISTANT PATHOGENS TO CAUSE A PANDEMIC

As we live in the era of antibiotics, the continuous use of antimicrobials increases the selection pressure on bacterial species to evolve and become untreatable, creating a hopeless situation [37]. Before the emergence of SARS-CoV-2, a "silent pandemic" was going on for more than three decades that caused more than 50,000 deaths each year in 2019. This resulted from multidrug-resistant (MDR) bacteria such as cephalosporin-resistant *K. pneumoniae, E. coli,* and carbapenem-resistant *Acinetobacter baumannii.* Studies have also shown that before the SARS-CoV-2 pandemic, the cases of pneumonia caused by antimicrobial-resistant Gram-negative bacteria were on the rise in many parts of the world [38]. Compared to other gram-negative bacteria, the *Acinetobacter* species acquire resistance much faster and become resistant to even new antimicrobials [39]. The most well-known infection caused by *Acinetobacter* species is ventilator-associated pneumonia. Due to their ability to survive in the hospital environment,

they also have the potential to cause nosocomial outbreaks [39].

Similarly, *Mycobacterium tuberculosis* was one of the most dangerous and dreaded bacterial illnesses before the advent of antibiotics. Thus, it can potentially result in a global epidemic [40]. According to WHO, 500,000 multidrug-resistant TB (MDR-TB) cases have been approximated amongst which 186,772 were only able to be diagnosed. Only 57 % of them were cured [41]. The use of antimicrobials increased during the COVID-19 pandemic. During the treatment of COVID-19, antibiotics are overprescribed, which increases the chances of secondary infections. Polly *et al*. [42] reported an increase in the incidence of Carbapenem-resistant *A. baumannii* (CRAB) and Methicillin-resistant *Staphylococcus aureus* (MRSA) infection both in ICUs and non-ICUs units. We are also facing antimicrobial resistance crises due to the failure of existing treatment strategies and the lack of new drugs. If the situation remains the same and we do not use available antimicrobials wisely, there are chances that we will return to the pre-antibiotic era of incurable diseases [43].

5. CHALLENGES IN TRADITIONAL ANTIBIOTICS DISCOVERY

Over the past 25 years, the challenges to discovering antibacterial drugs have kept the output of novel antibacterial drug classes at extraordinarily low levels. Resistance to the antibiotics can be caused by the failure of the drug to reach its target, either inactivated or altered, or by acquiring a target bypass system. For instance, the cell membranes of some microbial species are impermeable and prevent drug influx. In contrast, others produce enzymes that reside within or near the cell surface and inactivate the incoming drug.

The most important challenge to novel antibiotic agents is their development and marketing approval. Although they have the potential to address the deficiencies of existing classes of antibiotics and are vitally important to address the ever-increasing problem of bacterial resistance, a marked innovation gap in antibiotic development has been shown by examining the current industry pipeline over the past 20 years. There has been a decline in new antibiotic approvals, while the existing antibiotics are losing effectiveness more rapidly than they can be replaced. Difficulties associated with novel antibacterial discoveries and the reality that innovation of most new targets or chemical space approaches needs longer development. This indicates that novel solutions like nanoparticles have the greatest potential against bacterial resistance. Therefore, we must continue to apply what we have learned over the past decades and continue to strive to develop novel technologies against bacterial resistance [44, 45].

6. POTENTIAL OF NANOMATERIALS AS AN ALTERNATIVE TO TRADITIONAL ANTIBIOTICS

The discouraging financial incentives for commercial development result from the lack of antibiotics in the clinical pipeline. The widespread emergence of highly resistant strains could lead to a precarious situation and threaten the health system's viability, as seen with the current COVID-19 crisis. In the meanwhile, potential has been shown by several nano-antibiotics as alternatives to antimicrobial approaches [46].

Nanoparticles have been reported as a promising alternative to antibacterial agents in recent years. They possess several biomedical applications, such as antibacterial activity, applicability in tissue engineering, drug and gene delivery, and imaging. Furthermore, a possible relationship between the morphological characteristics of nanomaterials and the magnitude of their antibacterial potential has been reported by nanomaterial research. During in vitro studies, nanomaterials have been proven to demonstrate strong activity against several bacterial strains. These nanomaterials have also been used as a vehicle for drug delivery, pharmaceuticals, and antibodies [47, 48].

In nanotechnology, recent advances open new avenues to overcome the challenges by killing germs in bacterial infections without antibiotics. Advanced nanomaterials have presented antibiotic-free antibacterial strategies. Based on their mode of action, nanomaterials are classified as drug delivery agents for the delivery of natural compounds possessing antibacterial activity. Conventional antibiotics have the capability to potentially prevent the formation of new cell walls or chemically digest

the membranes of bacteria. However, nanomaterials can directly destroy the bacterial cell membrane through direct contact with bacterial cells. Regardless of the gram strain of bacteria (either gram-negative or gram-positive), the mechanism of antibacterial activity of nanoparticles lies in the physical harm to the bacterial cell membrane. Thus, nanomaterials have broad-spectrum antibacterial applications with little chance of resistance development. The other strategy of nanoparticles killing bacteria is generating toxic components, like reactive oxygen species (ROS) and reactive nitrogen species (RNS), which damage the intracellular proteins or genes by inducing lipid peroxidation of the bacterial cell membrane. To develop antibacterial nanomaterials that also include metal oxide nanoparticles efficiently generate ROS, tremendous efforts have been devoted [49].

Different nanoparticles are effective against resistant bacteria, and their antibacterial activities are size and shape-dependent. Nanoparticles like silver, gold, and Iron have shown a much more pronounced antimicrobial activity. Iron oxide nanoparticles show a repressive effect against bacteria like *S. aureus, S. enterica, P. mirabilis,* and *E. coli.* Similarly, zinc oxide nanoparticles have been reported to disrupt the cell wall of R. solanacearum indicating their good antibacterial potential. It is regarded as safe possessing light-activated oxidizing and catalytic effects. ZnO is found to be very effective when it comes down to the size range of nanometers. Due to its small size, ZnO becomes further effective in interacting with bacterial cells by penetrating them [50].

Furthermore, AgNPs have also been extensively tested against resistant pathogenic bacteria. It has been suggested that bacterial cells, when exposed to AgNPs, lose their DNA replication ability. The cell cycle halts at the G2/M phase due to DNA damage. Oxidative stress affects the cell, which is caused by the occurrence of ROS and inhibition of ATP synthesis. The release of silver ions from the AgNPs is another reason for bacterial cell death after exposure to these nanoparticles. It is believed that after penetration, the releasing atomic Ag0 and ionic Ag+ clusters inactivate the bacterial enzymes and cause cell death by producing hydrogen peroxide and other free radicals [51].

7. IMPORTANT PROPERTIES OF NANOMATERIALS THAT MAKE THEM EFFECTIVE

Nanoparticles exhibit interesting properties compared to their metallic counterparts. This means that the designed elements of nanomaterials play the most important role in making them effective [52]. Consequently, nanomaterials find too many applications in catalysis, diagnosis, electronics, sensors, and therapeutics. These properties include crystallinity, excellent stability, smaller size, surface plasmon resonance effect, unique shapes, and their higher surface-to-volume ratio [53]. These properties confer nanoparticles the extraordinary ability to be strongly antibacterial, antifungal, larvicidal, and antiprotozoal. More specifically, the unique size, crystal structure, and smaller size make nanoparticles superior to existing antibiotics which can ultimately lead to a reduced burden of antibiotic resistance [54].

Further, the nanoparticles have manageable morphology and good size dispersity [55]. Anisotropic is the main property of nanoparticles, which means their various crystal facets possess a different form of reactivity [53]. Metal nanoparticles, especially, make use of their stability, high surface-to-volume ratio, and improved electronic and optical properties to be more effective against pathogenic bacteria [56]. The optical properties of metallic salts are changed by changing the surface chemistry when converted to nanoform. The remarkable change in these properties mentioned above and the potential of customization of these properties have led nanoparticles to become one of the best avenues for fighting antibiotic resistance [57-59].

8. FROM TRADITIONAL NANOTECHNOLOGY TO NANOBIOTECHNOLOGY

Nanotechnology has rapidly developed as a significant field with applications in almost every aspect of life. The concept of the nanometer was first proposed by Richard Zsigmondy, the 1925 Nobel Prize Laureate in chemistry. Furthermore, the advancement to modern nanotechnology was led by Richard Feynman, the 1965 Nobel Prize Laureate in physics. He presented the concept

of employing matter at the atomic level. In its simplest form, nanotechnology is referred to the construction, design, and control of materials and particles with sizes less than 100 nm. Traditionally it gained the major attention of engineers and physical scientists because nanomaterials were employed in constructing computer chips and electronic devices. Today, progress in nanotechnology research has enabled scientists to develop techniques and systems for biological and medical research and applications that are referred to as nanobiotechnology [60, 61]. Nanobiotechnology enables to manipulate materials at a molecular and atomic level to synthesize ultra-small structures of biological importance [62]. It generally covers the applications of nanotechnology in rapid diagnosis and real-time monitoring, regenerative medicines, bioimaging techniques, directed and precise delivery of therapeutic agents, accurate therapy, and vaccine development [63].

In disease diagnosis and therapeutics, understanding the disease at a molecular level and then designing therapies accordingly using tools with such small dimensions is an ideal approach. These tools can be nanomaterials such as nanoparticles, nanoprobes, nanoconjugates, and nanocomposites [62]. The use of nanobiotechnological approaches can efficiently solve the problem of antibiotic resistance by fighting resistant bacteria. Various kinds of nanoparticles can be synthesized through biological means by using plant or microbial extract as a green media, where various biomolecules can act as reducing and capping agents. As a result, nanoparticles with high stability and increased dispersity are synthesized [50]. These nanoparticles can get attached to the bacterial cell wall and rupture it [64], destroy the cell organelles that disturb biochemical pathways [65], or generate reactive oxygen species that damage proteins and DNA [66].

Overall, nanoparticles possess excellent antibacterial properties as they can efficiently interact with bacteria, target multiple sites and pathways, and ultimately leads to bacterial cell death. Nanoparticles efficiently bind with biomolecules and form nanoconjugates with many antibacterial applications. Nanoparticles conjugated with nucleic acid aptamers specific to pathogen help in its rapid detection in the sample [67]. The combined application of antibiotics and nanoparticle conjugate potentially reduces the toxicity of both components as it reduces their amount and doses. It also restores the antibacterial property of antibiotics to which bacteria have developed resistance by increasing their absorption and bioavailability [7].

Nanomaterials also perform a significant role in the formation of vaccines with the ability to surpass mutations, potentially preventing the emergence of new outbreaks. Nanoparticles that mimic antigens, safely carry them, and deliver them to the targeted region in a controlled manner have extensively contributed to the field of vaccine development [68]. Conclusively, nanobiotechnology has enlightened new ways of fighting microbial diseases with more specificity and accuracy.

9. GREEN METALLIC NANOPARTICLES AGAINST PATHOGENIC BACTERIA

With the advancement of nanotechnology in the area of medicine and its use in various applications, it is not surprising to observe its role in managing the antibiotic resistance problem [69]. Embedded in the framework of green nanotechnology, developing new methods for synthesizing nanoparticles is extensively important [70]. Various methods (chemical, physical and biological), have been studied and reported to fabricate and synthesize nanoparticles with the required morphological characteristics, and functionalities [71]. It is urgent to develop better approaches to speed up the introduction of antimicrobial materials using green nanotechnology [72]. Nanoparticles can be synthesized through biological methods by self-assembling them into nanosized particles. They can efficiently interact with bacteria and destroy them in several ways (as shown in Figure 2). Green-synthesized nanoparticles are applicable in electronics, biological markers, and antimicrobials and possess the advantages of being safer, reproducible, and cheap, which can boost chemical reactions [73].

9.1 Silver Nanoparticles

Silver nanoparticles (AgNPs) are the most popular type due to their antimicrobial properties. AgNPs are versatile [74], and have been used in antimicrobial

gel formulations, AgNPs-aided dressings for wound healing [75], orthopedic operations [76], medical catheters [77], blood-contacting implants [78], endodontic filling materials [79], dental instruments [80] and coating of contact lenses [81]. AgNPs have many applications in products like building materials, antimicrobial coating, textiles, wound dressing, medical products, cosmetics, food, and antibacterial properties [82]. AgNPs synthesized through a chemical approach produce toxic and dangerous compounds that can harm the environment, require high energy and high pressure, and are very costly [83]. Alternatively, the synthesis of AgNPs through biological methods is environment-friendly, evading the use of poisonous and hazardous compounds [84]. Biological methods used to produce AgNPs include the employment of bacteria, fungi, yeast, and plants. Their extracts contain enzymes, proteins, amino acids, carbohydrates, and vitamins, which help to synthesize stable and dispersed AgNPs. The biological method also controls the shape and size of nanoparticles [85].

Plant-based silver nanoparticles have unique chemical, physical and biological properties. The effectiveness of these AgNPs evaluated in vitro has been well documented in the literature [86-88]. Readily acting and broad-spectrum bactericidal activity of plant-based AgNPs on both gram-negative and gram-positive strains of bacteria have been reported [89]. Due to the effective bactericidal activity of AgNPs aided with a faster healing rate due to the microbe-free environment, its use in biomedical applications has increased over the past few years. Plant-based AgNPs -impregnated dressings that have low cytotoxicity or no cytotoxicity are considered very safe for patients with serious wounds [90]. The oxidative stress-generating ability of Ag^{+} released by AgNPs has been reported. With the release of Ag^{+}, ROS generate that ultimately causes stress at molecular and cellular levels resulting in increased calcium levels in intracellular space, destruction of membranes, phosphatidylserine exposure in the outer membrane, DNA breakdown, and activation of caspase-like protein [91].

The study of Patra and Baek [92] suggested that using plant and plant extract-reduced AgNPs could potentially inhibit the growth of well-known pathogenic bacteria. The similar negative effect of bioactive AgNPs on the growth of *S. aureus* and *P. aeruginosa* were reported [93]. Following the penetration of AgNPs across the cell membrane of bacteria, several crucial steps take place. The silver ions disturb and halt the DNA's replication process leading to cell death [94].

9.2 Zinc Nanoparticles

In recent years, the antibacterial properties of zinc oxide nanoparticles (ZnONPs) have drawn substantial attention globally, predominantly since nanotechnology has been used to synthesize materials in the nanometer range [95]. As a result of the increased specific surface area generated by the reduction in particle size, ZnONPs exhibit attractive antibacterial properties. Moreover, ZnO is a non-toxic compound with several light-activated oxidative and catalytic effects on various chemical and biological species, making it an appropriate material for bio applications [96]. The ZnONPs are considerably biocompatible, and their rate of electron transport is high, so they are appropriate for use as biological membranes and in any other biological application in which they may have a great deal of function [97]. ZnO nanoparticles exhibit significant antimicrobial activity against several pathogenic bacteria, including *P. aeruginosa, S. pyogenes, Klebsiella, B. subtilis, S. aureus, M. tuberculosis, E. coli,* and *P. mirabilis*. The great antibacterial properties of nanoparticles are reflected in their toxicity, which is also, unfortunately, a major drawback of nanoparticles.

In comparison to soluble Zn compounds such as Zinc chloride, ZnONPs have a better antimicrobial effect, as they possess more active targeting potential, and they are capable of generating ROS inside a cell membrane, thus disrupting the integrity of cell membranes as well through in the denaturation of proteins, lipids, and also DNA [98]. An investigation reported that ZnONPs were bactericidal and not bacteriostatic in their effect on *Campylobacter jejuni* bacterial culture since no recovery of bacterial cells was observed after the replacement of nutrients with ZnONPs [99]. The antibacterial effects of spherical ZnONPs (70 nm) were examined against E. coli. The ZnONPs were administered at concentrations ranging from 3 to 12 mM for 24 hours, and full growth suppression

was seen at the 12 mM concentration. The ZnO-NPs destroy proteins and lipids on the membrane of the bacterial cells, causing the membranes to be damaged, resulting in leakage of the intracellular contents of the cells, and causing the death of the bacteria [100].

The antibacterial activity of date palm extract-stabilized spherical ZnONP (97 nm) synthesized on cotton fabric was demonstrated by El-Naggar *et al*. [101]. Compared with uncapped ZnONP and date palm extract, capped ZnONP was found to have higher antibacterial activity against various bacterial species such as *P. aeruginosa, B. subtilis, S. aureus,* and *E. coli*. Moreover, these capped ZnONPs showed no detectable cytotoxicity against human cell lines even 72 hours after treatment.

9.3 Gold Nanoparticles

Gold nanoparticles (AuNPs) are effective against a variety of bacteria and are known for their high surface area, simplicity in functional group modification, and non-specific antibacterial action [102]. AuNPs have shown particularly beneficial antibacterial activity. They are nontoxic, highly biocompatible, and have very stable chemical properties [103]. AuNPs are unlikely to create resistance than conventional antibiotics because they target a range of components in bacteria, including DNA and proteins; thus, they make it harder for bacteria to develop defense mechanisms that can withstand all harm [104]. The antibacterial effects of AuNPs are mainly based on biofilm and cytoderm disruption, formation of ROS, and release of metal ions that cause bacterial cell destruction [105]. Phytochemicals of *Clitoria ternatea* leaves have been extracted in methanol to synthesize AuNPs [106]. In the anti-biofilm test against *P. aeruginosa*, the biofilm formation rate was repressed up to 94.4 % by using a concentration of 100 µg/mL. Zhou *et al*. [107] examined the antibacterial efficacy of AuNPs against *M. tuberculosis* and *E. coli,* two Gram-positive and Gram-negative pathogens, respectively. They concluded that Gram-positive M. tuberculosis and Gram-negative *E. coli* were significantly inhibited by AuNPs. Another research work by Boomi *et al.* [108] used the leaf extract of *Croton sparsiflorus* to synthesize AuNPs showing a good zone of inhibition against *S. epidermidis* and *E. coli* around 30 mm and 26 mm.

9.4 Iron Nanoparticles

Iron nanoparticles (FeONPs) possess strong activity against various harmful bacteria and can be utilized as a substitute for antibiotics, much like other metallic nanoparticles [109]. Biogenic FeONPs are proven to be effective antimicrobials. When compared to silver and gold nanoparticles, FeONPs are substantially more affordable [110]. In addition, they are favored because they are less harmful to individuals than other nanoparticles, particularly silver, which may be toxic to various cell types. The semi-crystalline biogenic iron oxide (FeONPs) nanoparticles were synthesized from Tridax procumbens ranging in size from 80 to 100 nm and showed bactericidal action against the Gram-negative bacteria *P. aeruginosa* [111]. FeNPs made from *Moringa oleifera* leaf extract in a different study showed antibacterial efficacy against *E. coli, P. aeruginosa, S. aureus, Pseudomonas multocida,* and *Salmonella typhi* [112]. Biocompatible FeO-NPs were produced from P. granatum peel extract that was highly effective against *P. aeruginosa* [113]. Very stable FeNPs were biologically synthesized to suppress certain harmful bacteria using *Lantana camara* plant extract. These nanoparticles efficiently inhibited *K. pneumoniae, P. aeruginosa*, and *S. aureus* [114].

10. GREEN SYNTHESIZED LIPID NANOPARTICLES AGAINST RESISTANT PATHOGENS

Lipid nanoparticles (LNPs) are unique particulate systems for effective drug delivery. It possesses the combined advantages of nano polymers, liposomes, and emulsions and overcomes their limitations in drug delivery applications [115]. The size of LNPs lies between 50-1000 nm after drug encapsulation, and they are made of biocompatible and biodegradable materials able to encapsulate and carry both hydrophilic and hydrophobic therapeutic molecules [116]. These nano-carriers are considered safe, nontoxic, biocompatible, and easy to produce [117]. Due to the high possibilities of their surface modification, they can encapsulate various molecules. The solid matrix (at room and human body temperature) enables them to gradually release the encapsulated active therapeutic

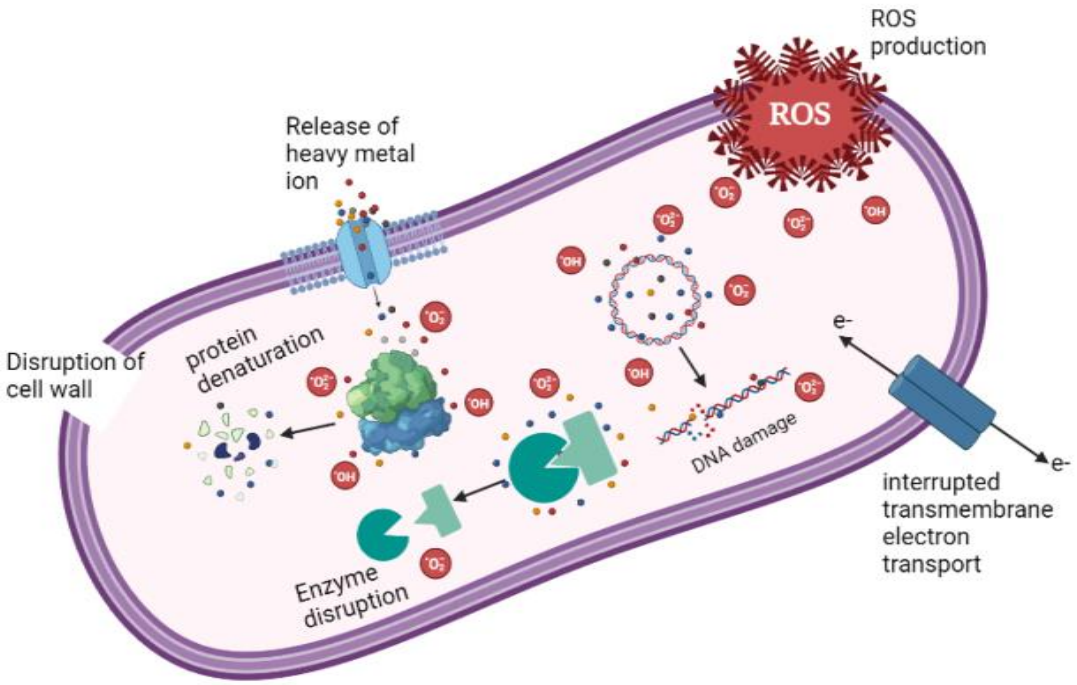

Fig. 2. Mechanisms through which nanoparticles interact with bacteria and disrupt them

ingredients in a controlled manner with enhanced intracellular permeability. The bioavailability of weakly soluble drugs can be enhanced with the use of such a particulate drug delivery system. Moreover, it can properly bio-distribute the drugs to the affected target areas [118, 119].

LNPs are green as their lipid component is derived from natural sources like purified triglycerides, glyceride compound mixtures, waxes, fatty acid esters, fatty alcohols, acylglycerols, and mixtures of acylglycerol esters. They are colloidal particles composed of a solid lipid matrix, surfactants (for stabilization), and active ingredients. Thus, the physiological lipid constituent in producing different LNPs makes it one of the primary drug delivery systems as it is biodegradable and biocompatible with minimum toxicity. LNPs were synthesized in the early 1990s in the search for the development of novel drug carriers. There are two major types of LNPs, namely solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs). SLNs are modifications of liposomes, polymeric nanoparticles, and emulsions to overcome their limitations. At the same time, NLCs are the next generation of SLNs with few improvements, including high stability and increased drug loading capacity. They can be synthesized through various methods such as high-pressure homogenization, hot and cold homogenization, solvent emulsification, evaporation or diffusion, supercritical fluid (supercritical fluid extraction of emulsions (SFEE)), ultrasonication or high-speed homogenization, and spray drying [120]. The resultant LNPs can be applied as drug carriers in treating many diseases.
LNPs loaded with antibiotics are effective against bacteria because they can directly fuse with the cell wall of bacteria and gradually release the loaded therapeutic agent in response to bacteria. The LNPs and antibiotics act synergistically, thus increasing the cumulative antibacterial effect [121]. Rifampicin-loaded LNPs were tested in a skin-wounded mouse model to treat skin infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA). The study revealed that rifampicin-LNPs are more effective against MRSA than antibiotics alone [122]. Similarly, in a recent study, a complex of silver nanoparticles with clotrimazole was loaded on SLNs to test it against MRSA. This complex with SLNs showed the highest antibacterial activity and

was represented to be a good nano-antibiotic [123]. Another promising nano-antibiotic composed of vancomycin conjugated with linoleic acid loaded on SLNs has been developed against MRSA.

The resultant complex showed an enhanced antibacterial effect in *in vitro* antimicrobial tests with a minimum inhibitory concentration of 15.62 µg/ml [124]. Antibacterial oligonucleotide therapeutics such as transcription factor decoys (TFDs) have also emerged as molecules that can avoid AMR. However, their safe delivery to the target site is challenging as it requires protection from nucleases. LNPs have also been used to encapsulate and safely deliver these therapeutic oligonucleotides to fight *E. coli* infection. Its safe delivery to the bacteria was verified with high efficacy. Thus, LNPs are an effective delivery tool for novel antibacterial agents that can cross the barrier of AMR [125].

Furthermore, it has been identified that LNPs can be bactericidal without any loaded drug. A study revealed that NLCs could interact with and inhibit resistant H. pylori without affecting gut microbiota [126]. NLC can also deliver vaccines to generate a protective immune response [127]. They have also been employed to systematically deliver therapeutic small-interfering RNAs [128]. Overall, LNPs are an effective tool in the fight against antibiotic resistance as they can increase the potential of existing antibiotics and help the alternatives of antibiotics to be efficient. Such active drug carriers can competently treat recalcitrant bacterial infections and protect the world from new outbreaks.

11. NANOPARTICLES-BIOMOLECULE CONJUGATES AS AN IMPORTANT TOOL

Various nanoparticles have been recognized and employed as effective antibacterial agents against resistant bacterial strains. Due to their known therapeutic potential, more complex nanoparticles have been designed using different strategies. One of these is the conjugation of nanoparticles with biomolecules. Biomolecules can efficiently interact with nanoparticles and provide them with specificity. A stable and convenient conjugation between nanoparticles and biomolecules such as vaccines, drugs, peptides, proteins, and nucleotides can be achieved [129].

Antimicrobial peptides are desirable alternatives to antibiotics because of their broad-spectrum activity and with little chance of resistance development. However, they possess some limitations, such as poor enzymatic stability and permeability to the target site. It has been reported that AMPs conjugated with nanoparticles can form potent antibacterial agents with an enhanced antibacterial potential of both components with a synergistic effect that can effectively fight antibacterial resistance [130]. AgNPs are reported to effectively interact and conjugate with AMPs after tagging cysteine residue to the terminals of peptides. This conjugate is an active antibacterial agent against MDR *K. pneumoniae* [131]. AMPs esculentin-1a derived from frog skin conjugated with AuNPs showed 15 times higher antibacterial activity than peptide alone against free and sessile *P. aeruginosa.* The conjugate had no toxic effect on human keratinocytes. Thus, it suggested an attractive alternative for treating epithelial infection [132]. Polymyxin B (a cationic AMP having high potential against Gram-negative bacteria) linked with AgNPs was tested against MDR strains. The results showed that the conjugate lowered MIC value compared to the control. The SEM study revealed massive damage to the cell membrane and leakage of the cell contents resulting in cell death [133]. Nanomaterials conjugated lysozymes have also proved efficiently active against resistant bacteria (Gram-positive and Gram-negative). Lysozymes were immobilized on chitosan nanofibers which showed enhanced antibacterial activity by increasing the catalytic cleavage reaction of peptidoglycans in the bacterial cell membrane [49].

In the progress of vaccine development, nanoparticles can be loaded with diverse molecules including nucleic acids, peptides, and proteins, to form antigenic sources to be recognized by immune cells. Nano-formulations protect the antigens from enzymatic degradation and allow safe and controlled delivery to the target site [134]. Nanoparticles coated with outer membrane vesicles (OMVs) of bacteria have been successfully developed as effective immunogenic agents. When tested in mouse models, the outer membrane coating of *E. coli* on AuNPs revealed the activation of B and T-cell immunity along with the activation

of dendritic cells [135]. Moreover, Shigella OMVs loaded poly(anhydride) nanoparticles have revealed greater mucosal defense compared to free OMVs in a mouse model [136].

12. CURRENT CHALLENGES TO NANOBIOTECHNOLOGY

Nanobiotechnology has emerged as a revolution in medical science and can potentially overcome the shortcomings of conventional biomedical strategies. The applications of nanobiotechnology have already been discussed in the previous sections. The research community has been using nanobiotechnology to solve the global issue of antibiotic resistance. It is clear from the discussion in the previous sections that nanobiotechnology can be a potential strategy for preventing COVID-19, like pandemics. Although, like every other technology, nanobiotechnology also has certain challenges to face.

Despite all the benefits of using bio-nanotechnology as a biomedical strategy, there are still some challenges to overcome. The biggest possible limitation of using nanomaterials antimicrobials is their potential toxicity, which is unfortunately poorly understood [137]. Most inorganic nanomaterials are metallic [138]. Metallic nanomaterials are not only mutagenic but can also be potential endocrine disruptors, and therefore, using nanomaterials leads to human health compromise [139]. Adverse biological responses can be initiated by nanomaterials cause of their genotoxic and carcinogenic nature [140]. There is a high risk associated with the use of nanomaterials. They can be accumulated in human bodies and may have adverse effects [141]. This threat of possible toxicological outcomes is forcing biomedical researchers to find a way to minimize metallic nanomaterials' toxicity. Nanomaterials-induced toxicity can lead to serious health issues in immunocompromised patients. Therefore, a very high risk is associated with applying nanomaterials to human bodies [142]. To assess the toxic effects of nanomaterials, nanobiotechnology experts tend to promote the safe design and utilization of nanomaterials, act like aliens in the bloodstream, and the human body search for different ways to get them out of the body, such as immune responses minimizing their efficiency [143]. To address this issue, scientists need to find a way for nanomaterials can overcome the forces driving them out of the body and achieve an increased safety-to-risk ratio.

The toxicity of metallic nanomaterials is not limited to human health. The excessive use of nanomaterials is also likely to have adverse environmental effects [144]. As metals are not easily degraded, improper disposal of metallic nanomaterials causes pollution [145]. Nanoparticles can easily enter the bodies of humans and other organisms through the skin due to their ultra-fine sizes [146]. They can also get suspended in the atmosphere and travel long distances [147]. Nanoparticles not only cause air pollution but can also have harmful effects on soil and groundwater [148]. This issue can be addressed by developing biodegradable nanomaterials such as polylactic acid (PLA) and polyglycolic acid (PGA) [149]. For this purpose, they need to evaluate the complete life cycles, such as fabrication, storage, distribution, application, and disposal of nanomaterials [150]. In this way, nanoparticle contamination and pollution can be controlled.

Another challenge to nanobiotechnology is the limited understanding of nanomaterials, their characteristics, and their potential toxic effects. The efficiency of nanomaterials is limited by their complex nano-systems [151]. Very little literature and published research are available about nanomaterials and their potential risks [152]. There is not a single FDA-approved nano-antibiotic available yet for human utilization despite their remarkable antimicrobial activity [153]. Nanobiotechnology experts, researchers, and developers need to work together to understand this revolutionizing technology better. Also, the knowledge associated with nanomaterials should not be preserved. It should be shared between experts, research bodies, and even nations worldwide because that is how the research community can work together as a team. Employing machine learning and artificial intelligence for modeling the ideal nanostructure designs and understanding the interaction between nanomaterials and living cells is also a challenge to nanobiotechnology [154]. If overcome these challenges efficacy and efficiency of nanomaterials will ultimately be increased. Nanomaterials could be better optimized as therapeutic agents for target drug delivery by overcoming these challenges,

and hence financially effective nanobiotechnology techniques will be developed. To launch the safe and proper commercialization of nanotechnology and its applications, authorities must make definite policies.

13. FUTURE IMPLICATIONS

As discussed in the sections above, different factors, such as over/misuse of antibiotics, their inappropriate prescriptions, and inappropriate utilization for livestock production, have led to the emergence of antibiotic-resistant pathogens. Various strains of bacteria, viruses, fungi, and parasites have developed resistance to most classes of the existing antibiotics and are therefore known as "superbugs" [155]. These superbugs can potentially cause various diseases that cannot be treated with the available classes and generations of antibiotics and can result in outbreaks and hence, pandemics [156]. However, nanotechnology has the potential to prevent such pandemics. Nanotechnology can overcome traditional antimicrobials' limitations and restore antibiotics' lost activity [157]. The threats of pandemics could be addressed by nanomaterials' antimicrobial potential and applications [158].

There are different nanotechnology-based tools available that can prevent pandemics by combating the antibiotic-resistance phenomenon of superbugs. This goal could be achieved by practicing the clinical applications of nanomaterials, such as diagnosis, prevention, drug delivery, vaccination, and treatment [159]. Nanoparticle-based biosensors have the ability to rapid detection of pathogens. For instance, the recently developed nano-biosensors for detecting SARS-CoV-2 [160]. FDA has approved 49 nano-based devices for the diagnosis of COVID-19 [161]. Many nanoscale biosensors have been created to diagnose infections such as HIV, influenza, etc. [162]. The nano-delivery systems can be used for accurate target drug delivery, a limitation of traditional drug carriers [163]. For example, liposomes, polymeric nanoparticles, nanocrystals, and dendrimers are potential drug carriers [164].

Similarly, the limited efficiency of traditional vaccines can be addressed by developing nanoparticle-based vaccines, which are much easier to design and synthesize [165]. Various vaccine nano-carriers have already been designed as vaccination tools [166]. All these nanomaterial-based tools and strategies offer an excellent chance of winning the fight against antibiotic resistance by tackling most superbugs and hence, preventing an outbreak from becoming a pandemic.

14. LIMITATIONS OF BIOGENIC NANOMATERIALS

Despite the vast variety of applications, the use of nanomaterials also has various limitations. As discussed in the previous sections, the potential toxicity of nanomaterials to both, health and environment cannot be ignored. More than 400 studies have been reported concerning the toxicity and eco-toxicity of nanomaterials [167]. As using nanomaterials as antimicrobials can result in serious health issues in immunocompromised patients [168], therefore, no FDA-approved nanomaterial-based drug is available for human use yet [153]. Nanomaterials are not only potentially toxic, but can also cause air, water, and soil pollution [169]. The limited understanding of the complex nano-systems of nanomaterials is also a subject of concern. Large-scale utilization and handling of nanomaterials is a very challenging task. The lack of logistic knowledge for developing green-nanomaterials is one of the primary limitations of biogenic nanomaterials [170]. However, the notable and successful applications of nanomaterials in nano-biomedicine outweigh their limitations.

15. CONCLUSION

The continuous development of multidrug-resistant pathogens, also now known as "superbugs," can lead to outbreaks and pandemics and is, therefore, a global threat. As the current antibiotics and strategies are struggling to cope with this issue, developing novel, cost-effective, eco-friendly, and more effective alternative strategies is necessary. Nanotechnology is one of these strategies. Nanomaterials-based tools such as nano-biomarkers, nano-biosensors, etc., can solve the problem of antibiotic-resistant and prevent pandemics as per their potential applications in different areas such as diagnostics, prevention, vaccination, and treatment. Although, this technology has certain challenges to overcome, for which researchers are coming up with effective solutions.

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17. CONFLICT OF INTEREST

The authors declared no conflict of interest.

18. REFERENCES

1. D.G.J. Larsson, and C.F. Flach. Antibiotic resistance in the environment. *Nature Reviews Microbiology* 20(5): 257-269 (2022).
2. B. Ribeiro Da Cunha, L.P. Fonseca, and C.R. Calado. Antibiotic discovery: where have we come from, where do we go? *Antibiotics* 8(2): 45 (2019).
3. B. Aslam, W. Wang, M.I. Arshad, M. Khurshid, S. Muzammil, M.H. Rasool, M.A. Nisar, R.F. Alvi, M.A. Aslam, M.U.I. Qamar, and D. Resistance. Antibiotic resistance: a rundown of a global crisis. *Infection drug resistance* 11: 1645 (2018).
4. A.R. Mahoney, M.M. Safaee, W.M. Wuest, and A.L. Furst. The silent pandemic: Emergent antibiotic resistances following the global response to SARS-CoV-2. *Iscience* 24(4): 102304 (2021).
5. T.M. Uddin, A.J. Chakraborty, A. Khusro, B.R.M. Zidan, S. Mitra, T.B. Emran, K. Dhama, M.K.H. Ripon, M. Gajdács, and M.U.K. Sahibzada. Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. *Journal of Infection Public Health* 14(12): 1750-1766 (2021).
6. N. Dey, C. Kamatchi, A. Vickram, K. Anbarasu, S. Thanigaivel, J. Palanivelu, A. Pugazhendhi, and V.K. Ponnusamy. Role of nanomaterials in deactivating multiple drug resistance efflux pumps–A review. *Environmental Research* 204: 111968 (2022).
7. A.M. Allahverdiyev, K.V. Kon, E.S. Abamor, M. Bagirova, and M. Rafailovich. Coping with antibiotic resistance: combining nanoparticles with antibiotics and other antimicrobial agents. *Expert review of anti-infective therapy* 9(11): 1035-1052 (2011).
8. M.C. Zambonino, E.M. Quizhpe, F.E. Jaramillo, A. Rahman, N. Santiago Vispo, C. Jeffryes, and S.A. Dahoumane. Green synthesis of selenium and tellurium nanoparticles: current trends, biological properties and biomedical applications. *International Journal of Molecular Sciences* 22(3): 989 (2021).
9. H. Chandra, P. Kumari, E. Bontempi, and S. Yadav. Medicinal plants: Treasure trove for green synthesis of metallic nanoparticles and their biomedical applications. *Biocatalysis Agricultural Biotechnology* 24: 101518 (2020).
10. M.T. Shaaban, M.F. Ghaly, and S.M. Fahmi. Antibacterial activities of hexadecanoic acid methyl ester and green-synthesized silver nanoparticles against multidrug-resistant bacteria. *Journal of basic microbiology* 61(6): 557-568 (2021).
11. D. Sharma, P. Shandilya, N. Saini, P. Singh, V. Thakur, R. Saini, D. Mittal, G. Chandan, V. Saini, and A. Saini. Insights into the synthesis and mechanism of green synthesized antimicrobial nanoparticles, answer to the multidrug resistance. *Materials Today Chemistry* 19: 100391 (2021).
12. S. Wahab, T. Khan, M. Adil, and A. Khan. Mechanistic aspects of plant-based silver nanoparticles against multi-drug resistant bacteria. *Heliyon* 7(7): e07448 (2021).
13. U. Anand, M. Carpena, M. Kowalska-Góralska, P. Garcia-Perez, K. Sunita, E. Bontempi, A. Dey, M.A. Prieto, J. Proćków, and J. Simal-Gandara. Safer plant-based nanoparticles for combating antibiotic resistance in bacteria: A comprehensive review on its potential applications, recent advances, and future perspective. *Science of The Total Environment:* 153472 (2022).
14. P. Hawkey. The growing burden of antimicrobial resistance. *Journal of antimicrobial chemotherapy* 62(suppl_1): i1-i9 (2008).
15. N.R. Naylor, R. Atun, N. Zhu, K. Kulasabanathan, S. Silva, A. Chatterjee, G.M. Knight, and J.V. Robotham. Estimating the burden of antimicrobial resistance: a systematic literature review. *Antimicrobial Resistance Infection Control* 7(1): 1-17 (2018).
16. C.J. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G.R. Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, and E. Wool. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* 399(10325): 629-655 (2022).
17. B. Harish and G. Menezes. Antimicrobial resistance in typhoidal salmonellae. *Indian journal of medical microbiology* 29(3): 223-229 (2011).
18. I. Frost, T.P. Van Boeckel, J. Pires, J. Craig, and R. Laxminarayan. Global geographic trends in

antimicrobial resistance: the role of international travel. *Journal of travel medicine* 26(8): taz036 (2019).
19. A.C. Palmer and R. Kishony. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nature Reviews Genetics* 14(4): 243-248 (2013).
20. J.M. Bello-López, O.A. Cabrero-Martínez, G. Ibáñez-Cervantes, C. Hernández-Cortez, L.I. Pelcastre-Rodríguez, L.U. Gonzalez-Avila, and G. Castro-Escarpulli. Horizontal gene transfer and its association with antibiotic resistance in the genus Aeromonas spp. *Microorganisms* 7(9): 363 (2019).
21. A. Russell. Whither triclosan? *Journal of Antimicrobial Chemotherapy* 53(5): 693-695 (2004).
22. R. Chuanchuen, R.R. Karkhoff-Schweizer, and H.P. Schweizer. High-level triclosan resistance in Pseudomonas aeruginosa is solely a result of efflux. *American journal of infection control* 31(2): 124-127 (2003).
23. L. Fernández and R.E. Hancock. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clinical microbiology reviews* 25(4): 661-681 (2012).
24. G.D. Wright. Molecular mechanisms of antibiotic resistance. *Chemical communications* 47(14): 4055-4061 (2011).
25. R.E. Hancock. The bacterial outer membrane as a drug barrier. *Trends in microbiology* 5(1): 37-42 (1997).
26. M.M. Fernandes, K. Ivanova, J. Hoyo, S. Pérez-Rafael, A. Francesko, and T. Tzanov. Nanotransformation of vancomycin overcomes the intrinsic resistance of gram-negative bacteria. *ACS applied materials interfaces* 9(17): 15022-15030 (2017).
27. J.P. Sarathy, V. Dartois, and E.J.D. Lee. The role of transport mechanisms in Mycobacterium tuberculosis drug resistance and tolerance. *Pharmaceuticals* 5(11): 1210-1235 (2012).
28. S. Singh, S. Datta, K.B. Narayanan, and K.N. Rajnish. Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. *Journal of Genetic Engineering Biotechnology* 19(1): 1-19 (2021).
29. J.-M. Pagès, M. Masi, and J. Barbe. Inhibitors of efflux pumps in Gram-negative bacteria. *Trends in molecular medicine* 11(8): 382-389 (2005).
30. F. Van Bambeke, E. Balzi, and P.M. Tulkens. Antibiotic efflux pumps. *Biochemical pharmacology* 60(4): 457-470 (2000).
31. W.C. Reygaert. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology* 4(3): 482 (2018).
32. S.F. Nadeem, U.F. Gohar, S.F. Tahir, H. Mukhtar, S. Pornpukdeewattana, P. Nukthamna, A.M. Moula Ali, S.C.B. Bavisetty, and S. Massa. Antimicrobial resistance: more than 70 years of war between humans and bacteria. *Critical Reviews in Microbiology* 46(5): 578-599 (2020).
33. R. Wheeler, R.D. Turner, R.G. Bailey, B. Salamaga, S. Mesnage, S.A. Mohamad, E.J. Hayhurst, M. Horsburgh, J.K. Hobbs, and S.J. Foster. Bacterial cell enlargement requires control of cell wall stiffness mediated by peptidoglycan hydrolases. *MBio* 6(4): e00660-15 (2015).
34. F.C. Tenover. Mechanisms of antimicrobial resistance in bacteria. *The American journal of medicine* 119(6): S3-S10 (2006).
35. A.L. Davidson, E. Dassa, C. Orelle, and J. Chen. Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiology molecular biology reviews* 72(2): 317-364 (2008).
36. T.J. Foster. Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS microbiology reviews* 41(3): 430-449 (2017).
37. E. Medina and D.H. Pieper. Tackling threats and future problems of multidrug-resistant bacteria. *How to overcome the antibiotic crisis*: 3-33 (2016).
38. D. Reynolds, J.P. Burnham, C.V. Guillamet, M. Mccabe, V. Yuenger, K. Betthauser, S.T. Micek, and M.H. Kollef. The threat of multidrug-resistant/extensively drug-resistant Gram-negative respiratory infections: another pandemic. *European Respiratory Review* 31(166) (2022).
39. V. Manchanda, S. Sanchaita, and N. Singh. Multidrug resistant acinetobacter. *Journal of global infectious diseases* 2(3): 291 (2010).
40. E. Banin, D. Hughes, and O.P. Kuipers. Bacterial pathogens, antibiotics and antibiotic resistance. *Federation of European Microbiological Societies* 41(3): 450-452 (2017).
41. S. Tiberi, M.J. Vjecha, A. Zumla, J. Galvin, G.B. Migliori, and A. Zumla. Accelerating development of new shorter TB treatment regimens in anticipation of a resurgence of multi-drug resistant TB due to the COVID-19 pandemic. *International Journal of Infectious Diseases* 113: S96-S99 (2021).
42. M. Polly, B.L. De Almeida, R.P. Lennon, M.F. Cortês, S.F. Costa, and T. Guimarães. Impact of the COVID-19 pandemic on the incidence of multidrug-

resistant bacterial infections in an acute care hospital in Brazil. *American journal of infection control* 50(1): 32-38 (2022).
43. T. Singhal. Antimicrobial Resistance: The'Other'Pandemic! *Indian Journal of Pediatrics:* 1-7 (2022).
44. M.N. Gwynn, A. Portnoy, S.F. Rittenhouse, and D.J. Payne. Challenges of antibacterial discovery revisited. *Annals of the New York Academy of Sciences* 1213(1): 5-19 (2010).
45. P.S. Hoffman. Antibacterial discovery: 21st century challenges. *Antibiotics* 9(5): 213 (2020).
46. L.L. Silver. Challenges of antibacterial discovery. *Clinical microbiology reviews* 24(1): 71-109 (2011).
47. G.V. Vimbela, S.M. Ngo, C. Fraze, L. Yang, and D.A. Stout. Antibacterial properties and toxicity from metallic nanomaterials. *International Journal of Nanomedicine* 12: 3941 (2017).
48. G.V. Vimbela, S.M. Ngo, C. Fraze, L. Yang, and D.A. Stout. Antibacterial properties and toxicity from metallic nanomaterials [Corrigendum]. *International Journal of Nanomedicine* 13: 6497-6498 (2018).
49. Y. Wang, Y. Yang, Y. Shi, H. Song, and C. Yu. Antibiotic-free antibacterial strategies enabled by nanomaterials: progress and perspectives. *Advanced Materials* 32(18): 1904106 (2020).
50. A. Singh, P.K. Gautam, A. Verma, V. Singh, P.M. Shivapriya, S. Shivalkar, A.K. Sahoo, and S.K. Samanta. Green synthesis of metallic nanoparticles as effective alternatives to treat antibiotics resistant bacterial infections: A review. *Biotechnology Reports* 25: e00427 (2020).
51. M.J. Hajipour, K.M. Fromm, A.A. Ashkarran, D.J. De Aberasturi, I.R. De Larramendi, T. Rojo, V. Serpooshan, W.J. Parak, and M. Mahmoudi. Antibacterial properties of nanoparticles. *Trends in biotechnology* 30(10): 499-511 (2012).
52. A. Gupta, S. Mumtaz, C.H. Li, I. Hussain, and V.M. Rotello. Combatting antibiotic-resistant bacteria using nanomaterials. *Chem Soc Rev* 48(2): 415-427 (2019).
53. K. Vijayaraghavan, S.K. Nalini, N.U. Prakash, and D. Madhankumar. One step green synthesis of silver nano/microparticles using extracts of Trachyspermum ammi and Papaver somniferum. *Colloids and Surfaces B: Biointerfaces* 94: 114-117 (2012).
54. J.M.V. Makabenta, A. Nabawy, C.-H. Li, S. Schmidt-Malan, R. Patel, and V.M. Rotello. Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nature Reviews Microbiology* 19(1): 23-36 (2021).
55. V. Vilas, D. Philip, and J. Mathew. Catalytically and biologically active silver nanoparticles synthesized using essential oil. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 132: 743-750 (2014).
56. U. Shedbalkar, R. Singh, S. Wadhwani, S. Gaidhani, and B.A. Chopade. Microbial synthesis of gold nanoparticles: Current status and future prospects. *Advances in Colloid and Interface Science* 209: 40-48 (2014).
57. V. Vidhu and D. Philip. Spectroscopic, microscopic and catalytic properties of silver nanoparticles synthesized using Saraca indica flower. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 117: 102-108 (2014).
58. R. Arunachalam, S. Dhanasingh, B. Kalimuthu, M. Uthirappan, C. Rose, and A.B. Mandal. Phytosynthesis of silver nanoparticles using Coccinia grandis leaf extract and its application in the photocatalytic degradation. *Colloids and Surfaces B: Biointerfaces* 94: 226-230 (2012).
59. M. Rashid and S. Sabir. Biosynthesis of Self-Dispersed Silver Colloidal Particles Using the Aqueous Extract of P. peruviana for Sensing dl-Alanine. *ISRN Nanotechnology* 2014: 7 (2014).
60. W.C. Chan. Bionanotechnology progress and advances. *Biology of Blood Marrow Transplantation* 12(1): 87-91 (2006).
61. J. Hulla, S. Sahu, and A. Hayes. Nanotechnology: History and future. *Human experimental toxicology* 34(12): 1318-1321 (2015).
62. W. Tan, K. Wang, X. He, X.J. Zhao, T. Drake, L. Wang, and R.P. Bagwe. Bionanotechnology based on silica nanoparticles. *Medicinal research reviews* 24(5): 621-638 (2004).
63. J. Li, M. Yao, Y. Shao, and D. Yao. The application of bio-nanotechnology in tumor diagnosis and treatment: a view. *Nanotechnology Reviews* 7(3): 257-266 (2018).
64. L. Wang, C. Hu, and L. Shao. The antimicrobial activity of nanoparticles: present situation and prospects for the future. *International Journal of Nanomedicine* 12: 1227 (2017).
65. M. Arakha, S. Pal, D. Samantarrai, T.K. Panigrahi, B.C. Mallick, K. Pramanik, B. Mallick, and S. Jha. Antimicrobial activity of iron oxide nanoparticle upon modulation of nanoparticle-bacteria interface. *Scientific reports* 5(1): 1-12 (2015).
66. B. Li and T. Webster. Bacteria antibiotic resistance:

New challenges and opportunities for implant-associated orthopedic infections. *Journal of Orthopaedic Research* 36(1): 22-32 (2018).

67. G. Singh, M. Manohar, A.A. Adegoke, T.A. Stenström, and R. Shanker. Novel aptamer-linked nanoconjugate approach for detection of waterborne bacterial pathogens: an update. *Journal of Nanoparticle Research* 19(1): 1-11 (2017).
68. Y. Shen, T. Hao, S. Ou, C. Hu, and L. Chen. Applications and perspectives of nanomaterials in novel vaccine development. *MedChemComm* 9(2): 226-238 (2018).
69. X. Wang, L. Yang, Z. Chen, and D.M. Shin. Application of nanotechnology in cancer therapy and imaging. *CA: a cancer journal for clinicians* 58(2): 97-110 (2008).
70. J.E. Hutchison. Greener nanoscience: a proactive approach to advancing applications and reducing implications of nanotechnology. *ACS nano* 2(3): 395-402 (2008).
71. S.S. Mughal and S.M. Hassan. Comparative Study of AgO Nanoparticles Synthesize Via Biological, Chemical and Physical Methods: A Review. *American Journal of Materials Synthesis Processing* 7(2): 15-28 (2022).
72. M. Nasrollahzadeh, M. Sajjadi, S.M. Sajadi, and Z. Issaabadi, *Green nanotechnology, in Interface science and technology.* 2019, Elsevier. p. 145-198.
73. X. Qu, J. Brame, Q. Li, and P.J. Alvarez. Nanotechnology for a safe and sustainable water supply: enabling integrated water treatment and reuse. *Accounts of chemical research* 46(3): 834-843 (2013).
74. J. Jain, S. Arora, J.M. Rajwade, P. Omray, S. Khandelwal, and K.M. Paknikar. Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. *Molecular pharmaceutics* 6(5): 1388-1401 (2009).
75. C. Rigo, L. Ferroni, I. Tocco, M. Roman, I. Munivrana, C. Gardin, W.R. Cairns, V. Vindigni, B. Azzena, and C. Barbante. Active silver nanoparticles for wound healing. *International journal of molecular sciences* 14(3): 4817-4840 (2013).
76. S.A. Brennan, C. Ní Fhoghlú, B. Devitt, F. O'mahony, D. Brabazon, and A. Walsh. Silver nanoparticles and their orthopaedic applications. *The bone joint journal* 97(5): 582-589 (2015).
77. P. Lackner, R. Beer, G. Broessner, R. Helbok, K. Galiano, C. Pleifer, B. Pfausler, C. Brenneis, C. Huck, and K. Engelhardt. Efficacy of silver nanoparticles-impregnated external ventricular drain catheters in patients with acute occlusive hydrocephalus. *Neurocritical care* 8(3): 360-365 (2008).
78. A. De Mel, K. Chaloupka, Y. Malam, A. Darbyshire, B. Cousins, and A.M. Seifalian. A silver nanocomposite biomaterial for blood-contacting implants. *Journal of Biomedical Materials Research Part A* 100(9): 2348-2357 (2012).
79. A. Bahador, B. Pourakbari, B. Bolhari, and F.B. Hashemi. In vitro evaluation of the antimicrobial activity of nanosilver-mineral trioxide aggregate against frequent anaerobic oral pathogens by a membrane-enclosed immersion test. *Journal of biomedical science* 38(1): 77-83 (2015).
80. M. Murphy, K. Ting, X. Zhang, C. Soo, and Z. Zheng. Current development of silver nanoparticle preparation, investigation, and application in the field of medicine. *Journal of nanomaterials* 2015 (2015).
81. M.D. Willcox, E.B. Hume, A.K. Vijay, and R. Petcavich. Ability of silver-impregnated contact lenses to control microbial growth and colonisation. *Journal of Optometry* 3(3): 143-148 (2010).
82. J. Talapko, T. Matijević, M. Juzbašić, A. Antolović-Požgain, and I. Škrlec. Antibacterial activity of silver and its application in dentistry, cardiology and dermatology. *Microorganisms* 8(9): 1400 (2020).
83. A.A. Yaqoob, K. Umar, and M.N.M. Ibrahim. Silver nanoparticles: various methods of synthesis, size affecting factors and their potential applications–a review. *Applied Nanoscience* 10(5): 1369-1378 (2020).
84. H.D. Beyene, A.A. Werkneh, H.K. Bezabh, and T.G. Ambaye. Synthesis paradigm and applications of silver nanoparticles (AgNPs), a review. *Journal of Sustainable materials and technologies* 13: 18-23 (2017).
85. L. Xu, W. Yi-Yi, J. Huang, C. Chun-Yuan, W. Zhen-Xing, and H. Xie. Silver nanoparticles: Synthesis, medical applications and biosafety. *Theranostics* 10(20): 8996 (2020).
86. A. Kumar and M. Jaiswal. Design and in vitro investigation of nanocomposite hydrogel based in situ spray dressing for chronic wounds and synthesis of silver nanoparticles using green chemistry. *Journal of Applied Polymer Science* 133(14) (2016).
87. S.N. El Din, T.A. El-Tayeb, K. Abou-Aisha, and M. El-Azizi. In vitro and in vivo antimicrobial activity of combined therapy of silver nanoparticles and visible blue light against Pseudomonas aeruginosa. *International journal of nanomedicine* 11: 1749

(2016).
88. D. Liang, Z. Lu, H. Yang, J. Gao, R. Chen, and Interfaces. Novel asymmetric wettable AgNPs/chitosan wound dressing: in vitro and in vivo evaluation. *ACS applied materials* 8(6): 3958-3968 (2016).
89. S. Iravani, H. Korbekandi, S.V. Mirmohammadi, and B. Zolfaghari. Synthesis of silver nanoparticles: chemical, physical and biological methods. *Research in pharmaceutical sciences* 9(6): 385 (2014).
90. J.L. Velázquez-Velázquez, A. Santos-Flores, J. Araujo-Meléndez, R. Sánchez-Sánchez, C. Velasquillo, C. González, G. Martínez-Castañon, and F. Martinez-Gutierrez. Anti-biofilm and cytotoxicity activity of impregnated dressings with silver nanoparticles. *Materials Science Engineering:* C 49: 604-611 (2015).
91. W. Lee, K.-J. Kim, and D.G. Lee. A novel mechanism for the antibacterial effect of silver nanoparticles on Escherichia coli. *Biometals* 27(6): 1191-1201 (2014).
92. J.K. Patra and K.-H. Baek. Antibacterial activity and synergistic antibacterial potential of biosynthesized silver nanoparticles against foodborne pathogenic bacteria along with its anticandidal and antioxidant effects. *Frontiers in microbiology* 8: 167 (2017).
93. B. Buszewski, A. Rogowska, V. Railean-Plugaru, M. Złoch, J. Walczak-Skierska, and P. Pomastowski. The influence of different forms of silver on selected pathogenic bacteria. *Materials* 13(10): 2403 (2020).
94. J. Lu, Y. Wang, M. Jin, Z. Yuan, P. Bond, and J. Guo. Both silver ions and silver nanoparticles facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes. *Water research* 169: 115229 (2020).
95. A. Sirelkhatim, S. Mahmud, A. Seeni, N.H.M. Kaus, L.C. Ann, S.K.M. Bakhori, H. Hasan, and D. Mohamad. Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. *Nano-micro letters* 7(3): 219-242 (2015).
96. S. Nadunga. A review on zinc and zinc oxide nanoparticles; biosynthesis, characterization and applications. (2022).
97. R. Salehi, M. Arami, N.M. Mahmoodi, H. Bahrami, and S. Khorramfar. Novel biocompatible composite (chitosan–zinc oxide nanoparticle): preparation, characterization and dye adsorption properties. *Colloids Surfaces B: Biointerfaces* 80(1): 86-93 (2010).
98. M. Kumar, A. Curtis, and C. Hoskins. Application of nanoparticle technologies in the combat against anti-microbial resistance. *Pharmaceutics* 10(1): 11 (2018).
99. Y. Xie, Y. He, P.L. Irwin, T. Jin, and X. Shi. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. *Applied environmental microbiology* 77(7): 2325-2331 (2011).
100. C. Wang, L.-L. Liu, A.-T. Zhang, P. Xie, J.-J. Lu, and X.-T. Zou. Antibacterial effects of zinc oxide nanoparticles on Escherichia coli K88. *African Journal of Biotechnology* 11(44): 10248-10254 (2012).
101. M.E. El-Naggar, S. Shaarawy, and A. Hebeish. Multifunctional properties of cotton fabrics coated with in situ synthesis of zinc oxide nanoparticles capped with date seed extract. *Carbohydrate polymers* 181: 307-316 (2018).
102. P. Singh, A. Garg, S. Pandit, V. Mokkapati, and I. Mijakovic. Antimicrobial effects of biogenic nanoparticles. *Nanomaterials* 8(12): 1009 (2018).
103. S. Senthilkumar, L. Kashinath, M. Ashok, and A. Rajendran. Antibacterial properties and mechanism of gold nanoparticles obtained from Pergularia daemia leaf extract. *Nanomed Res* 6(1): 00146 (2017).
104. C. Uruén, G. Chopo-Escuin, J. Tommassen, R.C. Mainar-Jaime, and J. Arenas. Biofilms as promoters of bacterial antibiotic resistance and tolerance. *Antibiotics* 10(1): 3 (2020).
105. E.O. Ogunsona, R. Muthuraj, E. Ojogbo, O. Valerio, and T.H. Mekonnen. Engineered nanomaterials for antimicrobial applications: A review. *Applied Materials Today* 18: 100473 (2020).
106. S. Vanaraj, J. Jabastin, S. Sathiskumar, and K. Preethi. Production and Characterization of Bio-AuNPs to Induce Synergistic Effect Against Multidrug Resistant Bacterial Biofilm. *Journal of Cluster Science* 28(1): 227-244 (2017).
107. Y. Zhou, Y. Kong, S. Kundu, J.D. Cirillo, and H. Liang. Antibacterial activities of gold and silver nanoparticles against Escherichia coli and bacillus Calmette-Guérin. *Journal of nanobiotechnology* 10(1): 1-9 (2012).
108. P. Boomi, G.P. Poorani, S. Selvam, S. Palanisamy, S. Jegatheeswaran, K. Anand, C. Balakumar, K. Premkumar, and H.G. Prabu. Green biosynthesis of gold nanoparticles using Croton sparsiflorus leaves extract and evaluation of UV protection, antibacterial and anticancer applications. *Applied Organometallic Chemistry* 34(5): e5574 (2020).
109. S. Saranya, K. Vijayarani, and S. Pavithra. Green

synthesis of iron nanoparticles using aqueous extract of Musa ornata flower sheath against pathogenic bacteria. *Indian Journal of Pharmaceutical Sciences* 79(5): 688-694 (2017).

110. U. Kamran, H.N. Bhatti, M. Iqbal, S. Jamil, and M. Zahid. Biogenic synthesis, characterization and investigation of photocatalytic and antimicrobial activity of manganese nanoparticles synthesized from Cinnamomum verum bark extract. *Journal of Molecular Structure* 1179: 532-539 (2019).
111. M. Senthil and C. Ramesh. Biogenic synthesis of Fe3O4 nanoparticles using tridax procurements leaf extract and its antibacterial activity on Pseudomonas aeruginosa. *Digest Journal of Nanomaterials Biostructures* 7(4) (2012).
112. S.O. Aisida, N. Madubuonu, M.H. Alnasir, I. Ahmad, S. Botha, M. Maaza, and F.I.J.a.N. Ezema. Biogenic synthesis of iron oxide nanorods using Moringa oleifera leaf extract for antibacterial applications. *Applied Nanoscience* 10(1): 305-315 (2020).
113. R. Irshad, K. Tahir, B. Li, A. Ahmad, A.R. Siddiqui, and S. Nazir. Antibacterial activity of biochemically capped iron oxide nanoparticles: A view towards green chemistry. *Journal of Photochemistry Photobiology B: Biology* 170: 241-246 (2017).
114. G. Jagathesan and P. Rajiv. Biosynthesis and characterization of iron oxide nanoparticles using Eichhornia crassipes leaf extract and assessing their antibacterial activity. *Biocatalysis agricultural biotechnology* 13: 90-94 (2018).
115. A.A. Date, M.D. Joshi, and V.B. Patravale. Parasitic diseases: liposomes and polymeric nanoparticles versus lipid nanoparticles. *Advanced drug delivery reviews* 59(6): 505-521 (2007).
116. K. Manjunath, J.S. Reddy, and V. Venkateswarlu. Solid lipid nanoparticles as drug delivery systems. *Methods Find Exp Clin Pharmacol* 27(2): 127-144 (2005).
117. S. Scioli Montoto, G. Muraca, and M.E. Ruiz. Solid lipid nanoparticles for drug delivery: pharmacological and biopharmaceutical aspects. *Frontiers in molecular biosciences* 7: 587997 (2020).
118. M. Harms and C. Müller-Goymann. Solid lipid nanoparticles for drug delivery. Journal of Drug Delivery Science Technology 21(1): 89-99 (2011).
119. E. Musielak, A. Feliczak-Guzik, and I. Nowak. Synthesis and potential applications of lipid nanoparticles in medicine. *Materials* 15(2): 682 (2022).
120. N. Naseri, H. Valizadeh, and P. Zakeri-Milani. Solid lipid nanoparticles and nanostructured lipid carriers: structure, preparation and application. *Advanced pharmaceutical bulletin* 5(3): 305 (2015).
121. C.R. Thorn, N. Thomas, B.J. Boyd, and C.A. Prestidge. Nano-fats for bugs: The benefits of lipid nanoparticles for antimicrobial therapy. *Drug Delivery Translational Research* 11(4): 1598-1624 (2021).
122. A. Walduck, P. Sangwan, Q.A. Vo, J. Ratcliffe, J. White, B.W. Muir, and N. Tran. Treatment of Staphylococcus aureus skin infection in vivo using rifampicin loaded lipid nanoparticles. *RSC advances* 10(55): 33608-33619 (2020).
123. R.S. Kalhapure, S.J. Sonawane, D.R. Sikwal, M. Jadhav, S. Rambharose, C. Mocktar, and T. Govender. Solid lipid nanoparticles of clotrimazole silver complex: an efficient nano antibacterial against Staphylococcus aureus and MRSA. *Colloids Surfaces B: Biointerfaces* 136: 651-658 (2015).
124. R.S. Kalhapure, C. Mocktar, D.R. Sikwal, S.J. Sonawane, M.K. Kathiravan, A. Skelton, and T. Govender. Ion pairing with linoleic acid simultaneously enhances encapsulation efficiency and antibacterial activity of vancomycin in solid lipid nanoparticles. *Colloids Surfaces B: Biointerfaces* 117: 303-311 (2014).
125. A. González-Paredes, L. Sitia, A. Ruyra, C.J. Morris, G.N. Wheeler, M. Mcarthur, and P. Gasco. Solid lipid nanoparticles for the delivery of anti-microbial oligonucleotides. *European journal of pharmaceutics biopharmaceutics* 134: 166-177 (2019).
126. C.L. Seabra, C. Nunes, M. Brás, M. Gomez-Lazaro, C.A. Reis, I.C. Gonçalves, S. Reis, and M.C.L. Martins. Lipid nanoparticles to counteract gastric infection without affecting gut microbiota. *European Journal of Pharmaceutics Biopharmaceutics* 127: 378-386 (2018).
127. X. Hou, T. Zaks, R. Langer, and Y. Dong. Lipid nanoparticles for mRNA delivery. *Nature Reviews Materials* 6(12): 1078-1094 (2021).
128. M.E. Gindy, B. Feuston, A. Glass, L. Arrington, R.M. Haas, J. Schariter, and S.M. Stirdivant. Stabilization of Ostwald ripening in low molecular weight amino lipid nanoparticles for systemic delivery of siRNA therapeutics. *Molecular pharmaceutics* 11(11): 4143-4153 (2014).
129. A. Masri, A. Anwar, N.A. Khan, and R. Siddiqui. The use of nanomedicine for targeted therapy against bacterial infections. *Antibiotics* 8(4): 260 (2019).

130. U. Rajchakit and V. Sarojini. Recent developments in antimicrobial-peptide-conjugated gold nanoparticles. *Bioconjugate chemistry* 28(11): 2673-2686 (2017).
131. I. Pal, D. Bhattacharyya, R.K. Kar, D. Zarena, A. Bhunia, and H.S. Atreya. A peptide-nanoparticle system with improved efficacy against multidrug resistant bacteria. *Scientific reports* 9(1): 1-11 (2019).
132. B. Casciaro, M. Moros, S. Rivera-Fernandez, A. Bellelli, M. Jesús, and M.L. Mangoni. Gold-nanoparticles coated with the antimicrobial peptide esculentin-1a (1-21) NH2 as a reliable strategy for antipseudomonal drugs. *Acta biomaterialia* 47: 170-181 (2017).
133. S. Ramesh, M. Grijalva, A. Debut, G. Beatriz, F. Albericio, and L.H. Cumbal. Peptides conjugated to silver nanoparticles in biomedicine–a "value-added" phenomenon. *Biomaterials science* 4(12): 1713-1725 (2016).
134. L.C.W. Lin, S. Chattopadhyay, J.C. Lin, and C.M.J. Hu. Advances and opportunities in nanoparticle- and nanomaterial-based vaccines against bacterial infections. *Advanced healthcare materials* 7(13): 1701395 (2018).
135. M. Anwar, F. Muhammad, B. Akhtar, M.I. Anwar, A. Raza, and A. Aleem. Outer Membrane Protein-Coated Nanoparticles as Antibacterial Vaccine Candidates. *International Journal of Peptide Research Therapeutics* 27(3): 1689-1697 (2021).
136. P. Angsantikul, S. Thamphiwatana, W. Gao, and L. Zhang. Cell membrane-coated nanoparticles as an emerging antibacterial vaccine platform. *Vaccines* 3(4): 814-828 (2015).
137. A.J. Huh and Y.J. Kwon. "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *Journal of controlled release* 156(2): 128-145 (2011).
138. V.K. Sharma, J. Filip, R. Zboril, and R.S. Varma. Natural inorganic nanoparticles–formation, fate, and toxicity in the environment. *Chemical Society Reviews* 44(23): 8410-8423 (2015).
139. I. Iavicoli, L. Fontana, V. Leso, and A. Bergamaschi. The effects of nanomaterials as endocrine disruptors. *International journal of molecular sciences* 14(8): 16732-16801 (2013).
140. S.H. Qari, A.F. Alrefaei, A.B. Ashoor, and M.H. Soliman. Genotoxicity and Carcinogenicity of Medicinal Herbs and Their Nanoparticles. *Nutraceuticals* 1(1): 31-41 (2021).
141. J. Zhao and V. Castranova. Toxicology of nanomaterials used in nanomedicine. *Journal of Toxicology Environmental Health, Part B* 14(8): 593-632 (2011).
142. D. Boraschi, L. Costantino, and P. Italiani. Interaction of nanoparticles with immunocompetent cells: nanosafety considerations. *Nanomedicine* 7(1): 121-131 (2012).
143. W.J.J.O.C.R. Abdussalam-Mohammed. Review of therapeutic applications of nanotechnology in medicine field and its side effects. *Journal of Chemical Reviews* 1(3): 243-251 (2019).
144. M. Bundschuh, J. Filser, S. Lüderwald, M.S. Mckee, G. Metreveli, G.E. Schaumann, R. Schulz, and S. Wagner. Nanoparticles in the environment: where do we come from, where do we go to? *Environmental Sciences Europe* 30(1): 1-17 (2018).
145. R. Saxena, M. Saxena, and A. Lochab. Recent progress in nanomaterials for adsorptive removal of organic contaminants from wastewater. *ChemistrySelect* 5(1): 335-353 (2020).
146. N.R. Panyala, E.M. Peña-Méndez, and J. Havel. Silver or silver nanoparticles: a hazardous threat to the environment and human health? *Journal of applied biomedicine* 6(3) (2008).
147. E. Kabir, V. Kumar, K.-H. Kim, A.C. Yip, and J. Sohn. Environmental impacts of nanomaterials. *Journal of Environmental Management* 225: 261-271 (2018).
148. A. Intisar, A. Ramzan, T. Sawaira, A.T. Kareem, N. Hussain, M.I. Din, M. Bilal, and H.M. Iqbal. Occurrence, toxic effects, and mitigation of pesticides as emerging environmental pollutants using robust nanomaterials–A review. *Chemosphere* 293: 133538 (2022).
149. L. Brannon-Peppas. Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery. *International journal of pharmaceutics* 116(1): 1-9 (1995).
150. F. Gottschalk, T. Sonderer, R.W. Scholz, and B. Nowack. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environmental toxicology chemistry* 29(5): 1036-1048 (2010).
151. M.A. Sadique, S. Yadav, P. Ranjan, S. Verma, S.T. Salammal, M.A. Khan, A. Kaushik, and R. Khan. High-performance antiviral nano-systems as a shield to inhibit viral infections: SARS-CoV-2 as a model case study. *Journal of Materials Chemistry B* 9(23): 4620-4642 (2021).
152. N. O'brien and E. Cummins. Ranking initial

environmental and human health risk resulting from environmentally relevant nanomaterials. *Journal of Environmental Science Health Part A* 45(8): 992-1007 (2010).

153. X. Yang, W. Ye, Y. Qi, Y. Ying, and Z. Xia. Overcoming multidrug resistance in bacteria through antibiotics delivery in surface-engineered nano-cargos: Recent developments for future nano-antibiotics. *Frontiers in Bioengineering Biotechnology*: 569 (2021).
154. H.M. Abdel-Mageed, A.E. Abd El Aziz, S.A. Mohamed, and N.Z. Abuelezz. The tiny big world of solid lipid nanoparticles and nanostructured lipid carriers: an updated review. *Journal of microencapsulation* 39(1): 72-94 (2022).
155. A. Alanis. Resistance to antibiotics: are we in the post-antibiotic era? *Archives of medical research* 36(6): 697-705 (2005).
156. W. Dodds, Disease now and potential future pandemics, in *The world's worst problems. 2019, Springer.* p. 31-44.
157. B. Das and S. Patra, *Antimicrobials: meeting the challenges of antibiotic resistance through nanotechnology, in Nanostructures for antimicrobial therapy.* 2017, Elsevier. p. 1-22.
158. L.D. Blackman, T.D. Sutherland, P.J. De Barro, H. Thissen, and K.E. Locock. Addressing a future pandemic: how can non-biological complex drugs prepare us for antimicrobial resistance threats? *Materials Horizons* 9(8): 2076-2096 (2022).
159. N. Zahin, R. Anwar, D. Tewari, M. Kabir, A. Sajid, B. Mathew, M. Uddin, L. Aleya, and M.M. Abdel-Daim. Nanoparticles and its biomedical applications in health and diseases: special focus on drug delivery. *Environmental Science Pollution Research* 27(16): 19151-19168 (2020).
160. M. Harun-Ur-Rashid, T. Foyez, I. Jahan, K. Pal, and A.B. Imran. Rapid diagnosis of COVID-19 via nano-biosensor-implemented biomedical utilization: a systematic review. *RSC advances* 12(15): 9445-9465 (2022).
161. C. Xu, C. Lei, S. Hosseinpour, S. Ivanovski, L.J. Walsh, and A. Khademhosseini. Nanotechnology for the management of COVID-19 during the pandemic and in the post-pandemic era. *National Science Review* 9(10): nwac124 (2022).
162. L. Krejcova, P. Michalek, M.M. Rodrigo, Z. Heger, S. Krizkova, M. Vaculovicova, D. Hynek, V. Adam, and R. Kizek. Nanoscale virus biosensors: state of the art. *Nanobiosensors in Disease Diagnosis* 4: 47-66 (2015).
163. S. Hua, E. Marks, J.J. Schneider, and S. Keely. Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. *Nanomedicine: nanotechnology, biology medicine* 11(5): 1117-1132 (2015).
164. Y.H. Choi and H.-K. Han. Nanomedicines: current status and future perspectives in aspect of drug delivery and pharmacokinetics. *Journal of Pharmaceutical Investigation* 48(1): 43-60 (2018).
165. M.S. Mufamadi, *Nanotechnology shows promise for next-generation vaccines in the fight against COVID-19*. 2020, Springer.
166. C. Constantin, A. Pisani, G. Bardi, and M. Neagu. Nano-carriers of COVID-19 vaccines: the main pillars of efficacy. *Nanomedicine* 16(26): 2377-2387 (2021).
167. R.D. Handy, R. Owen, and E.J.E. Valsami-Jones. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. 17(5): 315-325 (2008).
168. A. León-Buitimea, J.A. Garza-Cervantes, D.Y. Gallegos-Alvarado, M. Osorio-Concepción, and J.R. Morones-Ramírez. Nanomaterial-based antifungal therapies to combat fungal diseases aspergillosis, Coccidioidomycosis, Mucormycosis, and candidiasis. *Pathogens* 10(10): 1303 (2021).
169. N. Jan, N. Majeed, M. Ahmad, W.A. Lone, and R. John. Nano-pollution: Why it should worry us. *Chemosphere:* 134746 (2022).
170. S. Patil and R. Chandrasekaran. Biogenic nanoparticles: A comprehensive perspective in synthesis, characterization, application and its challenges. *Journal of Genetic Engineering Biotechnology* 18(1): 1-23 (2020).

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Review Article

Antiviral Pills against SARS-CoV-2 Virus to Combat Future Epidemic Threats in Pakistan

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Abstract: Antiviral pills are oral medications that treat infections through the inhibition of the viral growth and replication cycle. Paxlovid by Pfizer and Molnupiravir by Merck are the two pills effective for high-risk SARS-CoV-2 patients. Paxlovid works by inhibiting the replication cycle of SARS-CoV-2 using cysteine residues in-vitro. These cysteine residues inhibit the main protease of the virus by functioning as reversible covalent inhibitors. Molnupiravir works by introducing a high rate of mutations in the viral RNA causing the virus to become biologically unstable and non-functional. Both antiviral drugs can bridge the gap in the preparedness for viral outbreaks in low-income countries like Pakistan by mitigating the chances of fatality and inpatient treatment in high-risk, unvaccinated individuals. Pakistan has been plagued by various epidemics over the years however SARS-CoV-2 outbreak caused many deaths along with an economic crisis. The country lacks the resources to endure high inpatient treatment rates in case of SARS-CoV-2 infections, which is why the need for antiviral pills like Paxlovid and Molnupiravir is empirical to overcome epidemics and viral outbreaks. This work outlines the antiviral pills and their efficacy against SARS-CoV-2 with a focus on how these drugs can overcome significant gaps in epidemic preparedness and response in Pakistan. We aim to highlight how antiviral pills against SARS-CoV-2 can ensure resilience to future epidemic threats in Pakistan.

Keywords: SARS-CoV-2, Antiviral pills, Paxlovid, Molnupiravir, Epidemic, Pakistan

1. INTRODUCTION

The Middle East Respiratory Syndrome, Severe Acute Respiratory Syndrome, and other viral breakouts in the past have been caused by viruses of the Coronaviridae family [1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) had been identified as the source of a group of pneumonia cases reported in Wuhan, China, in December 2019 [1, 2]. This viral infection quickly spread around the world and was shortly classified as a worldwide pandemic by World Health Organization (WHO) [2–5]. In Pakistan, this pandemic has posed serious concerns regarding the country's weak healthcare infrastructure as the frequency of SARS-CoV-2 patients rose significantly. In order to prevent SARS-CoV-2, various vaccine types, including mRNA-based vaccines and viral vector vaccines, have been developed. While appropriate vaccinations have been approved to combat severe SARS-CoV-2 infections, the immediate requirement for orally administered antiviral drugs particularly designed to target SARS-CoV-2 persists [6, 7].

Vaccinations provide optimal protection against SARS-CoV-2, however, it is a challenge to vaccinate highly populous low-income countries. It is also difficult to transport and store vaccines in remote villages with limited resources. The Food and Drug Administration (FDA) has granted emergency use authorizations (EUAs) for a number of biological products & pharmaceuticals against SARS-CoV-2, such as Kineret, Evusheld, Actemra, Propofol-Lipuro 1 %, Baricitinib, and COVID-19 convalescent plasma, however, these authorizations are only allowed for inpatient treatments [8]. Following the outbreak, developing orally administered antivirals that can be consumed by outpatients became a top focus [7]. Two novel oral antiviral pills were approved by the US Food and Drug Administration (FDA)

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in 2021 for the treatment of outpatients with minimal to severe COVID-19 symptoms [9]. These two antiviral pills are Paxlovid (Pfizer) and Molnupiravir (Merck) which aim to reduce the frequency of reported deaths and the need for inpatient treatments [10].

The ribonucleoside analogues Beta-D-N4-hydroxycytidine (NHC), which is then modified intracellularly to its activated state Molnupiravirtriphosphate (MTP), is the main component in Molnupiravir. It functions by targeting RNA-dependent RNA polymerase (RdRp), an important enzyme in SARS-CoV-2 RNA replication and translation machinery. Molnupiravir introduces mutations in the viral RNA causing the virus to become biologically unstable and non-functional. On the contrary, Pfizer developed the antiviral pill nirmatrelvir marketed under the name, Paxlovid [11]. Paxlovid inhibits SARS-CoV-2 replication using cysteine residues in vitro. The cysteine residues inhibit the main protease of the virus by functioning as reversible covalent inhibitors. The description of the different characteristics of Paxlovid and Molnupiravir are illustrated in Table 1.

2. MATERIALS AND METHODS

Electronic databases such as Google Scholar, PubMed, and Science Direct had been browsed for publications dated between January 2020 to November 2022 with keyword phrases including "Paxlovid", "Molnupiravir'', "COVID-19", "SARS-CoV-2" "Antiviral pill", "MK-4482", "SARS-CoV-2", "EIDD-280", "nirmatrelvir plus ritonavir" "PF07321332", "Efficacy", "Safety" and "Pakistan''. The publications reporting the medical testing and experimental research on Molnupiravir and Paxlovid as antivirals against SARS-CoV-2 published between 2019 to 2022 met the criteria for inclusion. Non-relevant studies, non-English papers, and full-text unavailability were among the exclusion parameters. The process flow diagram for selecting publications for this article is given in (Figure 1).

3. PATHOPHYSIOLOGY OF SARS CoV-2

The four primary structurally important proteins found in coronaviruses are the spike, membrane, envelope, and nucleocapsid proteins. Extracellular matrix metalloproteinase inducer (EMMPRIN) and angiotensin-converting enzyme 2 are two examples of host cell surface receptors coronaviruses can use for entering human cells. RNA-dependent RNA polymerase (RdRp) controls viral RNA replication inside host machinery used for the proliferation of highly mutagenic and diverse virions [12]. Following the first interaction with coronaviruses,

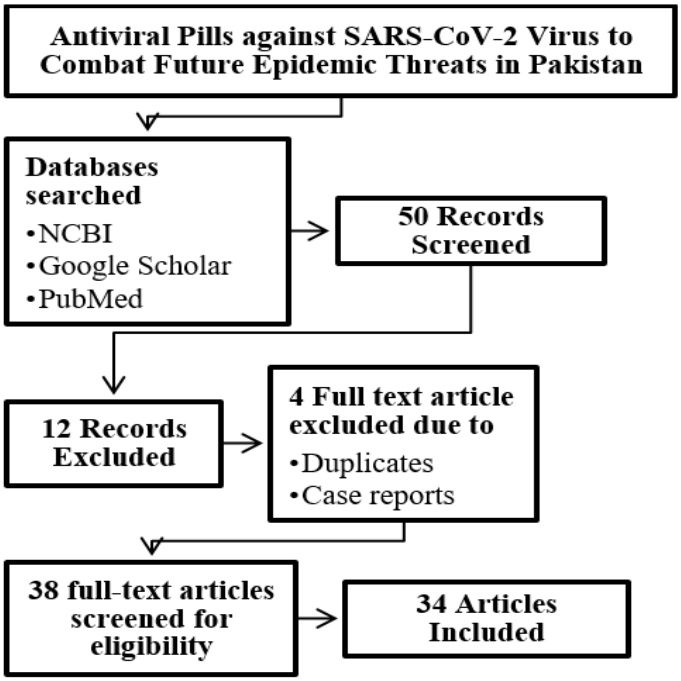

Fig. 1. The publication selection process summarized as a flow diagram.

Table 1. Different characteristics of Molnupiravir and Paxlovid

Characteristics	Molnupiravir	Paxlovid
Synonyms	Lagevrio; EIDD-2801; MK-4482; Molnupiravirum; Eidd 1931-isopropyl ester; WHO 11853	Nirmatrelvir; PF07321332; nirmatrelvir plus ritonavir; 7R9A5P7H32; WHO 12161
Class	Antivirals; esters; hydroxylamines; pyrimidinones; ribonucleosides; small molecules	Carboxamides; cyano compound; pyrrolidones; amines; small molecules; aza compounds; carbamate; fluorinated hydrocarbons; heterocyclic bicyclic compound; thiazoles
Mode of Action	SARS-CoV-2 RNA-dependent RNA polymerase inhibitor	Coronavirus-3C-like-proteinase inhibitor (nirmatrelvir); CYP3A-mediated metabolism of nirmatrelvir inhibitor (ritonavir)
Route of Administration	Oral	Oral
Pharmacodynamics	Molnupiravir hydrolyzes in cells to form N4-hydroxycytidine, it phosphorylates to Molnupiravir triphosphate and incorporates in viral RNA causing inactivating mutations, leading to new unstable virions.	Nirmatrelvir: attaches to the active site of SARS-CoV-2 main protease and blocks it, preventing it from processing polyproteins and halting viral multiplication. Ritonavir: prevents CYP3A-mediated nirmatrelvir metabolism, raising levels of nirmatrelvir in plasma; does not have activity towards SARS-CoV-2 main protease.
Adverse Effects	Diarrhoea, dizziness, headache, redness of the skin, vomiting, nausea.	Diarrhea, parageusia, high blood pressure, muscle ache
WHO ATC codes	J05 (Antivirals for Systemic Use)	J05 (Antivirals for Systemic Use)
EphMRA ATC code	J5B9 (Antivirals, others)	J5 (Antivirals for Systemic Use)
Molecular Formula	$C_{13}H_{19}N_3O_7$	$C_{23}H_{32}F_3N_5O_4$
Chemical formula	[(2R,3S,4R,5R)-3,4-dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidin-1-yl] oxolan-2-yl] methyl 2-methylpropanoate	(1R,2S,5S)-N-[(1S)-1-cyano-2-[(3S)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2S)-3,3-dimethyl-2-[(2,2,2-trifluoroacetyl)amino]butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide/1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate

interferons, immunoglobulins, and natural killer cells trigger an immune response. T lymphocytes, macrophages, and polymorphonuclear leukocytes, that have infiltrated the alveoli during the severe phases of SARS-CoV-2 infection produce cytokines like cachectin and interleukins IL-1, IL-6. Acute respiratory distress syndrome (ARDS) and multi-organ malfunction are caused by high levels of cytokines [11]. Because tissue factor is overexpressed in secondary hemostasis, hyper-inflammation is often linked to thrombophilia.

Following five days of incubation, clinical symptoms of SARS-CoV-2 begin to develop.

Pyrexia, coughing, along with exhaustion are the more common signs, but there are many others including respiratory phlegm, headaches, coughing up blood, diarrhea, lymphocytopenia, and dyspnoea. Upper and lower respiratory signs notably sternutation, pharyngitis, and runny nose, are indicative of SAR-CoV-2's distinct clinical symptoms. Contrary to MERS-CoV and SARS-CoV patients, who exclusively have diarrhea, people infected with SARS-CoV-2 generally exhibit diarrhea as well as other stomach problems [13]. SARS-CoV-2 spreads from individual to individual via aerosol particles and drops. When a virus enters the body it binds to the cell receptors of the host, and enters by either fusing with cell membrane or endocytic activity [14]. Transmission of the SARS-CoV-2 virus causes the activation of antigen-presenting cells such as accessory cells and phagocytes by pro-inflammatory substances. Major histocompatibility complexes I and II (MHC I and II) activate cellular and humoral response mediated by B and T lymphocytes against the SARS-CoV-2 antigens. As a result, antibodies and cytokines are produced. Increased virus multiplication in later phases of the disease compromises the stability of the epithelium and endothelium. Cell death and depletion of surface-active agents are brought on by the virus's invasion of type II pneumocytes in the lower respiratory system. Alveolar interstitial thickness increased vascular porosity, and edema is all results of ongoing inflammatory reactions. Hyaline membranes may develop in the alveolar cavities as a result of pulmonary edema.

Alveolar deterioration and failure are the outcomes of all these biochemical abnormalities, which in turn limits the exchange of gases [15].

4. MODE OF ACTION OF ORAL ANTIVIRALS AGAINST SARS-COV-2

Both Molnupiravir and Paxlovid target the SARS-CoV-2 Virus in different ways meaning that both drugs have different mechanisms of action that target the virus and halt its replication.

4.1 Molnupiravir

The medicine Molnupiravir, formerly identified as EIDD-2801, is an isopropyl ester class of drug produced using the ribonucleoside analogue Beta-D-N4-hydroxycytidine (NHC). It is subsequently transformed within cells into an activated state known as Molnupiravirtriphosphate (MTP). It works by inhibiting RNA-dependent RNA polymerase (RdRp), a key enzymatic protein involved in the SARS-CoV-2 RNA replication and translation process. This makes RdRp a prospective target protein for SARS-CoV-2 treatment [16, 17]. Molnupiravir can be orally administered due to higher bioavailability, unlike other monoclonal antibody therapy and ribonucleoside analogues that require in-patient intravenous dosing [18-20].

Molnupiravir works by preventing the enzymatic protein RdRp from functioning. This enzyme is among the Sixteen non-structural proteins (nsp), and it has an important role in catalyzing the viral RNA replicating machinery from existing templates. The activated state of Molnupiravir, Molnupiravirtriphosphate (MTP), is produced in the body and utilized as a substrate for RdRp. The host mitochondrial RNA polymerase could potentially integrate MTP as either cytosine or uracil analogue. When nucleoside triphosphate (NTP) and Molnupiravirtriphosphate (MTP) are present, SARS-CoV-2 RdRp incorporates MTP into the sub-genomic RNA or negative-stranded genome during positive-stranded genome transcription rather than cytosine or uracil nucleotide bases. In order to create a mutant positive-stranded genome and sub-genomic mitochondrial RNA, the acquired negative-stranded RNA containing MTP could be subsequently utilized as templates. Because of the existence of MTP in the negative-stranded genome, it results in mutations in the positive-stranded RNA, which therefore inhibits the development of functioning viral RNA and stops the virus from proliferating. Such a process is often renowned as an "error catastrophe" Molnupiravir has also been linked to genome alterations in humans by mammalian cell culturing studies [19, 21]. Figure 2 illustrates Molnupiravir's mode of action.

4.2 Paxlovid

The novel antiviral medication Paxlovid, formerly known as nirmatrelvir, was created by Pfizer [11]. The antiviral candidate Paxlovid is a modified version of lufotrelvir [22]. Lufotrelvir is a phosphate warhead-containing antiviral protease inhibitor [23]. On the contrary, Paxlovid was created for oral

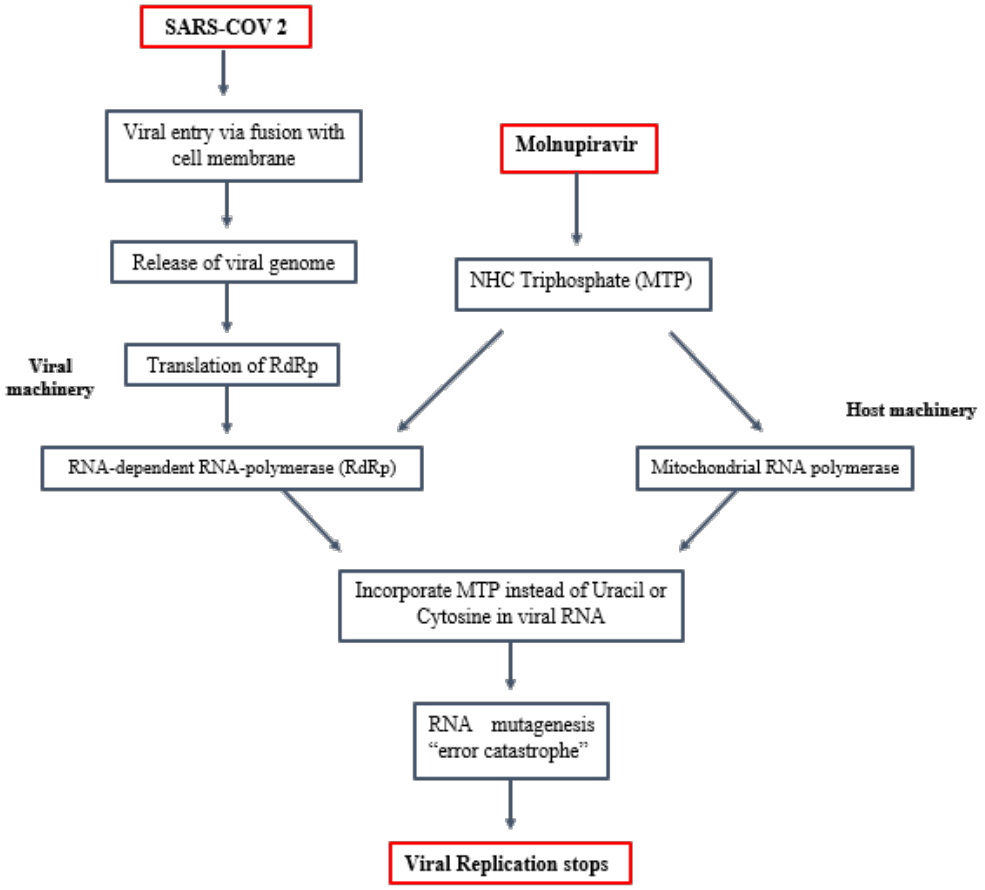

Fig. 2. Flow chart illustration of Molnupiravir mode of action

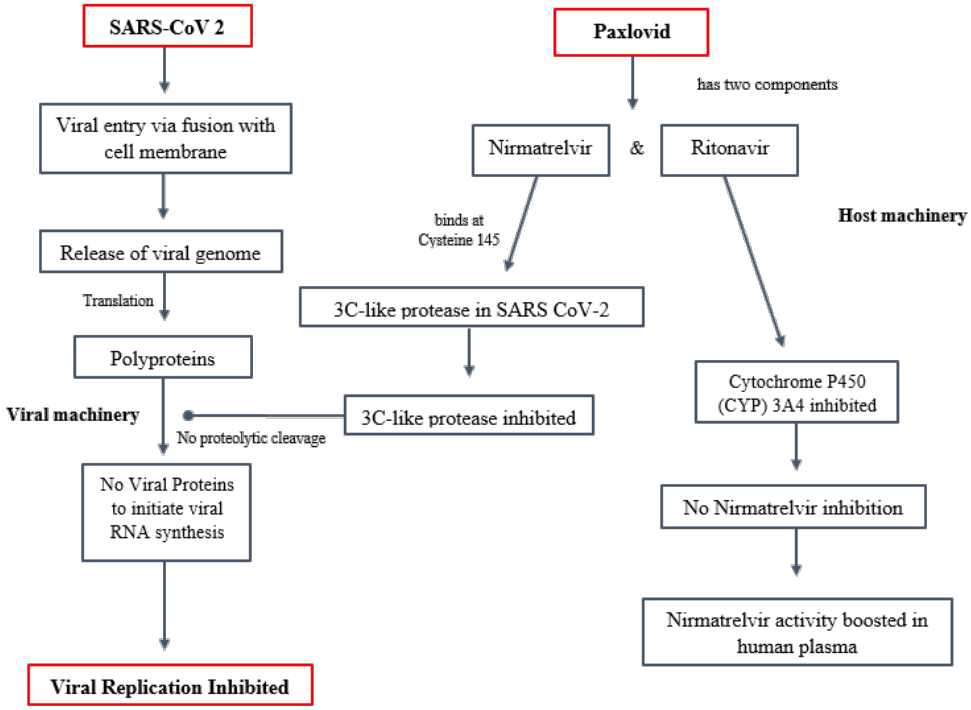

Fig. 3. Flow chart illustration of the mode of action of Paxlovid.

delivery by altering a tripeptide protein mimicking lufotrelvir [11].

Nirmatrelvir binds directly to the 3C-like protease (also referred to as SARS-CoV-2 Mpro active site) reversibly and exclusively hinders 3C-like protease activity by direct attachment to Cys145 [24]. Such type of protein inhibition prevents virus proliferation because the enzyme fails to digest polyproteins [16, 25]. When nirmatrelvir and ritonavir are given jointly, ritonavir pharmacokinetically enhances nirmatrelvir's activity and increases its half-life in the body.

Although ritonavir inhibits the protease activity of HIV, it cannot inhibit SARS-CoV-2 3C-like protease, which is why it is only used as an enhancer. It is reported to increase nirmatrelvir plasma concentration levels through the inhibition of cytochrome P450 (CYP) 3A4 inhibitor-mediated metabolism of nirmatrelvir [16, 25]. The increased absorption of Paxlovid appeared to become lower than the dose-proportional to steady state followed by two orally administered doses every day of Paxlovid (75 milligrams + 100 milligrams, 250 milligrams + 100 milligrams, and 500 milligrams + 100 milligrams) [10, 16]. By the second day, steady-state had reached (accumulation ≈ 2-fold). The median periods to maximum concentration levels of ritonavir were 3.98 h and nirmatrelvir were 3.00 h in normal and fit participants following one dosage of nirmatrelvir 300 milligrams with ritonavir 100 milligrams. Paxlovid could be taken with or without meals, however consuming it with fatty meals slightly enhanced nirmatrelvir absorption compared to fasting conditions [10, 16]. The mechanism of action of Paxlovid is given (Figure 3).

5. THERAPEUTIC TRIALS

5.1 Molnupiravir

From the four trials using Molnupiravir, the first phase of the research had been conducted on 130 competent participants to determine the drug's tolerability as well as the effective dosage range. The drug was proven to be non-toxic and generally tolerable. The uptake had not been affected in the fed state and the studied dosages were between 50 to 800 milligrams (maximum standard dosage 1600 milligrams/ day). administered twice for 5.5 days. When contrasted to the Molnupiravir cohort, the frequency of side effects had been greater compared to placebo; it was 43.8 % versus 35.4 % for one dosage and 50 % versus 42.9 % for several ascending dosages, correspondingly. In a single ascending dosage trial, headaches were most frequently observed, whereas diarrhea was more frequently observed in multiple ascending dose research [4].

The AGILE trial had been a Phase Ib/IIa dosage increment investigation that included 18 SARS-CoV-2 infected individuals. Individuals who had been infected with SARS-CoV-2 within 120 hours after the development of symptoms were grouped and arbitrarily assigned three dosage cohort groups (300, 600, and 800 milligrams) containing six individuals, respectively. According to the findings, minor side effects in individuals who got 800 milligrams (25 percent) and individuals who got normal treatment (83 percent). Molnupiravir 800 milligrams two times per day had been deemed to be nontoxic and tolerable, as plasma concentrations were in between the range [20].

For the Phase IIa research, 202 non-immunized individuals affected with SARS-CoV-2 disease were included. The individuals were given Molnupiravir doses of 200, 400, and 800 milligrams. In contrast to the placebo group (18 %), the initial antibody level was greater (35 %). The timeframe for viral inactivation was considerably shortened (14 to 15 days) with 800 milligrams doses of Molnupiravir in comparison to the placebo, but not using Molnupiravir 200 milligrams and 400 milligrams. In individuals who tested negative for antibodies, the viral inactivation was shortened (14 to 27 days). On day 28, the viral inactivation with 800 milligrams of Molnupiravir was at 92.5 %, 400 milligrams at 91.3 %, 200 milligrams at 78.7 %, and placebo at 80.3 %. In the Molnupiravir cohort, viral isolation from nasopharynx samples was much lower than in the placebo; on day 3 it was 1.9 % vs. 16.7 % and on day 5, it was 0 % vs. 11.1 %. A substantial change in both Molnupiravir dosages of 800 milligrams and 400 milligrams were observed [27].

The median duration for resolving SARS-CoV-2 disease was 8 days (6.0 to 12.0) for the

Molnupiravir 800 milligrams group versus 8 and a half days (7.0 to 11.0) for the placebo at a confidence interval of 95 percent. Individuals who were not admitted to the hospital and had low to intermediate symptoms, as well as a single determinant at minimum for the development of acute disease, participated in the MOVe-OUT experiment, a phase III research investigation. The overall probability of inpatient treatment for any reason or having passed away by day 29 had been found to be reduced with Molnupiravir (7.3 % [28 of 385 individuals]) in comparison to placebo (14.1 % [53 of 377 participants]) within the ITT cohort. The observed change was -6.8 percent at a confidence interval of 95 (-11.3 to -2.4; p = 0.001). Elevated viral titers (greater than 106 viral copies per milliliters) at the start were associated with relative risk differences of -5.4 (-9.9, -1.0) at CI 95 % [17].

The Molnupiravir cohort experienced only 6.3 % vs. 9.2% of SARS-CoV-2 related inpatient treatments or fatalities as contrasted with the placebo (difference, 2.8 percentage points; 95 percent confidence interval, 5.7 to 0.0), according to a previously defined supporting assessment. On day 29, one fatality was recorded in the Molnupiravir cohort whereas nine deaths were observed in the placebo. In 216 of 710 individuals present in the Molnupiravir cohort and 231 out of 701 in the placebo experienced negative side effects. In 77.6 % of nasopharynx swabs, measurable viral genome had been identified in the beginning. The nasopharynx SARS-CoV-2 levels changed on average by -0.33 at a 95 % confidence interval (0.50, 0.16) across a period [25].

5.2 Paxlovid

In the randomized, double-blind, placebo-controlled, phase II/III EPIC-HR study (NCT04960202), Paxlovid was efficient in lowering the likelihood of acute SARS-CoV-2 infection in high-risk adults having SARS-CoV-2 symptoms [12]. Individuals needed a single determinant at minimum for acquiring acute SARS-COV-2 infection, a COVID-positive test, and SARS-CoV-2 symptoms starting within five days after randomization to participate in the EPIC-HR study. Blood sugar, cardiac disease, autoimmune disease, high blood pressure, malignant tumors, obesity, severe respiratory, renal illness, being older than 60 years, etcetera were constituted as risk factors. Individuals who received a vaccine or were previously infected with SARS-COV-2 were not eligible for this trial.

EPIC-HR's cumulative effectiveness findings were similar to the findings of the planned interim assessment [12]. The modified intention-to-treat population was 697 in Paxlovid and 682 in placebo cohorts. Kaplan-Meier approximated the number of incidences of SARS-CoV-2 inpatient treatments or fatality on the 28th day to be 0.72 % with Paxlovid as compared to placebo which was at 6.53 % with a 95 % confidence interval, suggesting a reduced risk of hospitalization to 88.9 %. Paxlovid decreased the need for SARS-CoV-2 inpatient treatment or fatality due to any reason through day 28 by 87.8 percent compared to the placebo in individuals who were administered Paxlovid within five days of the onset of disease and had not received SARS-CoV-2 monoclonal antibodies. Modified intention-to-treat group had a population of 1039 while the placebo and Paxlovid groups had a population of 1046 in the Paxlovid and placebo cohort. The approximate incident rates were 0.78 % compared to 6.40 %. These rates have been comparable to those in the modified intention-to-treat groups when findings among all individuals given the drug within five days of the symptoms started (also those who got or were anticipated to get monoclonal antibodies) were evaluated. The effectiveness of Paxlovid had been the same in the modified intention-to-treat cohort 1 regardless of the categories grouped according to age (less and greater than 65 years), gender, diabetes, body mass index (underweight, normal weight, overweight, obese), time for the appearance of symptoms at the start of medication (less and greater than 3 days), and standard serological tests for the detection of SARS-CoV-2. Paxlovid was linked to approximately 0.9 log10 viral copies per milliliters decline in SARS-CoV-2 genome titers on the fifth day in assessable modified intention-to-treat patients versus placebo (p 0.001), and a comparable outcome had been observed in assessable modified intention-to-treat cohort 1 patient [12].

In an intermediate assessment out of the phase II/III Evaluation of Protease Inhibition for COVID-19 in Standard-Risk Patients study (NCT05011513) in outpatient, normal risk group having lab serological

tests confirming SARS-CoV-2, Paxlovid had no substantially reduced SARS-CoV-2 symptoms in comparison to the placebo group [11]. Those without vaccinations who were at a minimal probability of inpatient treatment or dying were also part of this study, along with individuals over 18 who were immunized but had a single determinant at minimum for COVID-19 progression. Paxlovid or a placebo was given to individuals randomly after twelve hours for five days. Enrollment was only at 45 % of what was anticipated at the point of intermediate assessment. Paxlovid failed to achieve the main objective of prolonged, self-reported relief from SARS-CoV-2 disease manifestations all for 4 days consecutively compared to placebo. Nevertheless, compared to the placebo, Paxlovid resulted in a 70 percent decrease in inpatient treatment as well as a drop in SARS-CoV-2 titers up to almost 10 times. After randomization, 2 out of 333 individuals in the Paxlovid cohort were admitted to the hospital, but there had been no cause-related fatalities. Only 329 patients in the placebo cohort 8 had been hospitalized but there were no fatalities. The remaining participants (80 %) verified the initial assessment's findings. On the other hand, 3 out of 428 patients were admitted for inpatient treatment after randomization, while 10 out of 426 placebo group patients were admitted to the hospital with no sustained fatality [11]. In comparison to other innovative medications, Paxlovid pills deliver the most optimistic clinical result reducing inpatient treatment and fatality by up to 89 % [26, 28]. It is one of the most anticipated methods to overcome epidemic threats [29–31].

6. ANTIVIRAL PILLS AGAINST EPIDEMIC THREATS IN PAKISTAN

Pakistan is always at an increased risk of transmission of vector-borne diseases, infectious diseases, or epidemics due to economic instability and climate change. COVID-19 is one of the outbreaks which resulted in serious threats. Pakistan's present situation is unsatisfactory because it has a large population and lesser healthcare amenities. Pakistan is a third-world country with lower economic resources to deal with the SARS-CoV-2 epidemic in comparison to the United States, China, Russia, and the United Kingdom. The needed numbers of healthcare facilities are unfortunately not being met, which is why controlling the spread of diseases and providing inpatient care is a challenge for Pakistan's government [32].

When it comes to vaccine hesitancy, Pakistan is not much different from the rest of the world. It is one of the two countries in the world, which could not eradicate poliovirus due to vaccine hesitancy prevalent in its population. Unfortunately, the COVID-19 vaccine has also come in the crosshairs of conspiracy theories and misperceptions in Pakistan and the saner voices have fallen prone to an astigmatic mentality [33].

The desire for better solutions, vaccine hesitancy, and better patient convenience from a less intrusive treatment led to the search for chemical molecules that could be administered as oral pills. Recently, Molnupiravir (EIDD-2801) and Paxlovid (PF-07321332), two potent oral antiviral pills were developed by Merck and Pfizer respectively [34].

7. CONCLUSION

As with several other respiratory illnesses, it is necessary to recall that immunization itself might not provide guaranteed protection, necessitating novel antiviral pills against SARS-CoV-2. In certain outpatient clinics, orally administered antiviral pills could be utilized effectively in the early phases of COVID-19 disease. Two highly effective orally administered antiviral pills for SARS-CoV-2 at the moment appear to be Paxlovid and Molnupiravir [35]. Approved medications for SARS-CoV-2 dramatically minimize mortality rate and inpatient care for individuals with light to moderate symptoms, according to initial investigations [36]. Both antiviral drugs can bridge the gap in the preparedness for viral outbreaks in low-income countries like Pakistan where vaccine hesitancy is one of the leading factors in combating disease. Lastly, possible impacts on cartilage and bones, observation of negative effects, as well as potential mutagenicity should be taken into consideration in the long run in prospective clinical studies of Paxlovid and Molnupiravir [35].

8. CONFLICT OF INTEREST

The authors declared that there is no conflict of interest and all authors contributed to the review and are liable

for the contents of the article.

9. REFERENCES

1. S. Tripathy, B. Dassarma, S. Roy, H. Chabalala, M. G. Matsabisa. A review on possible modes of action of chloroquine/hydroxychloroquine: repurposing against SAR-CoV-2 (COVID-19) pandemic. *International Journal of Antimicrobial Agents* 56: 2 (2020).
2. F. Wu, S. Zhao, B. Yu, Y.M. Chen, W. Wang, Z.G. Song, Y. Hu, Z.W. Tao, J.H. Tian, Y.Y. Pei, M.L. Yuan, Y.L. Zhang, F.H. Dai, Y. Liu, Q.M. Wang, J.J. Zheng, L. Xu, E.C. Holmes, and Y.Z. Zhang. A new coronavirus associated with human respiratory disease in China. *Nature* 579: 265–269 (2020).
3. European Centre for Disease Prevention and Control. Outbreak of acute respiratory syndrome associated with a novel coronavirus, China: first local transmission in the EU/EEA – third update. *ECDC: Stockholm* (2020).
4. C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, L. Zhang, G. Fan, J. Xu, X. Gu, Z. Cheng, T. Yu, J. Xia, Y. Wei, W. Wu, X. Xie, W. Yin, H. Li, M. Liu, Y. Xiao, H. Gao, L. Guo, J. Xie, G. Wang, R. Jiang, Z. Gao, Q. Jin, J. Wang, and B. Cao. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet* 395: 497–506 (2020).
5. K. Yuki, M. Fujiogi, and S. Koutsogiannaki. Covid-19 pathophysiology: A Review. *Clinical Immunology* 215 (2020).
6. E. Mathieu, H. Ritchie, E.O. Ospina, M. Roser, J. Hasell, C. Appel, C. Giattino and L. R. Guirao. A global database of COVID-19 vaccinations. *Nature Human Behaviour* 5: 947–953 (2021).
7. H. Fan, F. Lou, J. Fan, M. Li, Y. Tong. The emergence of powerful oral anti-covid-19 drugs in the post-vaccine era. *The Lancet Microbe* 3:2 (2022)
8. Emergency use authorization, U.S. Food and Drug Administration (2023). https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization (accessed 27 October 2022).
9. S. Rahman, M. Din, S. Khan, L. Yar, F. Zia, M. Idrees, and M. Ali. COVID-19 Vaccines: Pakistan's Perspective: COVID-19 Vaccines: Pakistan's Perspective. *Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences* 58: 11-17 (2021).
10. M. Kozlov. Why scientists are racing to develop more covid antivirals. *Nature* 601: 496–496 (2022).
11. D.R. Owen, C.M.N. Allerton, A.S. Anderson, L. Aschenbrenner, M. Avery, S. Berritt, B. Boras, R.D. Cardin, A. Carlo, K.J. Coffman, A. Dantonio, L. Di, H. Eng, R. Ferre, K.S. Gajiwala, S.A. Gibson, S.A. Greasley, B.L. Hurst, E.P. Kadar, A.S. Kalgutkar, J.C. Lee, J. Lee, W. Liu, S. W. Mason, S. Noell, J.J. Novak, R.S. Obach, K. Ogilvie, N.C. Patel, M. Petterson, D.K. Rai, M.R. Resse, M.F. Sammons, J.G. Sathish, R.S.P . Singh, C.M. Steppan, A.E. Stewart, J. B. Tuttle, L. Updyke, P.R. Verhoest, L. Wei, Q. Yang, and Y. Zhu. An oral SARS-COV-2 Mpro inhibitor clinical candidate for the treatment of COVID-19. *Science* 374: 1586–1593 (2021).
12. L. Zhang, D. Lin, Y. Kusov, Y. Nian, Q. Ma, J. Wang, A. V. Brunn, P. Leysenn, K. Lanko, J. Neyts, A. D. Wilde, E. J. Snijder, H. Liu and R. Hilgenfeld. A-ketoamides as broad-spectrum inhibitors of coronavirus and enterovirus replication: Structure-based design, synthesis, and activity assessment. *Journal of Medicinal Chemistry* 63: 4562–4578 (2020).
13. H.A. Rothan, and S.N. Byrareddy. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. Journal of Autoimmunity 109 (2020).
14. A. Parasher. Covid-19: Current understanding of its pathophysiology, clinical presentation and treatment. *Postgraduate Medical Journal* 97: 312–320 (2020).
15. F. Sanchez, J.G. Calvo, G. Mery, Y.E. Garcia, P. Vasquez, L.A. Barboza, M.D. Perez and T. Rivas. A multilayer network model of covid-19: Implications in public health policy in Costa Rica. *Epidemics* 39 (2022).
16. M. Imran, M.K. Arora, S.M.B. Ashdaq, S.A. Khan, S.I. Alaqel, M.K. Alshammar, M.M. Alshehri, A.S. Alshrari, A.M. Ali, A.M. Al Shammeri, B.D. Alhazmi, A.A. Harshan, Md. T. Alam, and Abida. Discovery, development, and patent trends on Molnupiravir: A prospective oral treatment for covid-19. *Molecules* 26:19 (2021).
17. F. Pourkarim, S.P. Anvarian, and H. Rezaee. Molnupiravir: A new candidate for Covid-19 treatment. *Pharmacology Research & Perspectives* 10:1 (2021).
18. C.X. Li, S. Noreen, L.X. Zhang, M. Saeed, P.F. Wu, M. Ijaz, D.F. Dai, I. Maqbool, A. Madni, F. Akram, M. Naveed, and J.H. Li. A critical analysis of SARS-COV-2 (COVID-19) complexities, emerging variants, and therapeutic interventions and vaccination strategies. *Biomedicine &*

Pharmacotherapy 146 (2022).

19. B. Malone, and E.A. Campbell. Molnupiravir: Coding for catastrophe. *Nature Structural & Molecular Biology* 28: 706–708 (2021).
20. A. Pouramini, F. Kafil, S. Hassanzadeh, S. Saifar, and H. R. Jhantigh. Molnupiravir; an effective drug in treating covid-19? *Journal of Preventive Epidemiology* 7:1 (2021).
21. F. Kabinger, C. Stiller, J. Schmitzova, C. Dienemann, G. Kokic, H.S. Hillen, C. Hobrtner, and P. Cramer. Mechanism of Molnupiravir-induced SARS-CoV-2 mutagenesis. *Nature Structural & Molecular Biology* 28: 740–746 (2021).
22. M. Marzi, M.K. Vakil, M. Bahmanyar, and E. Zarenezhad. Paxlovid: Mechanism of action, synthesis, and in silico study. *BioMed Research International* 2022 (2022).
23. R.L. Hoffman, R.S. Kania, M.A. Brothers, J.F. Davies, R.A. Ferre, K.S. Gajiwala, M. He, R.J. Hogan, K. Kozminsk, L.Y. Li, J.W. Lockner, J. Lou, M.T. Marra, L.J. Mitchell Jr., B.W. Murray, J.A. Nieman, S. Noell, S.P. Planken, T. Rowe, K. Ryan, G.J. Smith III, J.E. Solowiej, C.M. Steppan, and B. Taggart. Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. *Journal of Medicinal Chemistry* 63: 12725–12747 (2020).
24. M. Pavan, G. Bolcato, D. Bassani, M. Sturlese, and S. Moro. Supervised molecular dynamics (SUMD) insights into the mechanism of action of SARS-COV-2 main protease inhibitor PF-07321332. *Journal of Enzyme Inhibition and Medicinal Chemistry* 36: 1645–1649 (2021).
25. K. Anand, J. Ziebuhr, P. Wadhwani, J.R. Mesters, and R. Hilgenfeld. Coronavirus main proteinase (3CL pro) structure: Basis for design of anti-SARS drugs. *Science,* 300: 1763–1767 (2003).
26. Y.P. Hung, J.C. Lee, C.W. Chiu, C.C. Lee, P.J. Tsai, I.L. Hsu, and W.C. Ko. Oral nirmatrelvir/ritonavir therapy for covid-19: The dawn in the dark? *Antibiotics* 11:2 (2022).
27. J. Hammond, H.L. Tebbe, A. Gardner, P. Abreu, W. Bao, W. Wisemandle, M. Baniecke, V.M. Hendrick, B. Damle, A.S. Campos, R. Pypstra, and J.M. Rusnak. Oral Nirmatrelvir for high-risk, nonhospitalized adults with covid-19. *New England Journal of Medicine* 386: 1397–1408 (2022).
28. J.C. Frankel. Antiviral pills could change pandemic's course. *Science* 374: 799–800 (2021).
29. K. Fernando, S. Menon, K. Jansen, P. Naik, G. Nucci, J. Roberts, S. S. Wu, and M. Dolsten. Achieving end-to-end success in the clinic: Pfizer's learnings on R&D productivity. *Drug Discovery Today* 27: (2022).
30. E.G. McDonald, and T.C. Lee. Nirmatrelvir-ritonavir for COVID-19. *CMAJ* 194:6 (2022).
31. B. Malone, and E.A. Campbell. Molnupiravir: Coding for catastrophe. *Nature Structural & Molecular Biology* 28: 706–708 (2021).
32. A. Waris, U.K. Atta, M. Ali, A. Asmat, and A. Basit. COVID-19 outbreak: current scenario of Pakistan. *New Microbes and New Infections* 35 (2020).
33. M. Kashif. Perceptions and COVID-19 Vaccine Hesitancy among Pakistani Physiotherapists; A Qualitative Insight. *Pakistan Journal of Medical and Health Sciences* 15: 2302–2313 (2021).
34. P.O. Oladosu, N. Moses, O.P. Adigwe, and H.O. Egharevba. Potentials of medicinal plants with antiviral properties: The need for a paradigm shift in developing novel antivirals against COVID-19. *Journal of Advances in Medicine and Medical Research* 35 85–100 (2021).
35. S. Khiali, E. Khani, S.B. Rouy, and T. E. Maleki. Comprehensive review on Molnupiravir in COVID-19: a novel promising antiviral to combat the pandemic. *Future Microbiology* 17: 377–391 (2022).
36. T. Islam, M. Hasan, M.S. Rahman, and Md. R. Islam. Comparative evaluation of authorized drugs for treating Covid-19 patients. *Health Science Reports* 5: 4 (2022).

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Research Article

Public Health Laboratories Response to SARS-COV-2 Diagnostic Testing during COVID Pandemic in Pakistan

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Abstract: During COVID-19 Pandemic, diagnostic laboratories played a vital role in outbreak investigation, surveillance, patient monitoring, and therapeutic effectiveness, and hampered the transmission cycle globally. In Pakistan, the health department took an initiative to build BSL-III labs at divisional levels. A qualitative study was conducted among healthcare professionals from 13 major public health national-level laboratories through in-depth interviews with key informants to note down the challenges they faced during the COVID-19 pandemic during diagnostic testing. In this study, 77 % of public health laboratories faced sampling, administrative, and leadership issues. 53 % of laboratories have faced the unavailability of well-trained staff and human resources while both the biosafety and biosecurity protocols, and the lack of resources were compromised in 69 % of labs. Some lab staff (54 %) felt the wastage of resources in terms of excessive testing and fake sampling, while others (54 %) discussed a lack of training and work experience issues. As the majority of the technical lab staff was hired in temporary consultancy mode so 61 % of issues were related to late salaries. 38 % of issues were about fake reporting pressure from higher authorities. 69 % had issues with the continuous supply chain of kits, reagents, PPEs, etc. The work environment was not up to the mark of 69 %. High workload and mental health issues were faced by 92 %, while waste management was 23 %, shortage of lab space for massive testing by 38 %, and stigma and discrimination among healthcare workers and the general public due to involvement in COVID-19 testing were felt by 46 %.

Keywords: COVID-19, Pakistan, Healthcare system strengthening, Challenges of outbreak, Pandemic, Lesson Learned

1. INTRODUCTION

Human health remains at risk worldwide due to emerging infectious diseases. Scientists and researchers are always on the verge of war by coping with the multiple outbreaks of emerging infectious pathogens and their multiple serotypes like influenza, corona, dengue, chikungunya, cholera, tuberculosis, and many others right from the beginning of this world. In recent years, mortality rate percentages are declining due to good public health strategies, but still many new infectious diseases have been identified and registered, including Hantavirus pulmonary syndrome, AIDS, Ebola and Legionnaire virus [1].

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused a recent novel coronavirus disease outbreak that emerged in Wuhan China and became a pandemic in no time. Due to health emergencies, several national health organizations faced public health crisis which was threatening to world. A total of 4820714 infected cases were reported on the 18th of May, and the mortality rate was more than 316998 (7 %) [2]. In the last 2 decades, the world has faced 3 outbreaks of coronavirus; SARS-CoV-1 in 2003, MERS-CoV in 2012, and SARS-CoV-2 pandemic in 2019. To fight against such outbreaks and pandemics, the most practical and sensible approach is a robust response in form of early diagnosis and preventive strategies. For this purpose, better advanced diagnostic

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laboratories and effective surveillance programs are needed of the hour. Diagnostic laboratories play a pivotal role in outbreaks investigations through rapid detection of virus infection, rapid serological assays, and advanced molecular diagnostic techniques [3, 4]. These laboratories reported quick results and played a very important role in concluding the outbreak.

It is very important to break the transmission chain of COVID-19 in the population in the first place by diagnosis, reducing the number of suspects, and reducing the basic reproductive number. The viral transmission is decreased by controlling various factors such as COVID-19-positive individuals, infection severity, and viral shedding [5]. Due to compromised facilities of vaccination and treatment, the only possible method to decrease transmission of COVID-19 is the isolation of infected individuals to prevent transmission of the disease. Diagnostic testing for COVID-19 has been varying differently around the globe. The COVID-19 pandemic outbreak exposed some major vulnerabilities, limitations, and gaps in the healthcare departments of many countries.

Few developed countries in Asia showed prompt responses to control the COVID-19 pandemic. Singapore performed a wide screening program on patients with influenza, pneumonia, patients in ICU, and deaths with possible infection [6]. South Korea controlled the COVID-19 outbreak through extraordinary efforts of national testing and managed to perform 3 lac tests in the first 9 weeks after they diagnosed the first case of COVID-19 [7]. Hong Kong and Taiwan did the same job [8]. They implemented resource-intensive strategies that promoted diagnostic testing and isolation strategies to prevent transmission [8]. In the pressure of rapid transmission of COVID-19 across borders, the diagnostic testing was dependent on the type of test available, resources, and time of test results. Because the suspected cases were on high priority to assign them isolation to prevent spread in the community. Different diagnostic tests for COVID-19 are available and many more getting approval every day [9, 10]. Different diagnostic tests were available in Pakistan to cope with COVID-19 diagnosis such as, R-T PCR, LAMP, Lateral flow and ELISA. To fight with pandemic public health labs have been in deep need of reliable, accurate and fast testing for COVID-19 as given in Table 1. Graphical representation of the total number of tests performed by public sector laboratories of Pakistan shown in figure 1.

During the COVID pandemic, all molecular testing labs have had multiple diagnostic challenges worldwide [11]. The diagnostic labs face challenges when not only their healthcare workers are required

Table 1. Public Sector Laboratories and number of COVID-19 tests performed by laboratories during the COVID-19 pandemic in Pakistan (Reference: COVID-19 PORTAL HISDU Primary and Secondary Healthcare Department P&SHD)

S. No.	Public Sector Labs of Punjab Province Pakistan	Location	Covid-19 Tests Performed
1	Provincial Public Health Reference Laboratory, Lahore, Punjab, Pakistan	Lahore	3800000
2	National Institute of Health Islamabad	Islamabad	248799
3	Lahore General Hospital Laboratory	Lahore	51927
4	Jinnah Hospital Laboratory	Lahore	23320
5	Tb Bsl-3 Laboratory Lahore	Lahore	277637
6	University of Veterinary and Animal Sciences (Bsl-3)	Lahore	341244
7	Institute Of Public Health Lahore	Lahore	235510
8	Benazir Bhutto Hospital (BBH) Rawalpindi	Rawalpindi	557188
9	Allied Hospital Faisalabad	Faisalabad	940212
10	Trauma Centre (Bsl-3) Wazirabad	Wazirabad	397578
11	Nishtar Medical College	Multan	220104
12	KSMC Sialkot	Sialkot	240407
13	SHAHEEN Bsl-3 Lab Sargodha	Sargodha	507854
	Total No. of Tests		**79,41,780**

to deal with a high workload due to the increasing number of patients due to high transmission of an infection but also their own life remains at risk due to dealing with infectious pathogens [12, 13]. Diagnostic laboratories' fragility is significantly magnified during this pandemic. Reliability and accuracy of results remained a matter of concern for health authorities as false positive and false negative results not only affect the patient's health but can bring damage to public health policies, quality testing, emergency plans efficiency and pandemic control preventive measures [14].

In the present study, we tried to figure out the challenges faced by molecular-based public health labs during the COVID-19 pandemic in resource-limited settings. These challenges and lessons learned can be of utmost importance for any new pandemic preparedness and response. The knowledge of current diagnostic technologies and associated gaps in public health labs of developing countries can have a serious impact on global outbreaks.

This knowledge gap can be filled at the national level by a thorough assessment of issues faced by different diagnostic laboratories to tackle the COVID-19 pandemic. Identification of these gaps can halt the hidden deficiencies of any healthcare system and can facilitate removing those by ensuring improvements in the fight against any new pandemics such as COVID-19 global emergency.

2. METHODOLOGY

To access Pakistan's health system response to COVID-19 a qualitative large study was conducted. The data was collected using open-ended questions among healthcare professionals from 13 major public health national-level laboratories followed by in-depth interviews (IDI) among professionals. Collectively these laboratories performed almost 8 million SARS-CoV-2 PCR tests till now (from 1st March 2020 till 20th September 2022) which is such a huge number and contribution to the healthcare system of Pakistan in terms of tackling this COVID-19 severe pandemic and infection risks among the population. So, these labs also faced issues regarding preparedness and responses which are discussed in this study.

This study was conducted in two phases. In the first phase, open-ended questions among professionals were conducted to find out gaps related to Punjab Pakistan's health system response to COVID-19. According to the Strategic Preparedness and Response Plan (SPRP) from WHO COVID-19, different questions about 8 important points of the healthcare system were asked which include Country coordination, monitoring and planning, Risk communications and management, Surveillance, Entry points, National laboratories, Control and Prevention, Management of cases and Support & logistics [15]. Questions regarding these points were asked among a few healthcare

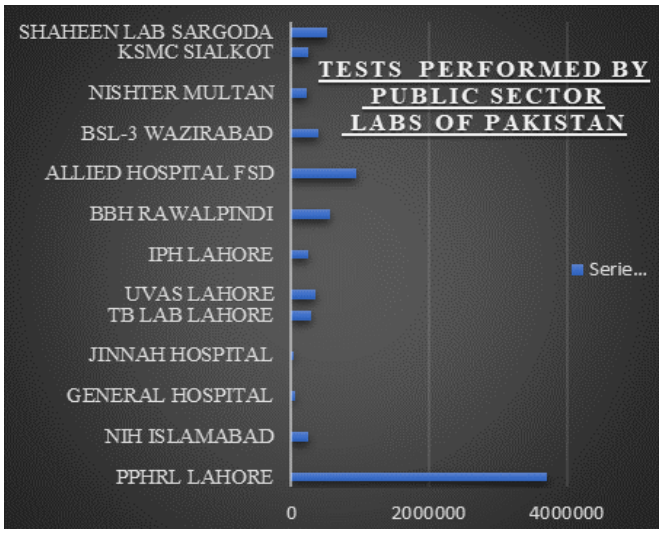

Fig. 1. Graphical representation of the total number of tests performed by public sector laboratories of Pakistan

professionals with various backgrounds to obtain their feedback for further improvement.

2.1 Data Collection

This study aimed to collect data from respondents from different laboratories with different experiences. The consent of respondents was taken before they participate in this study.

Respondents were allowed to share their experiences during the COVID-19 pandemic relevant to their background and expertise without any barrier. All responses were noted down in detail.

Few respondents based on their diverse backgrounds and expertise in the health system e.g. Health specialists, Lab in charge, public health specialists, focal persons, and health community representatives participated in this study. They also participated in the second phase of in-depth interviews.

2.2 Analysis of Data

The responses to open-ended questions were checked and analyzed. The content was arranged and content analysis was done independently by the research team followed by a series of group discussions. Gaps regarding health systems' preparedness and response during COVID-19 were marked, discussed, and noted.

3. RESULTS AND DISCUSSION

There are huge numbers of pre-analytical, analytical, and post-analytical challenges that are needed to be taken into consideration by public health authorities, medical lab technologists, and clinical microbiology laboratories to give accurate results shown in figure 2. It appears that the pre-analytical phase is the main source of errors in diagnostic laboratories accounting for approximately 46 % to 68.2 % of errors [16], It is observed despite continuous improvements during the whole testing process. Pre-analytical errors can result in compromised patient care, magnified financial burden, and unnecessary investigation in the healthcare system resulting in a compromised healthcare system [17].

3.1 COVID-19 Sample Collection Issues

During the Covid-19 pandemic, good sample collection materials and sample collection methods remained a hot topic as patients with COVID-19 had a high reservoir of the virus in their upper and lower respiratory tracks. Nasopharyngeal and oropharyngeal swabs are recommended for this purpose. According to the literature, nasopharyngeal swabs become a better option because they reach the exact area to be tested in the nasal cavity. But in China, oral swabs were mostly used instead of nasopharyngeal swabs frequently during the pandemic. The limit of detection accuracy from oral swabs is 30 % and from nasal swabs is 70 %. So, the COVID-19 infection detection with oral swabs was 32 %, and with nasal swabs was 63 %. Another reason to limit the sampling with nasal swabs was the unavailability of nasal swabs and viral transport media in Pakistan and also in those areas where extensive testing was required. Sampling issues which have been reported by different diagnostic labs were 77 % which contributed to compromised quality of diagnostic testing. These issues are an inappropriate and inadequate collection of the sample, sample mishandling, insufficient viral transport media in sampling vial, inappropriate conditions of sample transportation, prolonged sample storage, sample storage false temperature maintenance, presence of substances in the sample which can interfere with the such as cellular components due to additives and protocols issues occurring during sample preparation, manual sample preparation, pipetting issues, sample mismatch, inhibition and cross-contamination [18-20].

3.2 Leader Ship Issues

Governance and leadership are important components which influence the functioning of all main health systems. They depend on decisions made by the stakeholders in response to a pandemic [21-23]. There have been less effective coordination and strategic vision towards achieving a common goal, within and beyond the health systems among all the leadership. Seventy-seven percent of leadership and governance issues are reported from national labs of Pakistan because of high pressure from leadership, high workload, no appreciation, mismanagement, and less effective coordination.

Fig.2. Percentage of challenges in Laboratory Diagnosis faced by major public health laboratories of Pakistan

3.3 Biosafety, Biosecurity, and Lack of Resources Issues

Biosafety, biosecurity, and insufficient availability of resources issues are reported in 69 % of different national laboratories in Pakistan. This is a huge percentage level. Both of things are very important for qualitative working place. We should be prepared for coming pandemics to not repeat the same mistakes and should learn from the COVID-19 pandemic. Adding to this around 54 % of resources have been wasted. There must be a balance in the availability of resources in every lab to manage lack and waste of resources issues.

3.4 Mental Health, Salaries, and Workload Issues

Working under pressures in long duty shifts, getting exposed to COVID-19 infection, seeing people dying every day because of severe infection, leadership pressure, high workload, mismanagements, no salaries on time, short-term job contracts, fears of losing jobs, lack of resources and PPEs affected the mental health of health care workers to higher levels which are 92 %. This is a serious drawback of a pandemic. Mental health is very important for good well-being but in case, but if it affects a huge level the health of an individual is at risk. So, to prevent mental health issues a happy, motivated, and appreciative environment should be given to healthcare workers. Forty-six percent of the stigma of the population has been reported for healthcare workers who work in testing labs and 38 % of issues reported were related to late salaries.

Workload should be balanced between shifts and different labs working on the national level. Few labs receive very few samples and others receive thousands. The equal distribution of samples is very important in all the labs working to prevent workload. Ninety-two percent workload issue has been reported from national labs which is huge.

3.5 Assay Selection Issues

Amplification methods and deep sequencing methods played an important role in SARS-COV-2 diagnosis. Right after the SARS-CoV-2 outbreak, molecular diagnostic approaches were considered robust and primary diagnostic techniques for its detection but no one was sure what technique they should use for appropriate diagnosis. However, these approaches became a severe challenge for resources limited countries due to cost and shortages of diagnostic kits after its global outbreak. Thirty-eight percent of assay selection challenges were faced by different national laboratories in Pakistan. Molecular methods of deep sequencing e.g., next-generation sequencing and metagenomics now need

the hour for the identification of future variants of SARS-CoV-2. RT-PCR technique is mostly used to reduce time and cost. Multiple molecular methods are also in practice such as Multiplex isothermal amplification, loop-mediated isothermal amplification, microarray detection, and CRISPR (Clustered regularly interspaced short palindromic repeats) [18-20, 24].

3.6 Issues Related to False Results

In Pakistan, 38 % of labs reported the issue of false results. Molecular diagnostic techniques always remained the gold standard assays in the majority of infectious diseases diagnosis. RT-PCR method occasionally gives false positive and false negative results. But in the case of SARS-CoV-2, the situation remained twisted. Sometimes PCR results did not match with patients' signs and symptoms, leading to misunderstanding of false assay performance. On the other hand from a lab management perspective, quality test performance, kits reliability, and issues in sample collection are doubted. Nevertheless, these false results claim always lead to under or over-diagnosis of disease. A false positive result can have many consequences, it does not only lead to unnecessary treatment of patients but can also lead to social problems, as it makes the working of health professionals a question who are working in public laboratories facilities. In retrospect, a false negative result not only contributes to more spread of COVID-19 in the community but also to the patient's health remains at risk without any treatment. The precise and reliable results of diagnostic tests play a vital role in the diagnosis and management of the COVID-19 outbreak.

3.6.1 False Negative Results Issues

It is important to make precise and accurate approaches to the diagnosis of COVID-19 due to its high infection rate. False-negative results have harmful epidemiological effects to contain the outbreak and transmission of infection [25]. It is vital to reduce the number of false negative results for cohorts of patients in hospitals and determination of quarantine measures. Due to false negative results some patients who are hospitalized for other conditions unknowingly carry SARS-CoV-2. There is a need to differentiate recovered patients and silent carriers of SARS-CoV-2. This will help hospital management to sort out patients whom to discharge and whom to hospitalize.

3.7 Covid-19 Testing Results in Interpretation Issues

United States molecular diagnostic labs consider a sample confirm positive when initially both two targets N1 and N2 in CDC assay are present. A Cycle Threshold (CT) value less than 40 is defined as a positive test and 40 or more than 40 is considered as a negative test. CT value <40 of one of both targets, the test is considered as determinant and requires retesting for confirmation. In China, the assays for three targets, when there are two or more targets are positive, the test is considered positive. There have been some wrong practices during results interpretations due to a lack of experience, training, and CT value knowledge in laboratory staff. Low CT values for high viral load approaches should be used for results interpretation.

3.8 Laboratory Design Issues

Pakistan's government established BSL-3 laboratories at district levels due to a high number of patient samples during the COVID-19 pandemic. Each district designed its BSL-3 level lab in available resources and space. Healthcare workers reported many issues regarding lab design and space. Some labs were having huge spaces and some were very congested and inconvenient to work in them. Thirty-eight percent of labs reported this issue in Pakistan.

3.9 Test of Cure and Infectivity Issues

After recovering from COVID-19 disease and coming out from isolation the monitoring of patients with resolution of covid-19 pneumonia is not being done. It is a major concern as such patients enhance viral transmission. Even the discharged patients are shedding viable coronavirus and there are chances of transmission exist. It is recommended that the patient should remain isolated for at least 1 month. Two consecutive COVID-19 PCR tests with confirmed negative results should be conducted after cure and needs further investigation.

To increase diagnostic efficacy different approaches should be taken to overcome these

Table 2. Percentages of challenges and gaps reported by different healthcare laboratories.

Percentage of Labs	Gaps/Challenges
77 %	Sampling Issues
77 %	Administrative & Leadership Issues
53 %	Unavailability of Well-Trained Staff
53 %	Unavailability of Human Resource
69 %	Lack of Resources
69 %	Biosafety and Biosecurity issues
54 %	Wastage of Resources
92 %	Mental Health Issues
38 %	Shortage of Space
54 %	Fake Sampling
54 %	Lack of Training and Experience
61 %	Late Salaries
38 %	Fake Reporting Pressure
69 %	Supply chains of Kits, PPEs & Reagents
69 %	Work environment issues
92 %	High Work load
23 %	Waste Management
46 %	Stigma & Discrimination among HCW

challenges and gaps. Selecting the most appropriate source of sampling, as CDC recommends the nasopharyngeal swabs sampling which gives more accurate results [25-27]. Multiple diagnostic techniques should be used for confirmed results and to decrease the false negative results rate. Establishing a combined workflow of serological testing will help to achieve multidimensional, high-quality, reliable, and cost-effective diagnostic approaches for COVID-19 testing. The diagnostic testing should be done at different time points throughout the disease from hospitalization to weekly intervals [28] to decrease transmission. Governance and leadership are important components that expect to have effective coordination and strategic vision towards achieving a common goal, within and beyond the health systems among all the leadership. Strategic and timely coordination and communication is the main catalyst for resource optimization and distribution of workload. The expansion of laboratory testing capacity and enhancement in technology utilization through collaborative efforts between government agencies, universities, and industries would enable fast disease detection with diagnostic measures. With a centralized administrative system, COVID-19 responses such as health workforce mobilization and implementation of standardized operating procedures at all levels could be well-coordinated and synchronized effectively. However, such organized and managed commands could potentially restrict the local governments from making timely decisions for the best of their communities.

4. CONCLUSION

COVID-19 pandemic has dramatically highlighted the critical role of diagnostic technologies in the control of infectious diseases. The availability of established diagnostic technologies, which took decades to develop and optimize, has enabled scientists to plug and play in the design of SARS-CoV-2 diagnostics [29]. Different gaps and challenges have been faced by public health laboratories during the pandemic. Measures against the ongoing pandemic should be taken properly. Strategies should be established which are easier to administer and cover the challenges of pandemic diagnostic challenges. There is now a call for the development of ways to be rapidly implemented according to public health needs against any pandemic. Finally, the blinding speed with which SARS-CoV-2 has spread illustrates the need for preparedness and long-term investments in diagnostic testing.

5. CONFLICT OF INTEREST

No conflicts of interest between the authors and members of the potential conflicts of interest, counseling, expertise, working conditions, shareholding, and similar situations in any firm.

6. DECLARATION

The results of this manuscript are original. The same material is neither published nor under consideration elsewhere. The approval of all authors has been obtained before publication. In case the article is accepted for publication, its copyright will be assigned to the Pakistan Academy of Sciences. Authors must obtain permission to reproduce, where needed, copyrighted material from other sources and ensure that no copyrights are infringed upon.

7. REFERENCES

1. R. Joshi, R. Lodhe, S. Agrawal, and A. Jain. Hospital based infectious disease related proportional mortality study. *Journal of Global Infectious Diseases* 6: 10 (2014).
2. Info W: Coronavirus Update (2020) https://www.worldometers.info/coronavirus/ (accessed on 28 August 2020).
3. Z. Song, Y. Xu, L. Bao, L. Zhang, P. Yu, Y. Qu, H. Zhu, W. Zhao, Y. Han, and C. Qin. From SARS to MERS, thrusting coronaviruses into the spotlight. *Viruses* 11: 59 (2019).
4. R. Parreira. Laboratory methods in molecular epidemiology: viral infections. *Microbiology Spectrum* 6: 6-6 (2018).
5. P.L. Delamater, E.J. Street, T.F. Leslie, Y.T. Yang, and K.H. Jacobsen. Complexity of the basic reproduction number (R0). *Emerging Infectious Diseases* 25: 1 (2019).
6. K.D. Zhuang, A. Patel, B.S. Tan, F.G. Irani, A. Gogna, S.X. Chan, S.K. Sanamandra, T.T. Chong, S.P. Chng, and K.H. Tay. Outcome and distal access patency in subintimal arterial flossing with antegrade-retrograde intervention for chronic total occlusions in lower extremity critical limb ischemia. *Journal of Vascular and Interventional Radiology* 31: 601-606 (2020).
7. J.Y. Song, J.G. Yun, J.Y. Noh, H.J. Cheong, and W.J. Kim. Covid-19 in South Korea—challenges of subclinical manifestations. *New England Journal of Medicine* 382: 1858-1859 (2020).
8. Z. Li, Y. Yi, X. Luo, N. Xiong, Y. Liu, S. Li, R. Sun, Y. Wang, B. Hu, and W. Chen. Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *Journal of Medical Virology* 92: 1518-1524 (2020).
9. G. Webb, M.J. Blaser, H. Zhu, S. Ardal, and J. Wu. Critical role of nosocomial transmission in the Toronto SARS outbreak. *Mathematical Biosciences & Engineering* 1: 1 (2004).
10. T. Wong, T. Wallington, L.C. McDonald, Z. Abbas, and M. D.E. Christian, Low, D. Gravel, M. Ofner, B. Mederski, L. Berger. Late recognition of SARS in nosocomial outbreak, Toronto. *Emerging Infectious Diseases* 11: 322 (2005).
11. G. Lippi, and M. Plebani. A Six-Sigma approach for comparing diagnostic errors in healthcare—where does laboratory medicine stand? *Annals of Translational Medicine* 6: (2018).
12. C. Sheridan. Coronavirus and the race to distribute reliable diagnostics. *Nature Biotechnology* 382-384 (2020).
13. G. Lippi, M. Plebani, and M.L. Graber. Building a bridge to safe diagnosis in health care. The role of the clinical laboratory. *Clinical Chemistry and Laboratory Medicine* 54: 1-3 (2016).
14. G. Lippi, and M. Plebani. Laboratory abnormalities in patients with COVID-2019 infection. *Clinical Chemistry and Laboratory Medicine* 58: 1131-1134 (2020).
15. Organization WH: COVID-19 strategic preparedness and response plan: operational planning guidelines to support country preparedness and response. (2020).
16. M. Plebani. Errors in clinical laboratories or errors in laboratory medicine? *Clinical Chemistry and Laboratory Medicine* 44: 750-759 (2006).
17. M. Plebani, and P. Carraro. Mistakes in a stat laboratory: types and frequency. *Clinical Chemistry* 43: 1348-1351 (1997).
18. M. Espy, J. Uhl, L. Sloan, S. Buckwalter, M. Jones, E. Vetter, J. Yao, N. Wengenack, J. Rosenblatt, and F. Cockerill III. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Reviews* 19: 165-256 (2006).
19. T.R. Matos, M.A. de Rie, and M.B. Teunissen. Research techniques made simple: high-throughput sequencing of the T-cell receptor. *Journal of Investigative Dermatology* 137: e131-e138 (2017).
20. G.V. Zyl, J. Maritz, H. Newman, and W. Preiser. Lessons in diagnostic virology: expected and

unexpected sources of error. *Reviews in Medical Virology* 29: e2052 (2019).

21. L.O. Gostin, C.C. Mundaca-Shah, and P.W. Kelley. Neglected dimensions of global security: the global health risk framework commission. *Journal of the American Medical Association* 315: 1451-1452 (2016).
22. J. Hanefeld, S. Mayhew, H. Legido-Quigley, F. Martineau, M. Karanikolos, K. Blanchet, M. Liverani, E.Y. Mokuwa, G. McKay, and D. Balabanova Towards an understanding of resilience: responding to health systems shocks. *Health Policy and Planning* 33: 355-367 (2018).
23. J.V. Olmen, B. Marchal, W.V. Damme, G. Kegels, and P.S. Hill. Health systems frameworks in their political context: framing divergent agendas. *BMC Public Health* 12: 1-13 (2012).
24. G. Lippi, G. Lima-Oliveira, G. Brocco, A. Bassi, and G.L. Salvagno. Estimating the intra-and inter-individual imprecision of manual pipetting. *Clinical Chemistry and Laboratory Medicine* 55: 962-966 (2017).
25. H. Feng, Y. Liu, M. Lv, and Zhong J: A case report of COVID-19 with false negative RT-PCR test: necessity of chest CT. *Japanese Journal of Radiology* 38: 409-410 (2020).
26. S.M. Jung, A.R. Akhmetzhanov, K. Hayashi, N.M. Linton, Y. Yang, Yuan B, Kobayashi T, Kinoshita R, and H. Nishiura. Real-time estimation of the risk of death from novel coronavirus (COVID-19) infection: inference using exported cases. *Journal of clinical Medicine* 9: 523 (2020).
27. Clinical and virologic characteristics of the first 12 patients with coronavirus disease 2019 (COVID-19) in the United States. *Nature Medicine* 26: 861-868 (2020)
28. Mizumoto K, Kagaya K, Zarebski A, and Chowell G: Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Eurosurveillance* 25: 2000180 (2020).
29. K.K.W. To, O.T.Y. Tsang, C.C.Y. Yip, K.H. Chan, T.C. Wu, J.M.C. Chan, W.S. Leung, T.S.H. Chik, C.Y.C. Choi, and D.H. Kandamby. Consistent detection of 2019 novel coronavirus in saliva. *Clinical Infectious Diseases* 71: 841-843 (2020).

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Research Article

High Burden of Multidrug-Resistant Bacteria Detected in Different Water Sources can Spread the Antibiotic Resistance Genes in the Environment

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Abstract: Antibiotic-resistant bacterial infections are of global concern nowadays. Environmental sources like water and soil are playing a key role in spreading antibiotic-resistance genes to humans, animals, and other environments. Objective: The purpose of this study was to identify and report the presence of multidrug-resistant bacteria (MDRs) in environmental water sources that can direct the spread of resistant genes to other bacteria/environments. Methodology: Environmental water samples were collected from 2 livestock farms and a fish pond. Bacterial isolation and identification were carried out by following Burgey's manual of systematic bacteriology. Antibiotic susceptibility testing was done using the disk diffusion method and CLSI guidelines. Multiple antibiotic-resistant indexes were calculated. Whole genome sequences of previously reported bacteria were downloaded from NCBI to detect the resistance genes associated with phenotypic drug resistance and compared using the bioinformatics approach. Results: Microbial load was significantly high in all water sources. Following Genera were the most common: *Klebsiella, Escherichia, Proteus, Serratia, Acinetobacter, Enterobacter, Pseudomonas, Bacillus, Lactobacillus*, and *Staphylococcus*. Out of 10 classes of antibiotics, resistance against 8 classes were identified. Multiple Antibiotic Resistance (MAR) index range of isolated strains was between 0.4 and 0.9. Key Findings: Resistance against beta-lactam antibiotics was highest in our isolated strains with a MAR index of greater than 0.4 altogether. Conclusion: High burden of multidrug-resistant bacteria were detected in all water samples which can trigger the silent pandemic of antibacterial resistance.

Keywords: Antibiotic Resistance, Antibiotic Resistant Bacteria (ARBs), Antibiotic Resistance Genes (ARGs), Beta-lactam antibiotics, ESKAPE pathogens, MAR Index, Penicillins.

1. INTRODUCTION

Antimicrobial resistance (AMR) is increasing at an alarming pace in bacteria causing a major threat to existing options for antibiotics treatment. An enormous increase in antibiotic-resistant bacteria and antibiotic-resistance genes (ARGs) are universally found in human and animal infections and also in contaminated environments. This led to the emergence of a new term "Silent Pandemic of Antibiotic Resistance" [1]. Resistance to even the last regimes of antibiotics has been developed leaving very limited options or on occasion with no options at all making it impossible to treat antibiotic-resistant bacterial infections globally [2]. With each passing year, the number of deaths occurred by antibiotic-resistant bacterial infections is increasing significantly [3].

In 2019, nearly 4.95 million deaths have occurred due to antibiotic-resistant bacterial infections solely [4]. This number will increase exponentially in coming years and according to Balasegaram, this rise in infections due to antibiotic-resistant superbugs will leave humans with no choice even to treat very common bacterial infections in near future [5]. According to O'Neill, 2016, this silent pandemic will lead to more and more loss of precious human lives. In absence of any effective control measures, this pandemic

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will lead to around 10 million deaths and over 100 trillion dollars in monetary loss globally by 2050 [1]. According to research done by RAND Cooperation, the world population would have been 11-444 million more in absence of AMR as it would be in presence of drug-resistant superbugs in 2050 [1].

Mostly six (6) bacteria are involved in causing the deaths of humans in clinical cases named ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species). In 2019, ESKAPE pathogens have caused more than 1 million human deaths worldwide. [6]. The acquisition of resistance genes by these bacteria have been reported internationally to reduce the available antibiotic options to treat clinical infections [7]. These bacteria acquire resistance genes mostly through horizontal gene transfer mechanisms when present in a favorable environment along with a little role played by vertical transmission of genes from parents to daughter cells [5].

Intensive use of antibiotics as growth promoters and prophylactic use in animal farming, aquacultures, and clinical use of antibiotics in humans has been proposed to be the most common means of antibiotic resistance development and dissemination [1]. COVID-19 pandemic has also played a vital role in elevating drug resistance, as excessive and unnecessary use of antiseptics and sterilizers expedited the ARB propagation [8].

The unnecessary use and incomplete regimens of antibiotics in animals and humans lead to the survival of ARBs in presence of sub-lethal doses of antibiotics favoring the resistant superbugs to survive. These superbugs can then spread to humans through animal waste contaminated water, soil, and other environments where humans are in contact with animals and also by contaminated food animals like fish, cattle, etc. [1].

Epidemiological records of AMR are very imperative for AMR surveillance, for policy makers to ploy effective strategies, and to check the success of control measures adopted to combat AMR. Most of the recent and past studies are focused on clinical AMR cases. For effective surveillance of AMR, detection of ARBs and their antibiotic resistance patterns in different environmental sources is imperative to control the antibiotic resistance pandemic in the future. In this paper, we have investigated the presence of ARBs in contaminated water sources including fish pond water and drinking water of livestock rearing farms in Lahore Pakistan. This paper has helped us in pinpointing the presence of similar ARGs in pathogenic as well as in non-pathogenic bacteria confirming the spread of ARGs from environmental bacteria to human/ animal pathogens through HGT events.

2. MATERIALS AND METHODS

2.1 Water Sampling Sites

Water samples were collected from 3 different sources. One sample was collected from a fish pond located in Lahore while the other 2 samples were collected from small local livestock farms (drinking water samples of cattle) located at two different localities in the outskirts of Lahore. Topographical plots of all the sample collection sites (Figure 1).

2.2 Physical and Chemical Parameters of Water Samples

The sample's pH, temperature, colour, and odour were checked at sampling sites while taking samples. Sampling bottles containing samples were kept in ice containers before shipping to the lab for further analysis. On reaching the lab, the sample's electrical conductance (E.C), turbidity, and total dissolved solids were checked.

Chemical parameters of water quality testing were also checked to determine the safety of water samples for drinking by livestock animals.

2.3 Microbiological Testing

For microbiological analysis, samples were collected in sterile bottles. Serial dilutions of each sample were made and 100 µl of each dilution was spread on N-Agar plates in triplicate. These plates were incubated at 37 °C for 24 hours. After incubation, the number of colonies in each plate was recorded and the average at each dilution was calculated. Colony morphology of each unique and single colony on all plates was recorded and these marked colonies were further purified by using the quadrant streaking method.

2.4 Gram Staining and Biochemical Tests

Gram staining was done to find out the bacterial shape and gram's reaction. Smears were made, stained, and analyzed under a light microscope using an oil immersion lens. Based on gram staining results, each isolated strain was identified biochemically up to the genus level using Bergey's manual of systematic bacteriology.

2.5 Antibiotic Susceptibility Testing (AST)

Each identified strain was tested against at least 4 or more antibiotics classes following the Kirby Bauer disk diffusion assay. Each strain was diluted in accordance with 0.5 McFarland standard and then sterile cotton swabs were used for swabbing on Muller Hinton agar. After 16-18 hours of incubation at 37 °C, zones of inhibition diameters were measured and strains were categorized as resistant, intermediate, and sensitive against each tested antibiotic using the Clinical and Laboratory Standards Institute's (CLSI) guidelines. Multiple antibiotic resistance index was also calculated by dividing the no. of antibiotics to which a specific bacterial strain had shown resistance by the number of total tested antibiotics [9].

3. RESULTS

3.1 Physical and Chemical Parameters of Water Quality Testing

All the Physical parameters of water quality testing were normal for both drinking water samples of livestock farms whereas the fish pond water sample had a slightly unpleasant smell. Fish pond water samples also had a higher TDS and hence higher temperature and E.C. as well in comparison to other water samples (Table 1). All the chemical parameters of water samples were also in an acceptable range of drinking water (Table 2).

3.2 Microbiological Testing

There was a very high number of bacteria in the fish pond water sample as compared to the other 2 samples. Water sample from farm 1 has a lower colony count as compared to the sample from farm 2. Diversity of bacteria in drinking water samples from both farms was very low. Similar colonies in variable numbers were present on all petri dishes (Table S1). From the fish pond sample, 12 (52 %) colonies were selected for further study and characterization whereas, from livestock drinking water samples, 6 (26 %) and 5 (22 %) colonies were selected from farms 1 & 2 respectively. Colony morphology of each selected strain is summarized in Table S2. Pigmentation of selected and isolated strains varies from off-white, and white to yellow and orange. Their sizes range from pinpoint colonies to 35 mm. Elevation, texture, surface appearance, shape, margins, and opacity are also summarized in Table S2.

3.3 Gram Staining and Biochemical Characterization

In gram staining results, 9 % of strains were identified as gram-positive cocci, 13 % as gram-positive rods, and 78 % as gram-negative rods making them the most prevalent bacterial type. We have observed not a single gram-negative coccus from all three samples.

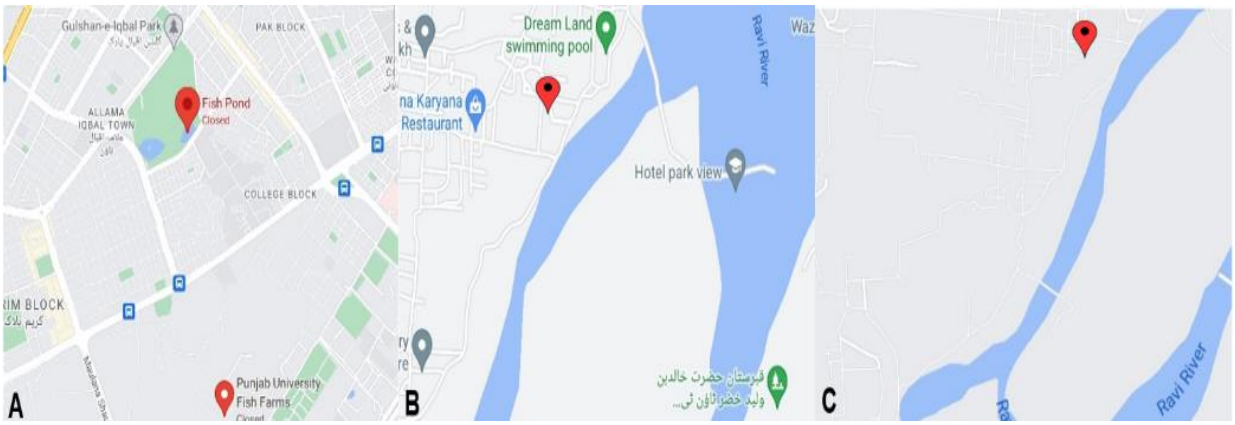

Fig 1. Topographical plots of the sample collection sites; A: Fish Pond water sample, B: Cattle farm 1 sample, C: Cattle farm 2 sample.

Table 1. Physical Parameters of Water Quality

Parameters	Units	Detection Limit	Reference Method	Permissible limits (PSQCA/NSDWQ, 2010)	Permissible limits (WHO)	Fish Pond	Cattle farm 1	Cattle farm 2
Color	-----	-----	Sensory Evaluation	5	5	Colorless	Colorless	Colorless
Odor	-----	-----	Sensory Evaluation	Odorless	Odorless	Pungent	Odorless	Odorless
Temperature	°F	41	Thermometer	NGVS	NGVS	68	62	60.6
Electrical Conductivity (E.C)	(µS/cm)	0.11	APHA, 22nd Edition	NGVS	NGVS	876	282	234
pH	-----	0.03	APHA, 22nd Edition	6.5-8.5	6.5-8.5	7.9	7.3	6.9
Turbidity	NTU	0.31	APHA, 22nd Edition	<5	0.5	7.1	1.25	0.9
Total Dissolved Solids (TDS)	ppm	-----	APHA, 22nd Edition	1000	1000	672	172	154

APHA: American Public Health Association
NGVS: No Guideline value set
NSDWQ: National Standards for Drinking Water Quality
PSQCA: Pakistan Standard Quality Control Authority
WHO: World Health Organization

Table 2. Chemical Parameters of Water Quality

Parameters	Units	Detection Limit	Reference Method	Permissible limits (PSQCA/NSDWQ, 2010)	Permissible limits (WHO)
Alkalinity as CaCO3	ppm	----------	APHA, 22nd Edition	NGVS	NGVS
Bicarbonate	ppm	5.0	APHA, 22nd Edition	NGVS	NGVS
Calcium	ppm	2.0	APHA, 22nd Edition	NGVS	200
Carbonate	ppm	5.0	APHA, 22nd Edition	NGVS	NGVS
Chloride	ppm	2.0	APHA, 22nd Edition	250	250
Hardness	ppm	5.0	APHA, 22nd Edition	500	500
Magnesium	ppm	1.0	APHA, 22nd Edition	NGVS	150
Potassium	ppm	0.02	APHA, 22nd Edition	NGVS	30
Sodium	ppm	1.57	APHA, 22nd Edition	NGVS	200
Sulphate	ppm	0.24	APHA, 22nd Edition	NGVS	250
Nitrate	ppm	0.03	APHA, 22nd Edition	10	10

APHA: American Public Health Association
NGVS: No Guideline value set
NSDWQ: National Standards for Drinking Water Quality
PSQCA: Pakistan Standard Quality Control Authority
WHO: World Health Organization

Biochemically, gram-positive cocci were further checked for the presence of catalase enzyme and all the isolates were positive for the catalase test confirming them to be Staphylococcus species and ruling out *Streptococcus species* (100 %). The yellow pigment was not observed in any of the gram-positive cocci colonies, so, ruled out the presence of *Micrococcus* organisms. Spore staining of gram-positive rods was done to find out the *Bacillus* species and 66.67 % of isolates were positive for endospore confirming them to be Bacillus species. No isolate was positive for acid-fast stain ruling out the presence of *Mycobacterium* species. Non-spore formers were further checked for the presence of catalase enzyme and 33 % of isolates were confirmed to be *Lactobacillus* by having catalase enzyme.

Oxidase test was performed for all the gram-negative rods; 33 % of strains were positive for oxidase. These oxidase-positive organisms were further evaluated for glucose fermentation. Approximately, 67 % of isolates were negative for glucose fermentation characterizing them to be *Pseudomonas* species whereas the remaining 33 % of the organism that were negative for glucose fermentation and positive for lactose fermentation were characterized as *Aeromonas* species. None of the isolates required sodium salts for their growth. A single strain that is gram-negative coccobacilli, oxidase negative, catalase positive, and oxidized glucose in OF test was identified to be an *Acinetobacter* specie. Remaining gram-negative isolates were characterized as pathogens of the family *Enterobacteriaceae*. They were further characterized using the API 20E strips. Proteus species were found to be 27 % whereas *Escherichia, Serratia, Enterobacter*, and *Klebsiella* species were all found to be 18 % approximately.

3.4 Antibiotic Susceptibility Testing

Various classes and multiple numbers of antibiotics were used for testing. CLSI guidelines (2019) were followed to characterize the strains as resistant, sensitive, and intermediately resistant to tested antibiotics. Resistance against eight out of 10 classes of antibiotics was observed. All verified strains were resistant to multiple classes of antibiotics confirming them to be multiple drug-resistant (MDR) strains. Out of all the tested strains against amoxicillin, ampicillin, piperacillin, piperacillin-tazobactam, tobramycin, cefuroxime, cefoxitin, meropenem, and linezolid was 100 %. Tested strains showed 100 % sensitivity towards doxycycline and clindamycin. AST results are summarized in Table 3.

3.5 MAR Index

MAR index of all the isolated strains was higher than 0.4 and ranges between 0.4 – 0.9. Bacteria isolated from livestock farms had the highest MAR index as compared to strains isolated from fish pond water samples (Figure 2).

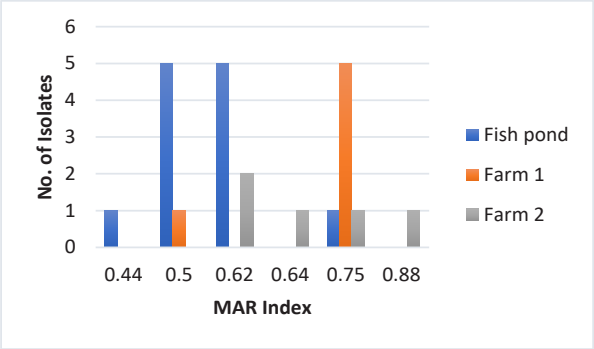

Fig. 2. Comparison of MAR Index of isolated strains

4. DISCUSSION

A large number of bacterial infections are caused by multidrug-resistant (MDR), extremely drug-resistant (XDR), or even total drug-resistant (TDR) organisms worldwide. AMR leads to greater than before morbidity cases, and early deaths in young individuals cause powerlessness. AMR is not only a threat to human lives but it is also a threat to the world economy [10]. To check the presence of ARBs in environments where food animals live, a water sample from a fish pond and 2 livestock farms was collected. Physical and chemical water quality parameters were checked as these were the drinking water samples of livestock animals. Visual impurities were not present in any sample indicated by colorlessness, odourlessness, normal pH range, and E.C. under permissible limits of WHO [11]. Chemical parameters of all the samples were also in the permissible range implying that these water samples are safe to drink by livestock animals [11]. Our main focus was on the biological contamination in water sources in accordance with the WHO plan to monitor and report the environmental ARBs

Table 3. Summary of Antibiotic Susceptibility Testing

Antibiotic Class	Antibiotic Generation	Antibiotic (Conc. In µg)	Abbreviation	No. of Isolates		
				Resistant	Intermediate	Sensitive
Penicillins	2nd	Amoxicillin (2)	A2	14	0	0
	2nd	Amoxicillin + Clavulanic acid (2+1)	AUG3	6	0	1
	3rd	Ampicillin (10)	AM10	7	0	0
	4th	Piperacillin 30	PRL30	9	0	0
	4th	Piperacillin-Tazobactam (30+6)	PTZ36	7	0	0
Aminoglycosides	-----	Gentamycin (10)	GM10	2	2	8
	-----	Tobramycin (10)	TN10	1	0	0
	-----	Amikacin (30)	AK 30	3	2	5
Tetracycline	1st	Tetracycline (30)	TE30	4	2	3
	2nd	Doxycycline (30)	DX30	0	0	1
Quinolones	2nd	Ciprofloxacin (1)	CIP1	10	3	0
	2nd	Levofloxacin (10)	LEV10	6	7	2
	3rd	Gatifloxacin (5)	GAT5	0	3	6
Cephalosporins	2nd	Cefuroxime (5)	CXM5	11	0	0
	2nd	Cefoxitin (10)	FOX10	14	0	0
	3rd	Cefotaxime+Clavulanic Acid (30+10)	CTC40	7	0	9
Macrolides	-----	Azithromycin (15)	ATH15	6	1	2
Carbapenems	-----	Meropenem (10)	MEM10	9	0	0
	-----	Imipenem (10)	IMI10	6	1	0
Lincosamide	-----	Clindamycin (2)	CD2	0	0	2
Chloramphenicol	-----	Chloramphenicol (30)	C30	0	4	5
Oxazolidinones	-----	Linezolid (10)	LZD10	1	0	0

along with clinical cases of ARBs [12]. Bacterial load was high in all samples, particularly in the fish pond water sample. The most frequent genus found in our study was *Pseudomonas* followed by *Proteus* (Figure 3). A study from Ghana also reported the high prevalence of *Pseudomonas* in environmental samples [13]. Another study reported the high prevalence of *Escherichia* and *Klebsiella* isolates [14]. A possible reason for the low bacterial load in the drinking water of livestock farms is that these samples were taken early in the morning when fresh water was given to animals. There are quite high chances of greater bacterial load if samples were taken in the afternoon or the evening. Isolated bacterial populations may also be subject to seasonal variation.

All isolates were verified against at least four different classes of antibiotics. *Pseudomonas* isolate had shown the resistance to most classes of antibiotics followed by genus *Escherichia*. MAR Index less than 0.2 is considered as safe, conversely, greater MAR Index is a signal of fecal contamination [15]. All the genus identified and studied in our paper had a MAR Index greater than 0.40. Table 4 representing contamination from a source where antibiotics are in use frequently and gratuitously [16]. This is a confirmation of antibiotics use as growth promoter and as prophylactic in livestock and fish farming irrelevantly.

Isolated strains from the fish pond had the higher resistance against multiple antibiotics followed by livestock farm 1 and the least resistance was observed in livestock farm 2 samples. Similar kind of results has also been reported in various studies worldwide. A study from Japan has reported the presence of all 6 clinically important pathogens in waste water treatment plants and all these isolates were highly resistant to multiple antibiotics [17]. Similarly, another study from South Africa also reported the presence of a large number of ARBs from various environmental sources including irrigation water, waste water, surface water, and drinking water (also in food items and vegetables) [18]. Researchers from Saudi Arabia had reported the presence of ESKAPE pathogens in nearly half of the hospital-acquired bacterial infections. All

Table 4. Genus wise MAR Index

Genus	Average MAR Index
Pseudomonas	0.68
Aeromonas	0.62
Acenitobacter	0.50
Escherichia	0.75
Proteus	0.56
Serratia	0.56
Enterobacter	0.81
Klebsiella	0.56
Staphylococcus	0.63
Bacillus	0.68
Lactobacillus	0.50

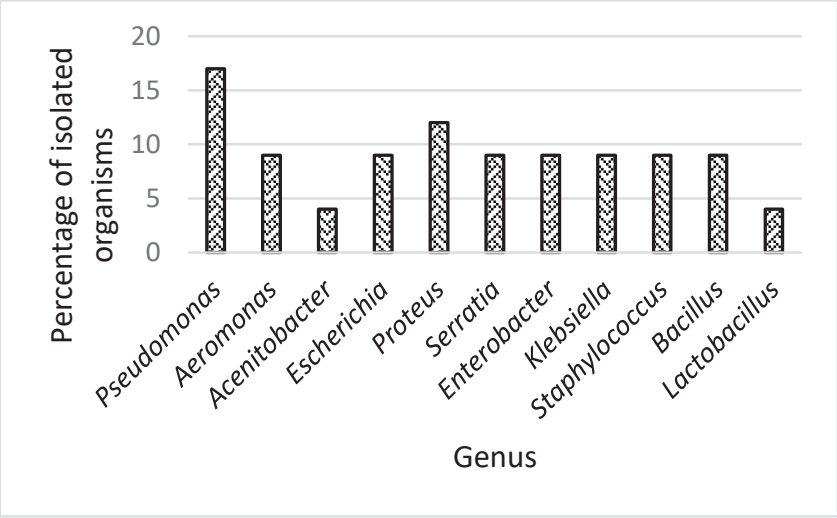

Fig. 3. Genus wise percentage of isolated strains

those pathogens were highly resistant to multiple antibiotics [19].

A study reported the presence of many distinguished MGEs in ESKAPE pathogens carrying resistance genes against Beta-lactam drugs and aminoglycosides [20]. This is a strong indicator of the spread of resistance against beta-lactam and other drugs in our as well as former studies by means of horizontal gene transfer mechanisms.

5. CONCLUSION

OA high burden of multidrug-resistant bacteria were isolated from all the water samples including bacteria from *Enterobacteriaceae, Pseudomonas, Aeromonas, Lactobacillus, Bacillus,* and *Staphylococcus*. Moreover, the MAR Index of all the isolated strains was greater than 0.4 indicating the unnecessary use and presence of antibiotics in selected environments. This means environmental water sources are playing a critical role in triggering the transmission of drug resistance through horizontal gene transfer mechanisms.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

Authors hereby declare no conflict of interest.

8. REFERENCES

1. M. Ye, J.Q. Su, X.L. An, and Y.G. Zhu. Silencing the silent pandemic: Eliminating antimicrobial resistance by using bacteriophages. *Science China. Life Sciences* 65: 1890-1893 (2022).
2. H. Carboné, M.S. Cotillas, E. Lacasa, P. Canizares, M.A. Rodrigo, and C. Sáez. Depletion of ARGs in antibiotic-resistance Klebsiella, Pseudomonas and Staphylococcus in hospital urines by electro and photo-electro disinfection. *Journal of Water Process Engineering* 49. 103035 (2022).
3. CDC. Antibiotic resistance threats in the United States, 2019. US Department of Health and Human Services, *Centres for Disease Control and Prevention, Atlanta* (2019).
4. C.J.L. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G.R. Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, and E. Wool. Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet* 399: 629-655 (2022).
5. M. Balasegaram. Learning from covid-19 to tackle antibiotic resistance. *ACS Infectious Diseases* 7: 693-694 (2021).
6. B.S. Lopes, A. Hanafiah, R. Nachimuthu, S. Muthupandian, Z.N.M. Nesran, and S. Patil. The role of antimicrobial peptides as antimicrobial and antibiofilm agents in tackling the silent pandemic of antimicrobial resistance. *Molecules* 27: 2995 (2022).
7. B.S. Lopes, N.J. Strachan, M. Ramjee, A. Thomson, M. MacRae, S. Shaw, and K.J. Forbes. Nationwide stepwise emergence and evolution of multidrug-resistant Campylobacter jejuni sequence type 5136, United Kingdom. *Emerging infectious diseases* 25: 1320 (2019).
8. J. Lu, and J. Guo. Disinfection spreads antimicrobial resistance. *Science* 371(6528): 474-474.
9. A.A.S. Sinegani, and N. Younessi. Antibiotic resistance of bacteria isolated from heavy metal-polluted soils with different land uses. *Journal of Global Antimicrobial Resistance.* 10: 247-255 (2017).
10. WHO. Glass whole-genome sequencing for surveillance of antimicrobial resistance. *World Health Organization, Geneva* (2020).
11. P.D. Patil, R.D. Sawant. Physico-chemical parameters for testing of water-a review. *International Journal of Environmental Sciences* 3: 1194 (2012).
12. M. Henderson, S.J. Ergas, K. Ghebremichael, A. Gross, and Z. Ronen. Occurrence of antibiotic-resistant genes and bacteria in household greywater treated in constructed wetlands. *Water* 14: 758 (2022).
13. H. Odoi, V.E. Boamah, Y.D. Boakye, and C. Agyare. Prevalence and phenotypic and genotypic resistance mechanisms of multidrug-resistant Pseudomonas aeruginosa strains isolated from clinical, environmental, and poultry litter samples from the Ashanti region of Ghana. *Journal of Environmental and Public Health* 2021: 9976064 (2021).
14. H. Tesfaye, H. Alemayehu, A.F. Desta, and T. Eguale. Antimicrobial susceptibility profile of selected Enterobacteriaceae in wastewater samples from health facilities, abattoir, downstream rivers and a

WWTP in Addis Ababa, Ethiopia. *Antimicrobial Resistance & Infection Control* 8: 1-11 (2019).

15. G.T. Gessew, A.F. Desta, and E. Adamu. High burden of multidrug resistant bacteria detected in little Akaki river. *Comparative immunology, microbiology and infectious diseases.* 80: 101723 (2022).
16. P.P. Amador, R.M. Fernandes, M.C. Prudêncio, M.P. Barreto, and I.M. Duarte. Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of class a and class c β-lactamases. *Journal of Environmental Science and Health, Part A* 50: 26-39 (2015).
17. M. Nishiyama, S. Praise, K. Tsurumaki, H. Baba, H. Kanamori, and T. Watanabe. Prevalence of antibiotic-resistant bacteria eskape among healthy people estimated by monitoring of municipal wastewater. *Antibiotics* 10: 495 (2021).
18. J. Denissen, B. Reyneke, M. Waso-Reyneke, B. Havenga, T. Barnard, S. Khan, and W. Khan. Prevalence of eskape pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. *International Journal of Hygiene and Environmental Health*. 244: 114006 (2022).
19. N.J. Ahmed, A. Haseeb, M.A. Hassali, E.E. Elsaid, and A.H. Khan. Antimicrobial resistance of eskape pathogens in a public hospital in Alkharj. *Latin American Journal of Pharmacy* 40: 442 (2021).
20. S. Das, S. Bombaywala, S. Srivastava, A. Kapley, R. Dhodapkar, and N.A. Dafale. Genome plasticity as a paradigm of antibiotic resistance spread in eskape pathogens. *Environmental Science and Pollution Research* 29: 40517-40529 (2022).

9. DECLARATION

Authors declare that:

(i) the results are original;
(ii) the same material is neither published nor under consideration elsewhere;
(iii) approval of all authors have been obtained; and
(iv) in case the article is accepted for publication, its copyright will be assigned to Pakistan Academy of Sciences.

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Research Article

Hematological and Biochemical Assessment of Children Infected with Measles Virus: 2022 Outbreak in Pakistan

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Abstract: Measles is a contagious disease caused by an RNA virus. Resurgence of measles after Covid-19 and its severity among children has led to many speculations about the Measles vaccination coverage and its efficacy. In this study, the clinical data of children <9 years (n=19) admitted at the Pakistan Institute of Medical Sciences (PIMS) in the measles ward was analyzed. The blood samples were processed for hematology and routine biochemistry tests. The results obtained were statistically analyzed on SPSS-21 software by using One-Way ANOVA for Complete Parameters (CP), Kruskal Wallis, and Mann-Whitney test for Differential leucocyte count (DLC) and Biochemical parameters. A $p<0.05$ was considered significant. The results suggest no significant difference in Complete blood parameters (CP) among non-vaccinated, partially vaccinated and fully vaccinated patients. Among DLC Basophils level was significantly different ($p=0.024$), being lower in partially vaccinated than non-vaccinated patients. Biochemical parameters showed that serum urea level was significantly different ($p=0.013$), showing a decline in fully vaccinated patients as compared to non-vaccinated patients. Moreover, a significantly higher level of Alkaline phosphatase as compared to the normal range was observed in fully vaccinated patients. However, lower levels of MCH, MCV, MCHC, RBC, Hb, eosinophils, and a higher level of RDW-CV were observed overall as compared to the normal range (healthy individuals). The results suggest improvements are needed in vaccination strategies for effectively controlling the disease. Anemic conditions in overall measles patients indicate poor health conditions. This study contains a limited sample size, further research on measles virus (MeV) mutations, and vaccine optimization could be helpful for the complete eradication of measles from Pakistan.

Keywords: Measles, Hematology, Biochemical parameters, Measles vaccination, Measles in children.

1. INTRODUCTION

Measles virus (MeV) is a very contagious virus that had been a cause of high fatality rates throughout the world before the advent of the Measles vaccine [1]. Measles virus is a single-stranded negative-sense RNA virus belonging to the family Paramyxoviridae and genus Morbillivirus [2]. There are other five genera belonging to this family including Rinderpest virus (RPV), Peste des Petitis Ruminant's Virus (PPRV), Canine Distemper Virus (CDV), Phocine Distemper Virus (PDV), Cetacean Morbillivirus (CeMV) that cause similar infectious diseases in Cattle, sheep and goats, Carnivores, seals and dolphins respectively [3].

The only reservoir of the measles virus is human beings. The transmission of MeV takes place via respiratory aerosols as it is an airborne virus. Moreover, it can also be transmitted via direct contact with the surface containing respiratory secretions from the infected person [2]. Measles virus has a high infectivity rate as one infected person can infect more than 12 persons on average [4]. As the virus makes its way to the human body the prodromal phase spans over 7-10 days and then MeV manifests itself in the form of cough, coryza, and fever followed by a rash on the face and the other parts of the body. These symptoms disappear usually with a decrease in viral load conferring lifetime immunity. However, in children with an underdeveloped immune system, it can take severe form developing pneumonia, otitis

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media, encephalitis, blindness, and other secondary bacterial and viral infections [5]. Sometimes if the virus persists in the nervous system, it can cause severe neurodegenerative diseases including subacute sclerosing panencephalitis (SSPE) measles inclusion-body encephalitis (MIBE). These complications have been reported to be associated with mutations in the viral F protein [6].

Measles has caused huge mortality globally before the availability of vaccines in 1963. Approximately 30 million cases were reported and greater than 2 million deaths annually. But with the advent of industrialization, improved lifestyle & nutrition, and the introduction of one dose of vaccine during the 1st year of life, the mortality rate has declined greatly. Also, better healthcare facilities and antibiotic therapies for measles-associated infections contributed to decreased death rates [2,7]. Measles virus is relatively stable as compared to the other viruses of this family as it is considered monotypic having a single serotype. It has been divided into eight clades from A to H based on different variable regions of the MeV genome and 24 genotypes [8].

A cost-effective live attenuated vaccine of measles is available and is usually given in two doses, the first dose at the 9th month and the second dose in the second year of life. Often the measles vaccine is incorporated into the mumps and rubella vaccine (MMR), and it is equally effective. Most of the countries have met WHO targets of measles complete immunization and have successfully eradicated measles but it still prevails in some African and Asian countries. Pakistan is also among those countries where there are still a significant number of measles cases [9]. B3 genotype has been reported in Pakistan previously from different areas including Sindh, Islamabad, and Khyber Pakhtunkhwa [10-12].

A recent spike in measles cases has been witnessed in Pakistan after Covid-19 pandemic in 2022. Several factors have been speculated for this re-emergence of measles. Despite complete vaccination of some patients, they still developed symptoms of measles. Our research analyzes the hematological and biochemical parameters of measles patients admitted at the Pakistan institute of medical sciences (PIMS) Islamabad to investigate the efficacy of MMR vaccination and the correlation of measles severity with different hematological and biochemical parameters.

2. MATERIALS AND METHODS

2.1 Inclusion/Exclusion Criteria

A Measles patients included in this study were 19 aged below 9 years admitted at the Pakistan Institute of Medical Sciences (PIMS) children's measles ward and were recruited for blood sampling. Only the patients whose vaccination status was known were included in this study and the rest of them were excluded. Written informed consent was obtained from the parents/guardians of young Children for the collection of clinical samples.

2.2 Clinical Specimen Collection

Children aged 1 month to 9 years having laboratory-confirmed measles and showing mild to severe symptoms of measles were enrolled in the current study. The data set of measles patients was divided into three subgroups based on their vaccination status including three Not vaccinated, eight partially vaccinated, and eight fully vaccinated. Patients who did not receive any dose of MMR vaccine at the time of admission at PIMS were considered as not vaccinated (n=3), and those who received one dose of vaccine at the time of admission at the hospital were assigned as partially vaccinated status (n=8) and the patients who had received two doses of MMR vaccine were considered fully vaccinated (n=8). Gender was also recorded but not used for analysis due to the limited number of samples (n=19).

Venous blood sample was successfully collected from all the patients in a 5 ml heparin vacutainer tube by the medical practitioner. Blood samples were processed for analyzing hematological parameters (CP, DLC) and routine biochemistry tests (BSR, total bilirubin, serum creatinine, SGPT (ALT), alkaline phosphatase, serum urea, calcium, potassium, sodium). The overall flow chart of the research has been shown in (Figure 1).

2.3 Blood Sample Processing

2.3.1 Hematological parameters

Blood was taken intravenously (1.5 ml) from the

measles patients into 5 ml EDTA tubes. The tubes were placed on the rotor to maintain the homogeneity of blood samples. The blood sample was fed to the probe of the TOSOH automated hematology analyzer and the results of CP (TLC, Platelets count, MHC, MCV, MCHC, RBC, Hb, PCV, RDW-CV, Platelet distribution width, mean platelet volume) and DLC (Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils, NRBC_per 100WBCs, Immature granulocytes) were recorded.

2.3.2 Biochemical parameters

Whole blood sample (2 ml) was taken from measles patients intravenously into a yellow top 5 ml glass tube. The blood was allowed to clot by setting it aside for 15-20 minutes. When the blood is fully clotted the tubes were centrifuged at 1000-2000 g for 10 minutes (Thermo-scientific LABOFUGE 200, Sweden). After centrifugation, the tubes were gently taken out and serum was obtained. The gel settled above the blood clot and the serum was separated into clear test tubes labeled with the patient's ID. The samples were placed in TOSOH G11 HPLC Analyzer, Japan and the report generated for the study parameters was recorded.

2.4 Statistical Analysis

Statistical analysis was performed on data sets obtained from laboratory tests. One-Way ANOVA was performed for comparing variations of TLC, Platelets count MCH, MCV, MCHC, RBC, Hb, PCV, RDW-CV, Platelets distribution width, and Mean platelet volume between three patients' groups. A p-value below 0.05 was considered

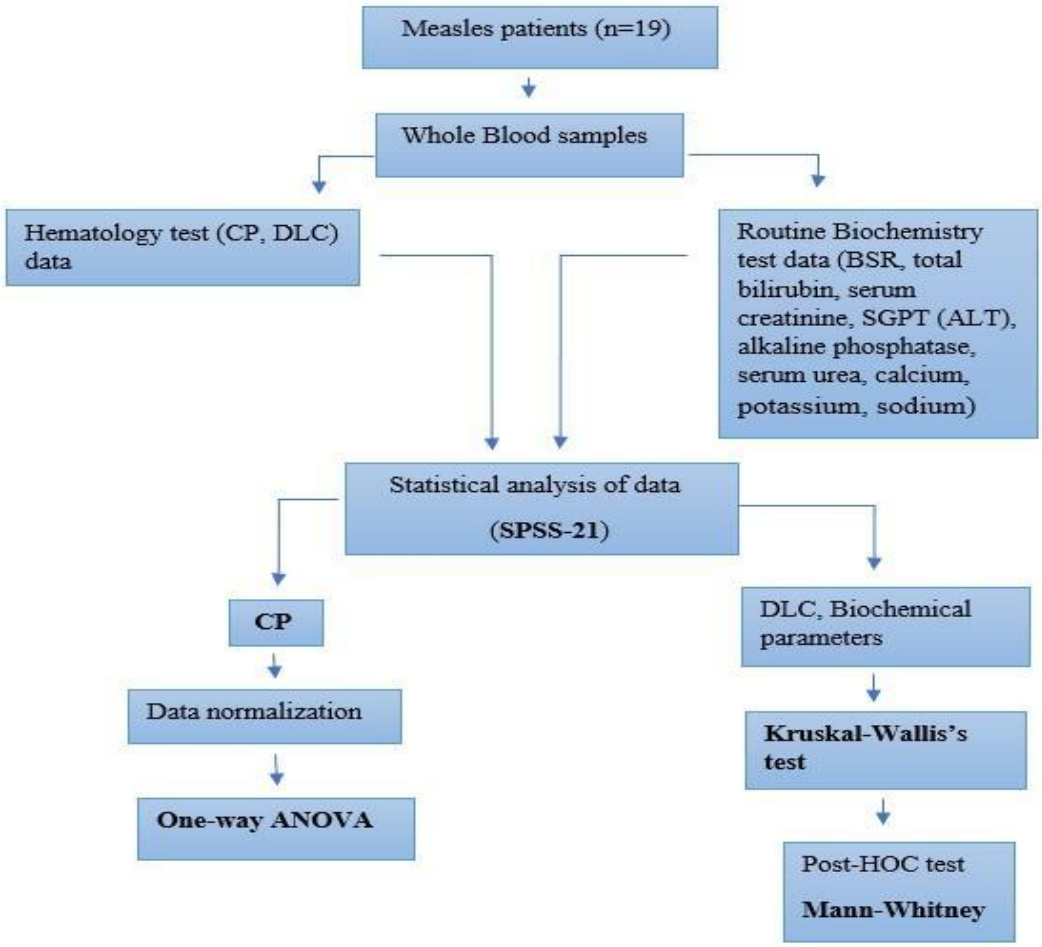

Fig. 1. Consort Diagram. Whole blood samples from nineteen patients were taken and processed for Haematology (Complete blood test, CBC) and routine biochemistry tests. The data obtained from the test results were analyzed in SPSS-21 software. Complete parameter (CP) data were analyzed by One-way ANOVA. Differential leucocyte count (DLC) and biochemical parameters were analyzed by the Kruskal-Wallis test and by the Mann-Whitney test.

significant. Non-parametric tests (Kruskal-Wallis, Mann-Whitney) were performed for DLC and Biochemical parameters.

3. RESULTS

The total number of measles patients recruited in the current study are 19 including six female and thirteen male patients from one month to nine years old children. Their vaccination status has been shown in Figure 2.

3.1 Hematological Parameters

3.1.1 Complete Blood Parameters (CP)

Our results showed that there was no significant difference between not vaccinated, partially-vaccinated and fully-vaccinated patients for TLC [F (2, 16)=0.284, p=0.756], Platelets count [F (2, 16)=2.172, p=0.146], MCH, [F(2, 16)=1.451, p=0.264], MCV [F(2, 16)= 1.174, p=0.335], MCHC [F(2, 16)=1.575, p =0.237], RBC [F(2, 16)=2.077, p=0.158], Hb [F(2, 16)=0.27, p=0.974], PCV [F(2, 16)= 0.150, p=0.862], RDW-CV, [F(2, 16)=3.257, p =0.065], Platelets distribution width [F(2, 16) =1.139, p=0.345], and Mean platelet vol. [F(2, 16) =0.260, p=0.774]. Lower levels of MCH, MCV, MCHC, RBC, and Hb (as compared to the normal range in healthy subjects) were observed in all three groups of measles patients. Moreover, there was a very high RDW-CV in all the groups as compared to the normal range (Table 1).

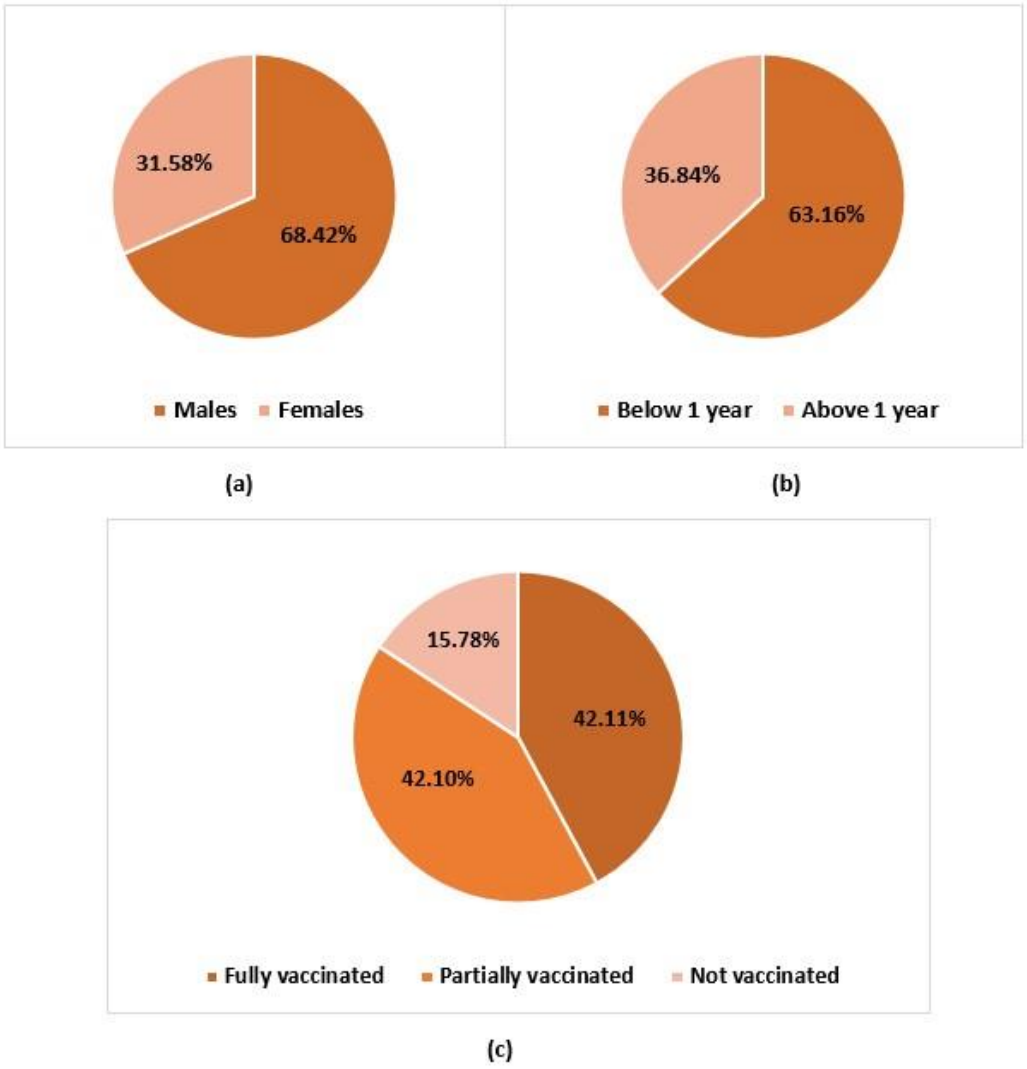

Fig. 2. (a) Gender ratio of measles patients, **(b)** The age ratio (upto-9 years)**, (c)** The ratio of the vaccination status of measles patients. (No dose=not vaccinated, 1 dose=partially vaccinated, 2 doses=fully vaccinated**).**

3.1.2 Differential Leucocyte Count

There was no significant difference ($p>0.05$) between neutrophils, lymphocytes, monocytes, eosinophils, NRBC_per100WBCs, immature granulocytes among not vaccinated, partially vaccinated, and fully vaccinated measles patients, except for Basophils ($p=0.024$) that was significantly different between non vaccinated and partially vaccinated patients (Figure 3) Moreover, a lower level of Eosinophils as compared to the normal range in healthy subjects prevailed in all the three patient groups (Table 2).

3.2 Biochemical Parameters

Statistical analysis of biochemical parameters including Blood Sugar Random (BSR), total bilirubin, serum creatinine, SGPT (ALT), alkaline phosphatase, serum urea, calcium, potassium, and sodium of three groups of measles patients showed no significant difference ($p>0.05$) among all the parameters except significantly different serum urea ($p=0.013$) between not vaccinated and vaccinated group. Moreover, a significantly higher level of Alkaline phosphatase as compared to the normal range was observed in fully vaccinated patients.

4. DISCUSSION

The symptoms and clinical manifestation of measles can vary in different patients depending on their age, nutritional status, and immunocompetency. The analysis of clinical data of measles patients (n=19) suggested that most of the measles patients were anemic with a lower level of MCH, MCV, MCHC, RBCs, and hemoglobin and a very high ratio of RDW-CV than the normal range among the healthy subjects (Table 1). The anemic condition ($Hb<11$) prevailed among all the patients irrespective of their vaccination status.

Our study is comparable with previous studies involving viral infections including dengue, hepatitis, HIV and Covid-19 led to a decrease in hemoglobin hence causing anemia in the infected patients [13-16]. Hemoglobin contains iron that carries oxygen to different organs of the body and when the concentration of hemoglobin decreases it causes hypoxia which could ultimately lead to organ dysfunction specifically targeting respiratory organs. Anemic conditions might have aggravated the respiratory problems in hospitalized measles patients, [17] as many of them (n=7) were put on ventilators for proper breathing. The genetic factor of the infected host can play a role in the viral induction of anemia [18]. The nutritional status and composition of the diet can also contribute towards anemia [19]. Some studies also suggest that the antiviral therapy or administration of antiviral drugs during treatment might induce anemia, by hemolysis or other related mechanisms [14]. Among all patient groups, there was no significant difference in hemoglobin level and other indicators of anemia, so we might infer that the vaccination did not affect the hemoglobin level, there might be some other reason including the viral induction of hemolysis, the poor nutritional status of children or the genetic factor of infected patients.

Measles vaccine in combination with other vaccines could be associated with mild adverse effects following the immunization (AEFI). Studies have reported the incidence of fever, neurological symptoms, agitation, nervousness, gastrointestinal diseases, thrombocytopenia, redness, swelling, local pain, lymphadenitis, etc [20]. Our results indicate a lower level of eosinophils in the patients (Table 2) that were partially vaccinated (one dose of vaccination). Some of these patients also showed symptoms of hyperpyrexia, skin rash, and excessive crying that are comparable with already reported studies [20].

The paradigm of eosinophils includes the destructive and inflammatory functions in cells. They are recruited because of T helper cells Th2 type reactions releasing cytotoxic granule proteins, different lipid mediators, and cytokines that promote parasite destruction, inflammation, and tissue damage. Under baseline conditions, eosinophils perform homeostatic, protective, and immunoregulatory functions in different organs of the body including the gastrointestinal tract, lungs, thymus mammary glands, and adipose tissues [21]. Eosinophils showed a decline (0.09 %) in partially vaccinated patients as compared to non-vaccinated (0.43 %) and then gradually increased (0.72 %) in fully vaccinated patients. With this transition in eosinophil levels, we can speculate that the previous history of administration of the MMR vaccine 1st dose could be associated with altered levels of eosinophils in measles patients that

Table 1. Comparison of complete blood parameters of not vaccinated, partially-vaccinated, and fully-vaccinated measles patients as compared to the normal range.

Parameters	Not vaccinated (n=3)	Partially vaccinated (n=8)	Fully vaccinated (n=8)	Normal range
	Mean ± SD	Mean ± SD	Mean ± SD	
TLC ($\times 10^9$/L)	12.8 ± 4.2	10.2 ± 1.9	9.4 ± 2.3	6-18
Platelet count (×1000/µl)	399 ± 73.5	442.3 ± 58.9	292.6 ± 44.7	200-550
MCH (pg)	18.3 ± 2.7	21.5 ± 1.3	22.1 ± 0.8	25-29
MCV (fl)	60 ± 8.3	68 ± 3.6	69.8 ± 2.3	72-84
MCHC (g/dL)	30.5 ± 0.5	31.5 ± 0.4	31.6 ± 0.3	32-36
RBC (million/ µl)	5.6 ± 0.3	4.9 ± 0.4	4.6 ± 0.1	6-18
Hb (g/dL)	10.2 ± 1.4	10.3 ± 0.4	10.1 ± 0.3	11-15.5
PCV	33.4 ± 4.1	32.6 ± 1.4	31.9 ± 0.9	30-38
RDW-CV (%)	20.8 ± 1.7	17.4 ± 0.8	16.7 ± 0.8	10-15
Platelets distribution width (%)	15.1 ± 0.3	15.5± 0.2	15.5 ± 0.12	15-17
Mean platelet vol.	8.6 ± 0.30	8.4 ± 0.4	8.2 ± 0.3	6.5-12

Abbreviations: TLC= total leucocyte count, **MCH**= mean corpuscular hemoglobin, **MCV**= mean corpuscular volume, **MCHC**= mean corpuscular hemoglobin concentrations, **PCV**=packed cell volume, **RDW-CV**= red cell distribution width-coefficient of variation.

Table 2. Comparison of differential leucocyte count in not vaccinated, partially-vaccinated, and fully-vaccinated measles patients as compared to the normal range.

Differential Leucocyte Count (DLC) %	Not vaccinated (n=3)		Partially vaccinated (n=8)		Fully vaccinated (n=8)		Normal range
	Mean	SE	Mean	SE	Mean	SE	
Neutrophils	56.6	11.6	54.9	6.3	48.8	7.9	30-60
Lymphocytes	34.1	12.2	38.5	6.0	43.1	6.8	25-55
Monocytes	8.5	0.9	6.4	1	7.2	2.5	2-10
Eosinophils	0.43	0.3	0.09	0.1	0.72	0.5	1-6
Basophils	0.3	0.1	0.1	0.03	0.2	0.03	0.0-2.0
NRBC_per100WBCs	0.6	0.6	0.07	0.05	0.00	0.0	0.00-2
Immature granulocytes	0.5	0.1	0.3	0.1	0.4	0.10	0.0-100

gradually recovered in fully vaccinated patients. The manifestation of allergy, inflammation, and breathing issues in partially vaccinated patients could be possibly related to AEFI.

Some other significant variations among the clinical data of measles patients were observed in the level of Alkaline phosphatase which was slightly higher than the normal range in fully vaccinated patients, and the level of serum-urea that remarkably declined from non-vaccinated toward fully vaccinated measles patients. Although the later one was found to be in the normal range in all the measles patients as compared to the healthy subjects. (Table 3) There is no direct evidence for the influence of MMR vaccination history on biochemical parameters in measles patients. But viral infections including hepatitis B and C have been reported to cause an increase in the level of serum Alkaline phosphatase (ALP) [22]. Alkaline phosphatase level in not vaccinated and partially-vaccinated patients lies under the normal range but are higher in fully-vaccinated patients as compared to the normal subjects. We can infer that vaccination

Table 3. Statistics for the comparison of biochemical parameters among three measles patients' groups as compared to the normal range.

Biochemical parameters	Not vaccinated (n=3)	Partially vaccinated (n=8)	Fully vaccinated (n=8)	Reference range
	Mean± SD	Mean± SD	Mean ± SD	
BSR (mg/dL)	124 ± 19.7	102.9 ± 7.8	121.6 ± 17.5	80-160
Total bilirubin (mg/dL)	0.4 ± 0.03	0.3 ± 0.02	0.4 ± 0.02	Upto-1.0
Serum creatinine (mg/dL)	0.3 ± 0.01	0.3 ± 0.03	0.3 ± 0.6	Upto-1.2
SGPT (ALT) (U/L)	25.7 ± 6.8	43.1 ± 11.2	40.5 ± 10.4	Upto-42
Alkaline phosphatase (U/L)	119.3 ± 0.7	123.9 ± 8.1	137.8 ± 22.7	Upto-135
Serum urea (mg/dL)	23.7 ± 2.7	27.6 ± 2.9	15.4 ± 1.5	12-50
Calcium (mg/dL)	9.3 ± 0.4	9.1 ± 0.2	8.7 ± 0.2	8.5-10.5
Potassium (m.mol/L)	4.2 ± 0.1	4.5 ± 0.1	4.3 ± 0.2	3.5-5
Sodium (m.mol/L)	133.6 ± 2.8	133.4 ± 2.2	135.5 ± 0.8	135-145

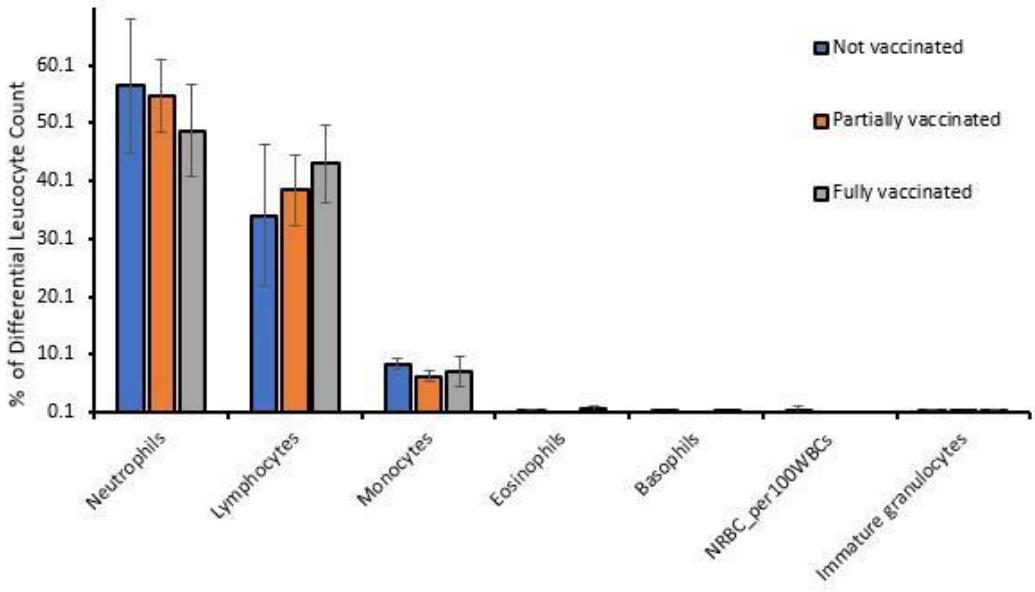

Fig. 3. Comparison of differential leucocyte count in not vaccinated, partially-vaccinated, and fully-vaccinated measles patients as compared to the normal range.

has somehow played a role in increasing ALP levels. ALP is a membrane-bound glycoprotein that promotes the hydrolysis of several kinds of phosphate monoesters. It is reported to be increased in patients with bone and liver diseases, but the level of ALP is slightly higher in infants and adolescents as compared to adults due to bone growth. A high level of ALP has been reported to be associated with respiratory infections [23]. In our case, complete MMR vaccination increases the level of ALP so we can say that the vaccination might have contributed to the aggravation of respiratory problems in measles patients despite curing the disease. Vaccination history affected the level of serum urea among measles patients by decreasing its level from non-vaccinated to completely vaccinated patients, but the fluctuation is under the normal range as compared to the healthy subjects, so we did not consider it under AEFI.

5. CONCLUSION

Despite the MMR vaccination administration/ campaigns, still, measles cases are being reported in the country. Our study indicated the suboptimal immunization conferred by the MMR vaccination. There can be several possible reasons including poor storage conditions of vaccines. The other possible reason could be malnourishment among the affected children (measles patients) as all the participants included in this study were <9 years so they might not be immunocompetent. Another possible reason behind it can be the variation/ evolution in the measles virus, as it is an RNA virus and these viruses usually have a relatively high rate of mutations. Further research could be conducted on a large set of measles patients with respect to their nutritional status, specific genetic factors, immunocompetency, and efficacy of MMR vaccination. The investigation for the optimization of immunization strategies and molecular characterization of the measles virus circulating among Pakistani patients could further highlight the unexplored aspects of measles prevalence, to meet the UN sustainable development goal of complete eradication of measles from developing countries such as Pakistan.

6 ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare no conflict of Interest.

8. REFERENCES

1. H.Y. Naim. Measles virus: A pathogen, vaccine, and a vector. *Human Vaccines and Immunotherapeutics* 11: 21–26 (2015).
2. P.A. Rota, W.J. Moss, M. Takeda, R.L. De Swart, K.M. Thompson, and J.L. Goodson. Measles. *Nature Reviews Disease Primers* 2:1 (2016).
3. B.K. Rima, and W.P. Duprex. Morbilliviruses and human disease. *Journal of Pathology* 208: 199–214 (2006).
4. S.A. Plotkin. Measles: Breakouts and Breakthroughs. *Journal of the Pediatric Infectious Diseases Society* 8(4): 289–290 (2019).
5. D.E. Griffin. Measles virus. *Fields Virology.* 56:285–291 (2013).
6. S. Watanabe, Y. Shirogane, Y. Sato, T. Hashiguchi, and Y. Yanagi. New Insights into Measles Virus Brain Infections. *Trends in Microbiology* 27: 164–175 (2019).
7. J.C.C. Lai, H.M.T.K. Karunarathna, H.H. Wong, J.S.M. Peiris, and J.M. Nicholls. Neuraminidase activity and specificity of influenza A virus are influenced by haemagglutinin-receptor binding. *Emerging microbes & infections* 8: 327-338 (2019).
8. M.V.T. Phan, C.M.E. Schapendonk, B.B. Oude Munnink, M.P.G. Koopmans, R.L. de Swart, and M. Cotton. Complete genome sequences of six measles virus strains. *Genome Announcements* 6: 11–12 (2018).
9. M.O. Mere, J.L. Goodson, A.K. Chandio, M.S. Rana, Q. Hasan, N. Teleb and J.P. Alexander. Progress Toward Measles Elimination — Pakistan, 2000–2018. MMWR. *Morbidity and Mortality Weekly Report* 68: 505–510 (2019).
10. S.S.Z. Zaidi, A. Hameed, M.S. Rana, M.M. Alam, M. Umair, U.B. Aamir, M. Hussain, S. Sharif, S. Shaukat, M. Angez, and A. Khurshid. Identification of measles virus genotype B3 associated with outbreaks in Islamabad, Pakistan, 2013–2015. *Journal of Infection and Public Health* 11: 540–545 (2018).
11. S.S.Z. Zaidi, A. Hameed, N. Ali, M. Umair, M.M. Alam, M.S. Rana, S. Sharif, U.B. Aamir, S. Shaukat, M. Angez, A. Khurshid, R. Akhtar, N. Mehmood

and N. Badar. A measles outbreak in Sindh, Pakistan caused by a genotype B3 virus. *Archives of Virology* 162: 3603–3610 (2017).

12. M. Ilyas, S. Afzal, J. Ahmad, S. Alghamdi and M. Khurram. The resurgence of Measles infection and its associated complications in early childhood at a tertiary care hospital in Peshawar, Pakistan. *Polish Journal of Microbiology* 69: 177–184 (2020).
13. G.S. Mahardhika, T.D. Tedjamartono, and I.P. Denny. Expanded Dengue Syndrome with Hemolytic Anemia: A Case Report. *IRMHS* 3: 37–49 (2020).
14. J.G. McHutchison, M.P. Manns, and D.L. Longo. Definition and management of anemia in patients infected with hepatitis C virus. *Liver International* 26: 389–398 (2006).
15. F. Masaisa, J.B. Gahutu, J. Mukiibi, J. Delanghe, and J. Philippé. Anemia in human immunodeficiency virus-infected and uninfected women in Rwanda. *American Journal of Tropical Medicine and Hygiene* 84: 456–460 (2011).
16. T.I. Hariyanto, and A. Kurniawan. Anemia is associated with severe coronavirus disease 2019 (COVID-19) infection. *Transfusion and Apheresis Science* 59: 102926 (2020).
17. S.J. Hemauer, A.J. Kingeter, X. Kingeter, M.S. Shotwell, P.P. Pandharipande, and L.M. Weavind. Daily lowest hemoglobin and risk of organ dysfunctions in critically ill patients. *Critical Care Medicine* 45: 479–484 (2017).
18. F. Morinet, M. Leruez-Ville, S. Pillet, and S. Fichelson. Concise review: Anemia caused by viruses. *Stem Cells* 29: 1656–1660 (2011).
19. A.N. Shah, J.S. Kothari, D.P. Desai, T. Savaliya, and R.B. Prajapati. Clinical and biochemical outcome in pediatric patients with iron deficiency anemia. *International Journal of Contemporary Pediatrics* 7: 2146-2151 (2020).
20. P. Bellavite, and A. Donzelli. Adverse events following measles-mumps-rubellavaricella vaccine: an independent perspective on Italian pharmacovigilance data. *F1000Research* 9: 1–18 (2021).
21. T. Marichal, C. Mesnil, and F. Bureau. Homeostatic eosinophils: Characteristics and functions. Frontiers of Medicine (Lausanne) 4:101–6 (2017).
22. H.A. Alemam, A.A. S.E. Omar Abadi, M.A. Salem, L. Elghawi, and A. Bashein. A comparative study of alkaline phosphatase level in serum of patients with end-stage renal disease, viral hepatitis (C), and (B). I*n The 4th Annual conference on theories and applications of basic and biosciences* 291-298 (2020).
23. Y. Sakurai, and T. Higashiguchi.Transient hyperphosphatasemia: Possible association with pediatric acute respiratory infection. *Pediatric Investigation* 5: 94–98 (2021).

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Research Article

Prescribing Pattern of Ampicillin and Cloxacillin: Sensitivity and Responsiveness in Pneumonia

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Abstract: Antimicrobial resistance (AMR) is now a global pandemic and a future threat to the existence of many clinical antibiotics. The excessive overuse in fisheries, poultries, and dairy farms and its irrational prescribing practices are the key factors that lead us to AMR explosions. The current main research objective is to evaluate the empirical practices of ampicillin along with cloxacillin, which are one of the running antibiotics in clinical practices, in most of the tertiary care hospitals in Khyber Pakhtunkhwa, Pakistan. In this study, the prescribing attitude, sensitivity and responsiveness of these two combinatory antibiotics (ampicillin-cloxacillin) in pediatric/adult pneumonia patients were evaluated in one of the public sector tertiary care hospitals in Mardan. Retrospective data was collected from pediatric ward A and medical A ward (adult), among which a total of n= 90 patient's prescriptions were evaluated for prescribing practices, WHO core indicators, polypharmacy as well as responsiveness and sensitivity of ampicillin and cloxacillin from hospital longevity. The ampicillin responsiveness was sorted out among all those patients that stayed for a long time in the hospital, and during which the antibiotic therapies were switched from time to time. A total of n= 90 pneumonia patients (40 % & 60 % female) cases were evaluated for ampicillin/cloxacillin (combination) hospital stay longevity and responsiveness. 46 % of patients were under the age of 1–20 years, whereas 31 % were adults between the age of 61–80 years. WHO indicators revealed, that in the prescribed medications (n=918 drugs total, among n=90 patients) 22.33 % of antibiotics were prescribed, where 31.37 % consisted of injectables. Among the antibiotics classes, 17 % of pneumonia patients received penicillin, among which 26.25 % were ampicillin + cloxacillin in the prescribing practices. Ampicillin + cloxacillin responsiveness in pneumonia patients was recorded from the hospital stay and longevity (days) of the patients during their empirical therapy. 46.98 % of pneumonia patients recovered within three days, whereas 40.96 % of patients recovered within six days with ampicillin + cloxacillin (combination therapy). While 10.84 % were stabilized within nine days, though, some patients (1.20 %), recovered after 12 days with ampicillin/cloxacillin (combination therapy). Thus, it may be concluded from the current studies, that the decrease in responsiveness to ampicillin/cloxacillin (combination therapy) and the increase in the hospital longevity of patients, may be an indication of antimicrobial resistance (AMR) in pneumonia patients. Though the studies are limited to a very specific number of patients, as well as only to the hospital longevity (stay) parameters of the patients in a tertiary care hospital. These studies should be subjected further to more extensive vigilant research.

Keywords: Ampicillin, Cloxacillin, Antibiotic responsiveness , Antimicrobial resistance (AMR), Hospital longevity.

1. INTRODUCTION

Around the world, pneumonia is a major factor in both morbidity and death among children. Incidence of pneumonia in children under the age of five is thought to be 120 million per year worldwide, with 1.3 million cases ending in death. Pneumonia should be defined as an acute infection of lung parenchyma by one or more than one pathogens, excluding the well-defined state of bronchiolitis,

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which is almost caused by a viral agent [1]. Different types of pneumonia are bacterial pneumonia, viral pneumonia, and mycoplasma pneumonia. Viral pneumonia, which accounts for around one-third of all occurrences of pneumonia, is brought on by viruses like the flu, whereas bacterial pneumonia is brought on by bacteria. Atypical pneumonia, also known as mycoplasma pneumonia, is brought on by bacteria and affects people of all ages, mostly smokers, alcoholic individuals, asthmatics and people with weak immune systems are at higher risk to be affected by and diagnosed with pneumonia [2]. One million children kill under the age of 5 every year due to pneumonia and are responsible for 15 % of all pediatric fatalities, with 90 - 95 % of these deaths taking place in underdeveloped nations. Only 15 nations account for 2/3 of pneumonia episodes in children under the age of five mostly in the south- Asia and sub -Saharan African countries [3]. In Pakistan, mortality rate for meningitis and pneumonia range from 16 - 37 % and 10 - 30 %, respectively. Notably, the mortality rate among young infants is higher in underdeveloped nations (10 - 40 %) due to worse access to the health care system. Mortality rates were 3 % for children under the age of five, 14 % for people in the range of 5 to 65, and 24 % for adults over the age of 65 years [4, 5].

In preschool children with pneumonia, antimicrobial treatment is not typically advised (because of viral infections). Streptococcus pneumoniae is still the most usually implicated pathogen, amoxicillin or amoxicillin-clavulanate is the most recommended first-line antimicrobial treatment for community-acquired pneumonia (CAP) in children [6]. The ketolides, vancomycin, and the more recent anti-pneumococci fluoroquinolones (gemifloxacin, levofloxacin, moxifloxacin, trovafloxacin, and pefloxacin), as well as the newly available oxazolidinone linezolid, are all effective against drug resistance *Strepto coccus Pneumoniae* (DRSP) [7]. Since children are frequently the targets of infections with a variety of etiologies, ranging from the more common chest infections to the less common meningitis, they are thought to be the most frequent receivers of antibiotics than any other category of patient.

The discovery of antibiotics and the emergence of AMR goes side by side, since its discovery to date. AMR is a future pandemic and considers a global threat to the existence of many antibiotics [5]. Rising AMR is one of the greatest threats to global public health since it raises morbidity, mortality, and costs while reducing the selection of antimicrobials that may be used as possible treatments [8]. The proper administration of antibiotics in children is crucial because there are few antibiotic formulations acceptable for this demographic. In several countries, studies of antibiotic prescribing trends in primary care facilities for children have revealed improper antibiotic usage ranging from 19.6 - 79.8 %. Children, on the other hand, are special drug-using groups, as well as their organs and functions, are underdeveloped. They have a distinct digestive system, insufficient liver and renal metabolism, and insufficient blood-brain barrier function absorption of antibiotics. The distribution, metabolism, and excretion are all poorer than in adults. As a result, more emphasis should be placed on the antibiotics that are being used inappropriately in this group [9]. Resistance in bacteria developed, either through their drug-protein target modification, enzymes, or through genetic evolutions [10].

To preserve the efficacy of antimicrobials, the worldwide mostly implemented program and approaches comprised of public awareness campaigns and antimicrobial guidelines; though, further tactics and strategies concentrated on vaccination, and varying protocols around recommending and repayment [11, 12]. It includes rational antimicrobial usage, regulation of antibiotic over-the-counter access, improved hand hygiene, and improved infection prevention and control. The need is for a thorough knowledge of resistance mechanisms as well as innovation in novel medications and vaccines. To tackle antimicrobial resistance, a multidisciplinary, coordinated regulatory strategy is required [13]. Drug regulatory authority of Pakistan (DRAP) has also adopted various strategies and policies for the control of AMR various policies and strategies [14-17].

Reform is required to overcome policy implementation difficulties. Today, declining antibiotic efficiency poses a danger to human and animal health, and hence to global development. Deaths from drug-resistant illnesses are expected to

rise from 700,000 to 10 million per year, with costs estimated to reach $100 trillion by 2050 [18]. The Centers for Disease Control (CDC) in the United States declared in 2013 that the human race had entered the "post-antibiotic" age. Additionally, the rising prevalence of antimicrobial resistance traits in bacteria is an evolutionary reaction to the widespread use of antimicrobials [19]. The emergence of AMR in modern human civilization will increase the use of older, less efficient infection-control measures on an individual level. Such procedures, such as debridement, disinfection, amputation, and isolation, will result in a lengthier, more intrusive, and less effective treatment of infections [20].

The prescribing practices of antibiotics in our healthcare settings are mostly on empirical therapies (without culture sensitivity tests), which may be due to the either expensive or may be due to severity of diseases, for which the patient mostly needs urgent antimicrobial therapies. There is no proper method to identify or diagnose antibiotic sensitivity or responsiveness in healthcare settings. The only observations are from prescribing practices and attitude and the patient response to the respective antibiotics. In public sector hospitals, patients' antimicrobial therapies changed due to non-responsiveness to antibiotics, which seems to be AMR [17].

So the current study is also an approach to observe the clinical prescribing practices and attitude of one of the running antibiotics ampicillin and cloxacillin in public sector hospitals. Pediatric therapies start in most cases with these antibiotics and then changed due to non-responsiveness and sensitivities. This way, patient hospital stay (longevity) is prolonged, which may increase the hospital stay time, cost, and other associated consequences which may affect both patients and physicians.

2. MATERIALS AND METHODS

2.1 Study Design and Setting

The current study of ampicillin and cloxacillin responsiveness in pediatric pneumonia was conducted in the paeds ward and medical A ward in a tertiary care public sector hospital of Mardan, KP, Pakistan. It is the 4th largest tertiary healthcare unit with 550 beds in Pakistan that has offering tertiary healthcare facilities to the local community and nearby cities, towns, and villages (Figure 1).

2.2 Data Collection

The two months' retrospective data were accessed from patient records (January-Feburaray, 2022). The patient's medical records, age, sex, dates

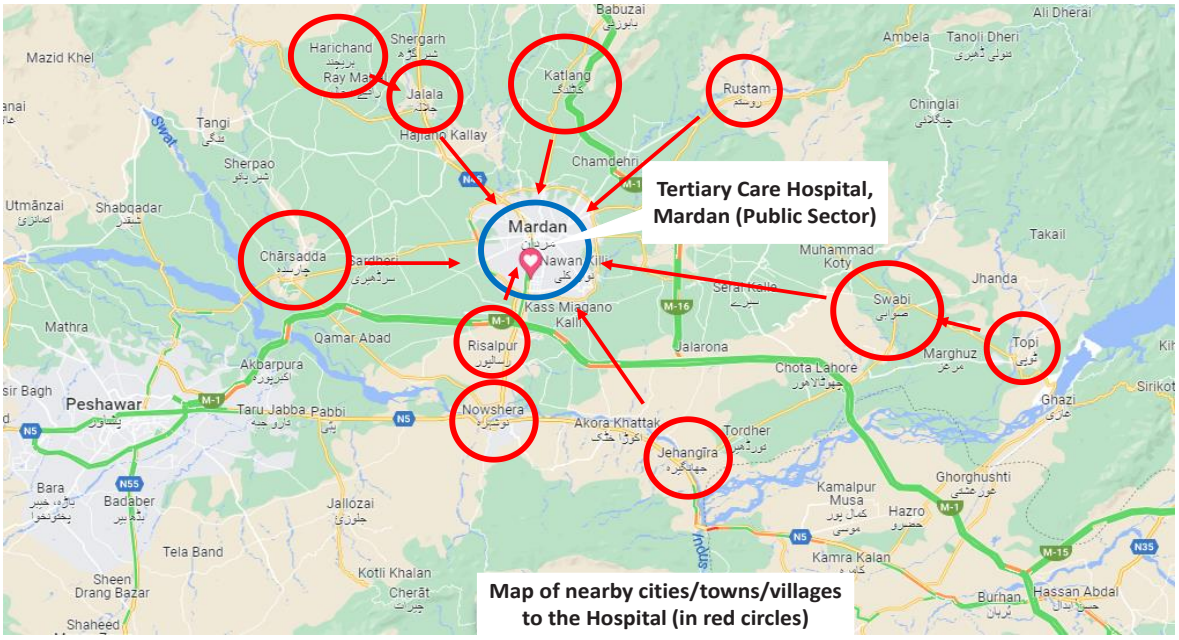

Fig. 1. Cities and districts within the vicinity of the tertiary care hospital, Mardan (copyright google map with modification)

of admission and discharge, medical history, presentation of signs and symptoms, and initial categorization of pneumonia during the hospital stay, treatment schedules, and hospital stay (longevity) were observed.

2.3 Inclusion/Exclusion criteria

All those patients (of all ages) who were diagnosed with pneumonia, and were under medication therapies, stayed for more than one day in the hospital ward, were included in the study. While patients who expired in the hospital or those with incomplete information were excluded from the studies.

2.4 Prescribing practices and WHO core indicators

Prescriptions are a legal document/consent between a physician and patients on medication management in a healthcare setting, on basis of which medications are administered by the pharmacist to the patients. In the current study, all prescription patterns in medical wards A and B in tertiary care hospitals, Mardan were evaluated for all those indicators that may assist in the rational use of medicines as well as the antibiotic prescribing attitude that comes under standard guideline procedures. Similarly, prescriptions writing was coordinated with the WHO core indicator utilized for each prescription, which includes the total number of items per prescription, drugs with generic names in percent, percent of prescriptions with antibiotics, injectable per encounters percentage of encounters, and EDL of 2020 was utilized. WHO baseline indicators standard guidelines for prescription writing were mentioned in Table 1, it should be noted, that WHO core indicators are recommended or proposed standard guidelines, they are not implemented by the drug regulatory authority of Pakistan (DRAP) or Pakistan medical and dental council (PMDC).

2.5 Hospital longevity and ampicillin/ cloxacillin responsiveness

Hospital stay and longevity of patients being affected by pneumonia were calculated from the date of admission and discharge of the patients. Where the ampicillin/cloxacillin responsiveness was observed from the antibiotic therapy from the patient history chart. During the medication schedules, antibiotics therapy was changed due to non-responsiveness and patient instability. As in the case of children, mostly empirical therapy was followed, no CSTs were conducted in most practices, as they need urgent treatment and CSTs took more than 72 hrs. Therefore, based on symptoms and lab findings, empirical therapy was started for proper treatment on physician directives.

2.6 Statistical analysis

Graphs were plotted and appropriate statistically significant differences were assessed by Student t-test and for multiple comparisons and co-relations using graph pad prism software 8.4.2 (679) version. The differences were significant statistically when * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** $p < 0.0001$, where ns indicated non-significant.

Table 1. WHO core indicators standard values for prescribing practices/attitude.

WHO Core indicators	Standard values (recommended/proposed)
Total number of drugs per encounter	1.6-1.8 (~2)
Total number of Injectables (%)	13.4-24
Total number of Antibiotics (%)	20-26.8
Total number of drugs Prescribed on the generic name (%)	100
Total number of drugs from EDL (%)	100

3. RESULTS

3.1 Study area and locations

Mardan is one of the important and second largest cities of KP, while the 19th biggest city of Pakistan, which is located in the valley of Peshawar, has a population of 358, 604 inhabitants (2017 Census). Mardan is located at an altitude of 283 m and in the southwest of the district at 34°12′0 N 72°1′60 E. To the south, Risalpur, West Charsadda, Yar Hussain to the east, and Takhtbahi and many more districts in vicinities are shown in Figure 2. Where the tertiary care hospital is a 550-bed medical complex and teaching hospital that provides basic health to nearby cities, towns, and villages.

3.2 Patient demographics and locality

A total of n= 90, retrospective patient data were collected from both pediatric ward A and medical ward A (adults), within two months, Figure 2 and Figure 3 show gender-wise and age-wise information of all patient details collected respectively. The highest ratios of pneumonia were recorded among paeds (1-20 years) and adults (60-80 years).

3.3 Prescribing practices and WHO core indicators

Table 2 shows that the total number of drugs prescribed to the patients are n= 918 of which 370 (40.26 %) were prescribed to females and 548 (59.74 %) were prescribed to male patients. Figure 4 shows various dosage forms prescribed, among which injectables were prescribed in high percentages. Figure 5 shows the percentage of drugs per encounter (polypharmacy) in prescribing practices, whereas detail is given in Table 3.

3.4 Ampicillin/cloxacillin sensitivity and responsiveness in pneumonia patients

Table 4 shows the number of total drugs prescribed to pneumonia patients and their percentages, where Figure 6 shows, the number of patients and their hospital stay, while Figure 7 shows the hospital stay or longevity of patients using ampicillin/cloxacillin prescribing, its effects during the hospital stay and their responsiveness in pneumonia patients. Figure 8 shows the percentage of patients (longevity), who received ampicillin /cloxacillin therapy for pneumonia treatment during the hospital stay. It should be noted that a total of n=83 out of 90 pneumonia patients received ampicillin + cloxacillin therapy.

4. DISCUSSION

As per WHO core indicators findings, in this study, a total of n= 918 drugs were prescribed among all n=83 pneumonia patients, which makes an encounter of 10.21/ prescription, which is much higher than the recommended and proposed value of WHO core indicator (1.6–1.8). Though,

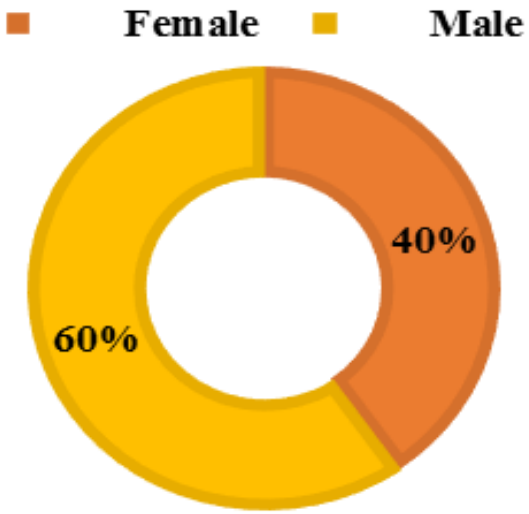

Fig. 2. Gender-wise pneumonia patient percentage collected

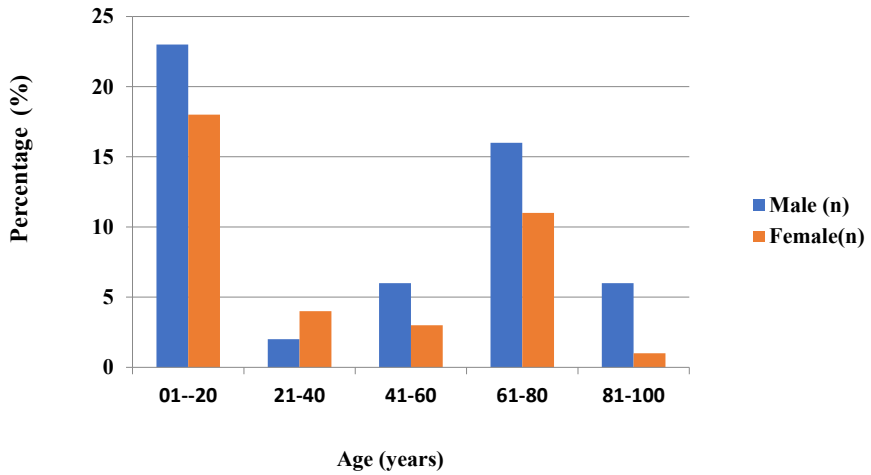

Fig. 3. Age wise distribution of patients in both pediatric and medical ward A.

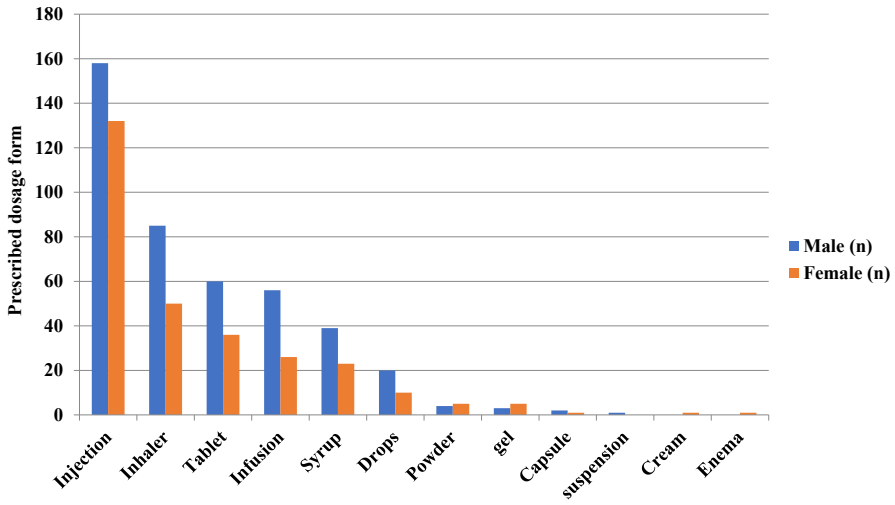

Fig. 4. Dosage form prescribed among the male and female pneumonia patients

Table 2. WHO Core indicator extracted from patient prescription in pediatric A and medical ward A (adults) of a public sector tertiary care hospital

WHO Core indicators			
WHO indicators	**Frequency (*n*)**	**Average number of drugs prescribed**	**Standard values recommended by WHO**
Total number of drugs per encounter	918	10.20	1.6-1.8 (~2)
Total number of Injectable (%)	288	31.37 %	13.4-24 %
Total number of Antibiotics (%)	205	22.33 %	20-26.8 %
Total number of drugs Prescribed on the generic name (%)	127	13.83 %	100 %
Total number of drugs from EDL (%)	898	97.82 %	100 %

*EDL: essential drug list

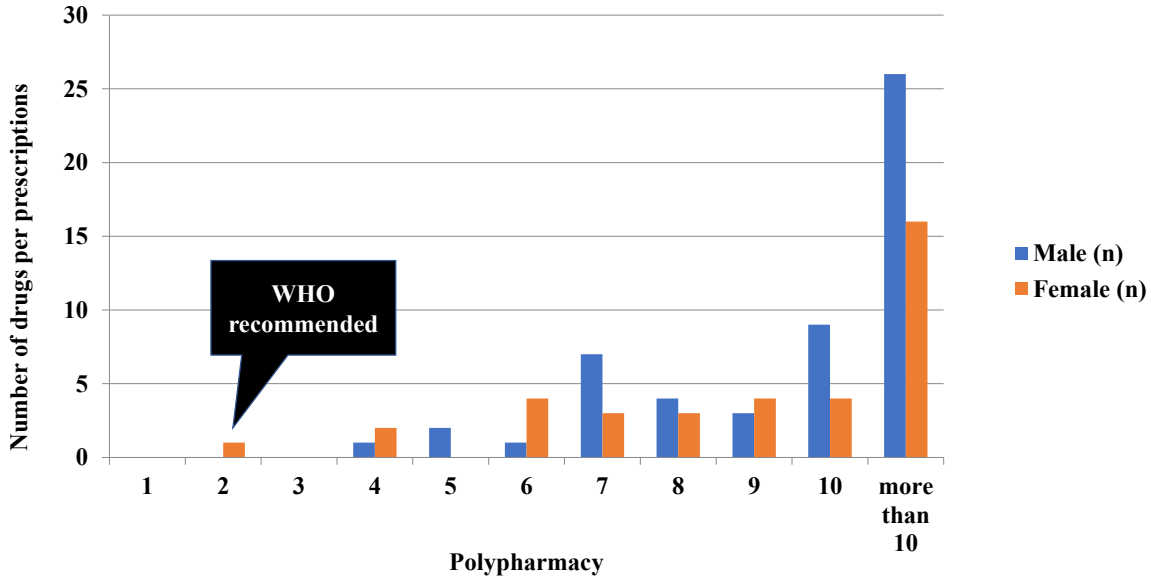

Fig. 5. Polypharmacy and number of drugs per prescription.

Table 3. Percentage of the class of antibiotics prescribed among pneumonia patients

Class of Drug	Male (n)	Female (n)	Total (*n*)	Percentage (%)
Other Antibiotics	42	33	75	36.58
Cephalosporin	44	31	75	36.58
Penicillin	**21**	**14**	**35**	**17.08**
Quinolones	8	4	12	5.86
Macrolide	4	2	6	2.92
Aminoglycoside	2	0	2	0.98
Total	121	84	205	100

Furosemide	8	0.87146
Zinc sulphate	13	1.416122
Miconazole	9	0.980392

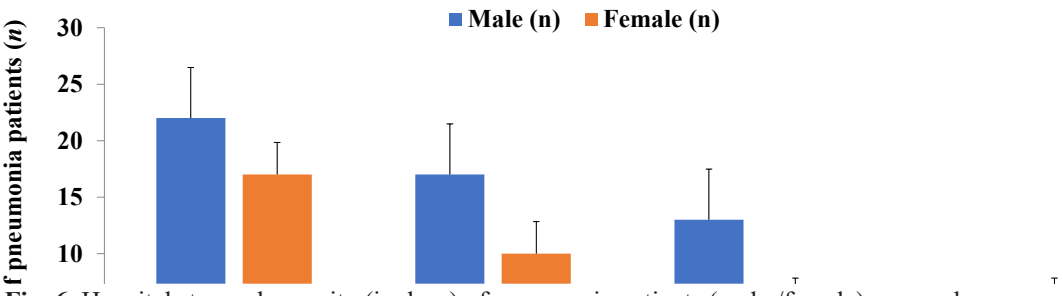

Fig. 6. Hospital stay or longevity (in days) of pneumonia patients (male /female), n=number

Table 4. Percentage of drugs prescribed to pneumonia patients

Prescribed medications	**Frequencies**	**Percentage**
Total number of Drugs prescribed in n=90 prescriptions	918	100%
Average no of Drugs/case	10.20	……
Ampicillin + Cloxacillin	**241**	26.25272
Paracetamol	148	16.122
Clarithromycin	40	4.357298
Cefotaxime	79	8.605664
Ceftriaxone	25	2.723312
Ceftazidime	22	2.396514
Cefoperazone +Sulbactam	26	2.832244
Ibuprofen	26	2.832244
Acefylline	15	1.633987
Amikacin	15	1.633987
Linezolid	57	6.20915
Dexamethasone	143	15.57734
Hydrocortisone	40	4.357298
Captopril	5	0.544662
Midazolam	6	0.653595
Furosemide	8	0.87146
Zinc sulphate	13	1.416122
Miconazole	9	0.980392

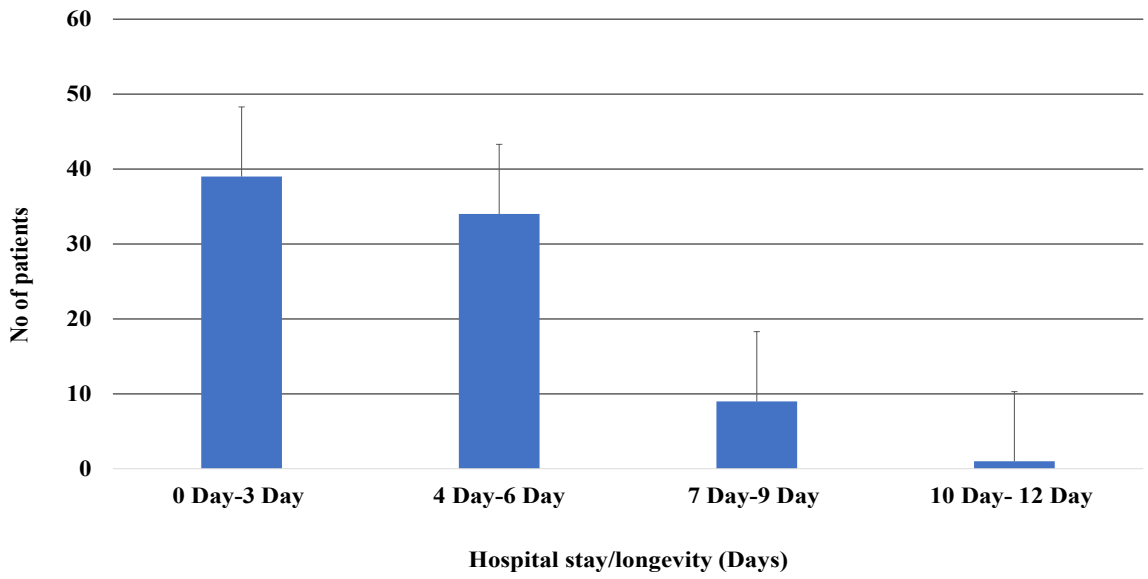

Fig. 7. Hospital longevity (days) among patient using ampicillin / cloxacillin and then therapy switched over to another antibiotic therapy.

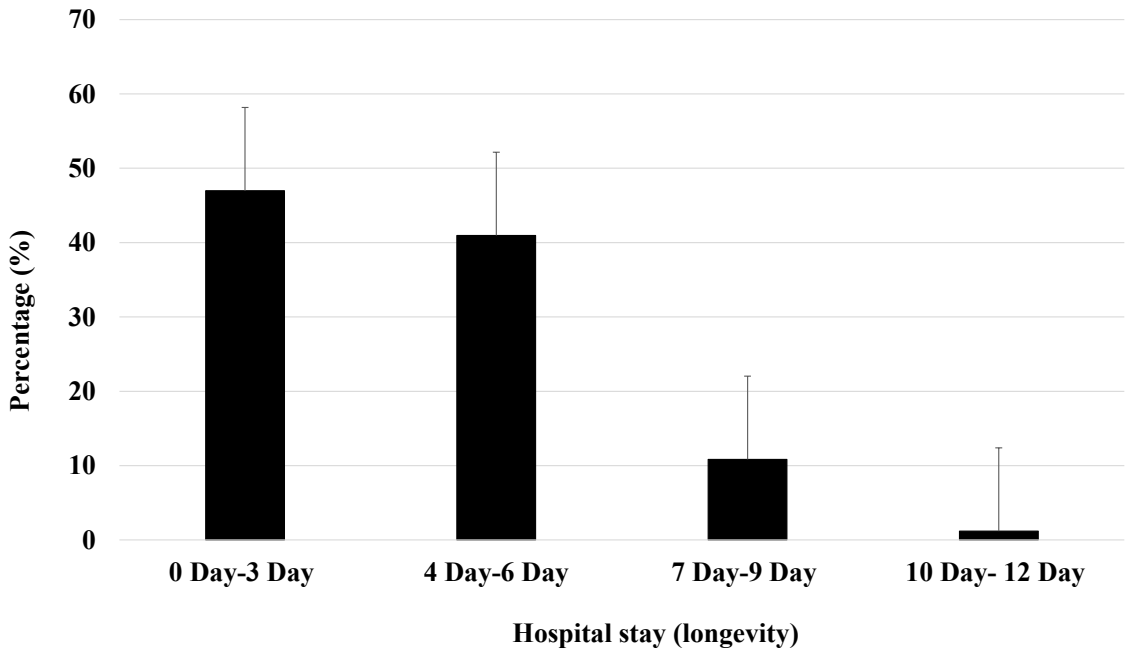

Fig. 8. Percentage of pneumonia patients receiving ampicillin /cloxacillin therapy and hospital stay (Note: *n*=83 pneumonia patients received ampicillin/ cloxacillin)

it's very common practice in Pakistan, where an average prescription may contain more than 5–7 drugs, which leads to polypharmacy or multidrug prescriptions. Polypharmacy may have serious consequences, such as enhancing the ADR, s drug interaction and decreasing the compliance rate in patients. Similarly, regarding the prescribing practices and attitude, the average no of antibiotics prescribed to the patients was 22.3 %, which is the same as that of WHO recommended value (20 - 26 %), and lower than 70 % in a study found in South West Ethiopia [24, 25].

According to WHO core indicators, antibiotics prescribing was 22.33 %, which is somewhat near the standard recommended guidelines (20 - 22.8 %). As, mostly, pneumonia patients are kept on injectable antibiotic therapies to achieve prompt therapeutic responses. Though higher injectables and antibiotic therapies are rational approaches, the main problem as to antimicrobial responsiveness and sensitivity, which may prolong our hospital stay [24, 26-29]. Here in our studies, regarding the antibiotics' responsiveness and hospital stay longevity, first evaluated the most highly prescribed antibiotics. In this regard, cephalosporins were prescribed to 36.58 % of pneumonia patients, whereas penicillin was prescribed to 17.08 %, which were mostly ampicillin and cloxacillin (combinations). Among the number of prescribed antibiotics, ampicillin/ cloxacillin (combinations) were highly prescribed (26.25 %) in pneumonia patients. Patients were initially kept on ampicillin/cloxacillin therapies and were mostly stabilized within three Days (n=39 patients), where n=34 patients recovered within six Days. While hospital stays responsiveness of patients n=9, longevity reached nine days while receiving ampicillin /cloxacillin therapy, whereas n=1 patients reached up to twelve days, while receiving the same therapy. So, patients receiving ampicillin /cloxacillin empirical therapies and associated hospital stay and longevity may be considered an indicator for AMR resistance, less responsiveness, and hospital longevity indicates weak responsiveness to the infections and may proceed for culture analysis. From the current findings, in pneumonia patients, it was observed 46.98 % of pneumonia patients (including both males and females), recovered within three days with empirical therapy of ampicillin /cloxacillin, while 40.96 % people show responsiveness within six days of their hospital stay, where 10.84 % patients, the hospital stay longevity increased up to nine days. Still, 1.2 % of the people's hospital stay increased up to 12 days. Further extensive studies should be carried out on its culture sensitivity tests

for sensitivity and responsiveness of ampicillin/ cloxacillin in both inpatients and outpatients.

5. CONCLUSION

From current findings, it can be concluded, that the responsiveness of ampicillin /cloxacillin combinations in reference to hospital stay and longevity is an alarming state in the existing therapy of pneumonia, especially in public sector hospitals in KP, Pakistan. Increasing hospital stay and longevity among patients may be an indication of less responsiveness and sensitivity, which may be due to AMR. Children and adults as well as both male and female genders are at equal risk of AMR, which needs further extensive research and proper evaluation for its control and prevalence. Secondly, hospital stay longevity also indicates the feeble and low responsiveness of antibiotics in hospital patients who are affected by bacterial infections. During their therapies, various antibiotics were changed, due to weak responsiveness to antibiotics. Therefore, keep in mind, all physicians and healthcare professionals should avoid empirical therapies, they should follow CSTs for sensitivity and resistance of most antibiotics and should also keep vigilant approaches for hospital stay and longevity, especially among pneumonia patients. The current, studies have limitations, as this was only confined to pneumonia, as well to a single public sector hospital, such studies should be extended to various bacterial infections as well to many more teaching hospitals and medical complexes in future endeavors.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

8. REFERENCES

1. G. Mackenzie. The definition and classification of pneumonia. *Pneumonia* 8: 1-5 (2016).
2. Y. Rong, L. Tang, Y. Tian, J. Liu, and Z. Wu. Identification and classification of pneumonia disease using a deep learning-based intelligent computational framework. *Neural Computing and Applications* 1-14 (2021).
3. D.A. Rodrigo, J.P Yarzabal, J.P Cruz, J.P. Schmidt, J. Kleijnen, and J Johannes. Epidemiology of community-acquired pneumonia and implications for vaccination of children living in developing and newly industrialized countries: A systematic literature review. *Human vaccines & immunotherapeutics* 12: 2422-2440 (2016).
4. I.F.N. Hung, T. Tantawichien, Y. H. Tsai, S. Patil, and R. Zotomayor. Regional epidemiology of invasive pneumococcal disease in Asian adults: epidemiology, disease burden, serotype distribution, and antimicrobial resistance patterns and prevention. *International Journal of Infectious Diseases* 17: e364-e373 (2013).
5. M.I. Hutchings, A.W. Truman, and B. Wilkinson. Antibiotics: past, present and future. *Current Opinion in Microbiology* 51: 72-80 (2019).
6. V. Clifford, M. Tebruegge, M. Vandeleur, and N. Curtis. Question 3: can pneumonia caused by penicillin-resistant Streptococcus pneumoniae be treated with penicillin? *Archives of Disease in Childhood* 95: 73-77 (2010).
7. C. Cillóniz, C. Dominedò, and A. Torres. Multidrug resistant gram-negative bacteria in community-acquired pneumonia. *Annual Update in Intensive Care and Emergency Medicine* 2019: 459-475 (2019).
8. K. Hayat, Khezar, M. Rosenthal, A. H. Gillani, J. Chang, j. Wenjing , C. Yang, M. Jiang, M. Zhao, and Y. Fang. Perspective of key healthcare professionals on antimicrobial resistance and stewardship programs: a multicenter cross-sectional study from Pakistan. *Frontiers in Pharmacology* 10: 1520 (2020).
9. W. Wang, Y. Chang, S. Yu, X. Zhou, L. Wang, X. He, and H. Zhou. Antibiotic prescribing patterns for children's outpatient department of primary care institutions in Southwest China. *BMC Primary Care* 23: 269 (2022).
10. T.V Shanbhag, S. Shenoy, and V. Nayak, *Pharmacology for Dentistry, E-book. Published by Elsevier Health Sciences* (2021).
11. G. Birgand, E. Castro-Sánchez, S. Hansen, P. Gastmeier, J.C Lucet, E. Ferlie, and R. Ahmad. Comparison of governance approaches for the control of antimicrobial resistance: analysis of three European countries. *Antimicrobial Resistance & Infection Control* 7:1-12 (2018).
12. R.V Katwyk, Susan, J.M Grimshaw, M. Nkangu, R.

Nagi, M. Mendelson, M. Taljaard, and S.J. Hoffman. Government policy interventions to reduce human antimicrobial use: a systematic review and evidence map. *PLoS Medicine* 16: e1002819 (2019).

13. R.R. Uchil, G.S. Kohli, V.M. KateKhaye, and O.C. Swami. Strategies to combat antimicrobial resistance. *Journal of Clinical and Diagnostic Research* 8: ME01 (2014).
14. S. Zikria, M.A. Hassali, F.K. Hashmi, B. Godman, and F. Saleem. Antimicrobial dispensing practices and determinants of antimicrobial resistance: a qualitative study among community pharmacists in Pakistan. *Family Medicine and Community Health* 7: (2019).
15. M.S. Khan, A.D. Bagale, A. Mateus, Z. Sultana, R. Hasan, and J. Hanefeld. What are the barriers to implementing national antimicrobial resistance action plans? A novel mixed-methods policy analysis in Pakistan. *Health Policy and Planning* 35: 973-982 (2020).
16. R. Jabeen, U. Rabbani, and N. Abbas. Situation analysis of antimicrobial resistance control, policy and regulation-a reflection of Pakistan. *Pakistan Journal of Pharmaceutical Sciences* 34: (2021).
17. C. Esmita, M. McKee, R. Ahmad, M. Balasegaram, C. Bonaconsa, G. B. Merrett, and R. Busse et al. Optimising antimicrobial use in humans–review of current evidence and an interdisciplinary consensus on key priorities for research. *The Lancet Regional Health-Europe* 7: 100161 (2021).
18. J. Dušan, J. Littmann, A. Zorzet, and O. Cars. Antimicrobial resistance—a threat to the world's sustainable development. *Upsala Journal of Medical Sciences* 121: 159-164 (2016).
19. C.A Michael, D.D. Howes, and M. Labbate, The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in Public Health* 2: 145 (2014).
20. R.D Smith, M.R. Keogh-Brown, and T. Barnett. Estimating the economic impact of pandemic influenza: an application of the computable general equilibrium model to the UK. *Social Science and Medicine* 73: 235-244 (2011).
21. L.Y. Tsung, Y.Y. Jeng, T.L. Chen, and C.P. Fung. Bacteremic community-acquired pneumonia due to Klebsiella pneumoniae: clinical and microbiological characteristics in Taiwan, 2001-2008. *BMC Infectious Diseases* 10: 1-7 (2010).
22. R. Asghar, S. Banajeh, J. Egas, P. Hibberd, I. Iqbal, M.K Bwalya, Z. Kundi, P. Law, W. MacLeod, I.M Radovan, and G. Mino. Chloramphenicol versus ampicillin plus gentamicin for community acquired very severe pneumonia among children aged 2-59 months in low resource settings: multicentre randomised controlled trial (SPEAR study). *British Medical Journal* 336: 80-84 (2008). .
23. W. Fonseca, K. Hoppu, L.C Rey, J. Amaral, and S. Qazi. Comparing pharmacokinetics of amoxicillin given twice or three times per day to children older than 3 months with pneumonia. *Antimicrobial Agents and Chemotherapy* 47: 997-1001 (2003).
24. A.H Mariam, Y. Raghavendra, and E.M Bobasa. Evaluating rational drug use with the help of World Health Organization's core indicators in Bule Hora Hospital, Southern Ethiopia. *Encounters* 7: 11 (2015).
25. D.B. Mamo, and B.K. Alemu. Rational drug-use evaluation based on World Health Organization core drug-use indicators in a tertiary referral hospital, Northeast Ethiopia: a cross-sectional Study. *Drug, Healthcare and Patient Safety* 12: 15 (2020).
26. M. Atif, M.R. Sarwar, M. Azeem, D. Umer, A. Rauf, A. Rasool, M. Ahsan, and S. Scahill. Assessment of WHO/INRUD core drug use indicators in two tertiary care hospitals of Bahawalpur, Punjab, Pakistan. Journal of Pharmaceutical Policy and Practice 9: 1-8 (2016).
27. B.D. Mekonnen, M.Z. Ayalew, and A.A. Tegegn. Rational drug use evaluation based on world Health organization core drug use indicators in Ethiopia: a systematic review. *Drug, Healthcare and Patient Safety* 13: 159 (2021).
28. J.T.M. Shrestha, S. Tiwari, D.K Kushwaha, P. Bhattarai, and R. Shrestha. Drug Prescription in the Department of Medicine of a Tertiary Care Hospital according to the World Health Organization/ International Network for Rational Use of Drugs Core Indicators: A Descriptive Crosssectional Survey. *Journal of the Nepal Medical Association* 59: 745 (2021).
29. C. Yue, S. Chusri, R. Sangthong, E. McNeil, J. Hu, W. Du, D. Li et al. Clinical pattern of antibiotic overuse and misuse in primary healthcare hospitals in the southwest of China. *PLoS One* 14: e0214779 (2019).

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Research Article

Knowledge, Attitude and Practice towards Covid-19 in Different Universities across Khyber Pakhtunkhwa, Pakistan

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Abstract: There have been 6,566,610 fatalities and 626,337,158 reported cases of COVID-19 worldwide. Pakistan presently has over 1,573,922 confirmed cases and 30,625 deaths. A survey-based study was performed from January to September 2022 among different university communities to find out their KAP level. Data was collected in Google Forms through a questionnaire. The Independent t-test, Multinomial regressions, and Non-parametric Mann-Whitney test were used to assess the level of significance (p-value ≤0.05). 317 out of 605 participants were male (52.5 %), the majority of participants were 15-29 years old (72.7 %), unmarried/divorced (71.6 %), have no children (82.1 %), residing in the urban area (54.9 %) and possess a college/university degree (59.5 %). The majority of participants correctly answered five out of six knowledge questions (M = 4.96, SD = 1.03) and correctly identified the primary symptoms of COVID-19 (94.4 %) along with the proper identification of mode of transmission (95.2 %) while 1.8% wrongly replied and 2.1 % did not reply. A knowledge test revealed the significant frequency of misconception, with nearly half of the respondents (46.3 %) assuming that illness might be contracted by eating or coming into touch with wild animals. Wearing a facial mask is highly practiced (M = 3.59, SD = 0.91), followed by avoiding crowded places (M = 3.44, SD = 0.95) and practicing hand hygiene (M = 3.36, SD = 1.04). Females, the elderly, and the less educated, on the other hand, have less understanding of COVID-19, making them especially susceptible to the pandemic. It is proposed that further awareness might contribute to a favorable attitude and practice.

Keywords: Knowledge, Attitude, Practice, Covid-19, Pandemic, Preventive Strategies

1. INTRODUCTION

Millions of people were infected by COVID-19 worldwide, since it first surfaced in Wuhan, China, in December 2019 [1]. January 30, 2020, was the day when the COVID-19 outbreak was declared a Public Health Emergency of International Concern (PHEIC), asking governments to take rapid and

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decisive measures against the virus [2, 3]. Unlike previous corona outbreaks [4], this extremely infectious zoonotic virus [5, 6] from an unknown animal source has gone from a local flu-related severe acute respiratory syndrome [4, 7] to a pandemic endangering millions of lives in a matter of weeks. COVID-19 has wreaked havoc on global public health by putting an undue burden on the world's healthcare systems.

As it spreads through social interactions [8, 9], billions of people have already been put on lockdown, which has affected hundreds of thousands of people in just a few months, causing them economic and psychological stress [4].

There have been 6,566,610 fatalities and 626,337,158 reported cases of COVID-19 worldwide. A total of 12,830,378,906 doses of vaccine have been given as of October 26, 2022 [2]. The pandemic has put a strain on global health systems. A near-universal opinion confirms that combating COVID-19 will require a multisectoral response that includes participation not only from other sectors but also from communities and the general public [10]. While much has been reported about the nature and scope of the healthcare response [11], little is known about how people view COVID-19, particularly in low- and middle-income countries [12].

Simple personal protection measures like masks or hand washing, as well as more complex interventions like lockdowns, restrictions on public gatherings, and crowded place shutdowns, can only be successful if the population has sufficient knowledge and appropriate attitudes to ensure their effective implementation [13]. This is made more difficult in areas where education is not uniform and where historical, regional, and religious customs can hinder the implementation of such initiatives. For resource-strapped in low- and middle-income countries, the only hope is to empower communities so that they can take both short- and long-term preventative measures [14].

Pakistan presently has over 1,573,922 confirmed cases and 30,625 deaths (as of October 30, 2022), with community transmission accounting for 92 % of infections [15]. It also has a weak health system, a digital gap, and poor literacy rates, all of which point to the necessity for a comprehensive communication strategy to safeguard the public against COVID-19. Considering the above, we conducted a survey to determine the level of KAP and their determinants of COVID-19 among different university communities.

2. MATERIALS AND METHODS

2.1 Study design

A cross-sectional survey-based study was conducted from January - September 2022 to determine the level of KAP and its factors towards COVID-19 among different university communities.

2.2 Data Collection and Management

A structured questionnaire was established in a Google Form by reviewing the scientific literature and available information on COVID-19 that are relevant. The questionnaire was written in English. There were three sections in the questionnaire: Socio-demographic information, Responses to Knowledge items, and Responses to knowledge, attitudes, and practices.

Convenience samplings were used in this research. The Google Form link was posted on several platforms after a call for participation. Other social media platforms were used to maximize engagement.

2.3 Data Analysis

For data analysis, Google Form responses were copied to Excel File and imported into IBM SPSS version 25 for Windows. Participants' demographic characteristics were described by descriptive statistics, including standard deviation (SD), frequency, percentage, and mean. Numbers and percentages were given for categorical data, while mean and standard deviation were provided for continuous data. The Independent t-test, Multinomial regressions, and Non-parametric Mann-Whitney test were used to estimate the significance levels. A p-value ≤ 0.05 was determined to be statistically significant.

2.4 Ethical Considerations

Online informed consent was obtained on the initial page of the form by responding to a "No or Yes" question from respondents before the collection of data. Respondents were provided adequate privacy during the data collection. The confidentiality of their information was protected. They were asked to give correct responses without any hesitation and free of bias.

3. RESULTS

The survey received 728 responses, and after eliminating those with missing information, 605 respondents were considered for the final analyses of which 317 were male (52.5 %; Table 1). The age of the majority of participants was 15-29 years (n=440; 72.7 %). Most of the participants were unmarried/divorced (n=433; 71.6 %), have no children (n=497; 82.1 %), and residing in the urban area (n=332; 54.9 %). The monthly household income of the majority of individuals was 121-250. Table 1 shows that none of the participants of the current study possess a high school or below education level (n=0) while most of them are college/university degrees (n=360; 59.5 %).

The majority of participants correctly answered five out of six knowledge questions (M = 4.96, SD = 1.03). The majority of respondents (94.4 %) correctly identified the primary symptoms of COVID-19, and 95.2 % properly identified the method of transmission—respiratory droplets of infected people—while 1.8 % wrongly replied and 2.1 % did not reply. A knowledge test revealed the significant frequency of misconception, with nearly half of the respondents (46.3 %) thinking that illness could be contracted from eating or interacting with wild animals (Table 2). The majority of responders (87.9 %) indicated that wearing a generic medical mask aids in prevention, although 6.4 % gave inaccurate information and 5.5 % were unsure.

The majority of responders correctly answered about five out of six knowledge questions

Table 1. Survey respondents' descriptive statistics

Sociodemographic Characteristics	Total (n=605)	
	n	%
Gender		
Female	288	47.6
Male	317	52.4
Age (year)		
15-29	440	72.7
30-49	161	26.6
50+	4	.7
Marital status		
Married	172	28.4
Unmarried/Divorced	433	71.6
Presence of children		
None	497	82.1
1-2	86	14.2
3-5	22	3.6
Residence		
Urban	332	54.9
Rural	273	45.1
Monthly household income (USD)		
Up to 120	150	24.8
121-250	165	27.3
251-375	53	8.8
376-500	105	17.4
Above 500	127	21.0
Education level		
High School or below	0	0
Postgraduate degree	245	40.5
College/University degree	360	59.5

(M = 4.96, SD = 1.03). The respondent's knowledge regarding COVID-19 was appreciable. The majority of the respondents were aware of the transmission of SARS-CoV-2 through respiratory droplets of infected people (95.2 % answered correctly, 1.8 % incorrectly, and 2.1 % reported that they did not know). Respondents in a knowledge item "believing that contracting an infection from eating or coming into touch with wild animals" showed a significant incidence of misconception (46.3 % answered correctly, 51.1% incorrectly, and 2.1 % reported that they did not know). Around 69 % of respondents knew correctly that not all people with COVID-19 have severe cases, 25.5 % Incorrectly answered and 4.6 % answered they did not know as shown in Table 2.

The perceived susceptibility of respondents to COVID-19 infection was close to "neither high nor low"(score = 3) (M = 3.16, SD = 1.09); The mean severity was greater than the mean perceived susceptibility, which was just higher than "neither high nor low"(score = 3) (M = 3.29, SD = 0.94). Both social distancing (M = 3.49, SD = 0.69) and beliefs about the efficacy of preventive measures (M = 3.51, SD = 0.68) were high. Wearing face masks was the most performed practice. (M = 3.59, SD = 0.91), followed by Avoiding crowded places (M = 3.44, SD = 0.95) and practicing hand hygiene (M = 3.36, SD = 1.04) (Table 3).

4. DISCUSSION

The current study analyzed the characteristics of KAP in relation to COVID-19 and discovered certain demographic factors linked with KAP.

Table 2. Responses to Knowledge items

S. No.	Knowledge items	Correct	Incorrect	Do not Know
1	Fever, tiredness, dry cough, myalgia, and shortness of breath are the most common clinical signs of COVID-19.	94.4	.7	4.1
2	There is currently no specific cure for COVID-19, however initial diagnosis and supportive treatment can make the majority of patients recover.	92.1	3.5	4.5
3	COVID-19 does not affect everyone in the same way. Only older persons with persistent disorders are more severely affected.	69.9	25.5	4.6
4	COVID-19 is spread through close contact with or consumption of wild animals.	46.3	51.1	2.1
5	Ordinary individuals can protect themselves from SARS-CoV-2 infection by wearing regular medical masks.	87.9	6.4	5.5
6	SARS-CoV-2 transmits through respiratory droplets produced by infected patients coughing and sneezing.	95.2	1.8	2.1

Table 3. Responses of knowledge, attitudes, and practices

Variable	Range	M	SD
Knowledge			
Knowledge	2-6	4.96	1.03
Attitudes			
Perceived risk			
Perceived susceptibility	1-5	3.16	1.09
Perceived severity	1-5	3.29	0.94
Efficacy belief of precautionary behavior			
Practicing personal hygiene	1-4	3.51	0.68
avoiding crowded places	1-4	3.49	0.69
Practices			
Wearing facial masks	1-4	3.59	0.91
Practicing hand hygiene	1-4	3.36	1.04
Avoiding crowded places	1-4	3.44	0.95

Recently, analogous analyses to analyze KAP against COVID-19 in respective populations were undertaken in South Korea [16], China [13], India [17], Bangladesh [18], United States [19], and Iran [20]. The majority of the 728 participants (52.4 %) were men between the ages of 15 and 29. A comparable survey done in Iran by [20] indicated a greater proportion of female subjects (52.5 %) than men, with age findings similar to the current research. The findings of our study regarding gender, age, and education level were almost similar to the survey conducted in India [17]. It was discovered that the majority of people (71.6 %) were single. The findings contradicted the findings of [20], where respondents were married (55.3 %). The findings revealed that sociodemographic characteristics such as gender, age, literacy, and so on influenced KAP toward COVID-19.

The present poll included a higher proportion of urban participants (n=332; 54.9 %) than rural ones (n=273; 45.1 %). A recent study included a disproportionately high proportion of metropolitan participants (95 %) [20]. The rural population has a lower COVID-19 knowledge level than the urban population. These findings are also consistent with previous studies that looked at the relationship between sociodemographic variables and the level of knowledge during the COVID-19 epidemic in China [16] and Hong Kong [21]. Generally, people live in cities because of their occupations, and the average household income per month was between 121 and 150 USD. People's jobs were associated with their presence in congested regions, indicating the necessity for a careful evaluation of the scenario before resuming the jobs, since this might contribute to the spreading of COVID-19.

The majority of participants (59.5 %) have a college/university degree, while none have an education above high school. In terms of illiteracy, the true knowledge level of COVID-19 in the general population of Pakistan may be lower. In this study, the mean knowledge score was lower among jobless people, which corresponds to statements by Clements (2020) [19], as the knowledge level was lower in participants with lower wages. The poor knowledge among persons with lower income and education levels may imply a link between socioeconomic variables and understanding of COVID-19. Attitudes, particularly effectiveness beliefs, are expected to have a strong and persistent influence on performing preventive behaviors, meaning that encouraging preventive behaviors against COVID-19 would include boosting both knowledge and efficiency perceptions among the general population [16]. Similar to data that effectiveness beliefs are key determinants of prevention measures, this study revealed that for people to engage in cautious behaviors after receiving information, they must trust that these practices will be beneficial.

As indicated by Nakhostin-Ansari et al. (2020) [20], greater emphasis should be placed on informing and equipping lower socioeconomic communities against COVID-19, as they are the most susceptible group in this pandemic. By comparing the KAP toward COVID-19 and socio-demographic data, it was shown that unmarried younger men, those with a higher education level, and inhabitants of urban areas had a good understanding of COVID-19.

5. CONCLUSION

Females, the elderly, and the less educated, on the other hand, have less understanding of COVID-19, making them especially susceptible to the pandemic. It is proposed that further awareness might contribute to a favorable attitude and practice. Knowledge and awareness of COVID-19 among the general population could lead to greater trust in health authorities and adherence to health recommendations which could help in controlling a pandemic like COVID-19.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

7. REFERENCES

1. N. Zhu, D. Zhang, W. Wang, X. Li, B. Yang, J. Song, X. Zhao, B. Huang, W. Shi, R. Lu, and P. Niu. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine* 382: 727-733 (2020).
2. WHO. WHO Coronavirus (COVID-19) Dashboard (2022). https://covid19.who.int/ (accessed 30 October 2022).
3. S. Rahman, M. Din, S. Khan, L. Yar, F. Zia, M. Idrees, and M. Ali. COVID-19 Vaccines: Pakistan's Perspective: COVID-19 Vaccines: Pakistan's

Perspective. Proceedings of the Pakistan Academy of Sciences: *B. Life and Environmental Sciences* 58: 11-17 (2021).

4. M.E. El Zowalaty, and J.D. Järhult. From SARS to COVID-19: A previously unknown SARS- related coronavirus (SARS-CoV-2) of pandemic potential infecting humans – Call for a One Health approach. *One Health* 9: 100124 (2020).
5. C.R. MacIntyre. Global spread of COVID-19 and pandemic potential. *Global Biosecurity* 1: (2020).
6. W. Zhang, R.H. Du, B. Li, X.S. Zheng, X.L. Yang, B. Hu, Y.Y. Wang, G.F. Xiao, B. Yan, Z.L. Shi, and P. Zhou. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. *Emerging Microbes & Infections* 9: 386-389 (2020).
7. C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, L. Zhang, G. Fan, J. Xu, X. Gu, and Z. Cheng. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet* 395: 497-506 (2020).
8. Z.A. Memish, R. Steffen, P. White, O. Dar, E.I. Azhar, A. Sharma, and A. Zumla. Mass gatherings medicine: public health issues arising from mass gathering religious and sporting events. *The Lancet* 393: 2073-2084 (2019).
9. R.M. Anderson, H. Heesterbeek, D. Klinkenberg, and T.D. Hollingsworth. How will country-based mitigation measures influence the course of the COVID-19 epidemic? *The Lancet* 395: 931-934 (2020).
10. X. Qian, R. Ren, Y. Wang, Y. Guo, J. Fang, Z.D. Wu, P.L. Liu, and T.R. Han. Fighting against the common enemy of COVID-19: a practice of building a community with a shared future for mankind. *Infectious Diseases of Poverty* 09: 8-13 (2020).
11. X. Lin, X. Wang, Z. Peng, R. Wang, P. Wang, Z. Li, Y. Zhou, H. Lei, C. Li, W. Liu, and L. He. Current Situation of the Corona Virus Disease Under Active Control and Prevention in China, Outside Hubei Province: A Real-World Epidemiological Analysis. *SSRN Electronic Journal* (2020).
12. J. Hopman, B. Allegranzi, and S. Mehtar. Gestión de COVID-19 en países de ingresos bajos y medianos. *JAMA Network* 10: (2020).
13. B.L. Zhong, W. Luo, H.M. Li, Q.Q. Zhang, X.G. Liu, W.T. Li, and Y. Li. Knowledge, attitudes, and practices towards COVID-19 among Chinese residents during the rapid rise period of the COVID-19 outbreak: a quick online cross-sectional survey. *International Journal of Biological Sciences* 16: 1745-1752 (2020).
14. M. Kelley, R.A. Ferrand, K. Muraya, S. Chigudu, S. Molyneux, M. Pai, and E. Barasa. An appeal for practical social justice in the COVID-19 global response in low-income and middle-income countries. *The Lancet Global Health* 8: e888-e889 (2020).
15. Government of Pakistan. See the Realtime Pakistan and Worldwide COVID-19 Situation! (2022). https://covid.gov.pk/ (accessed 30 October 2022).
16. Q. Li, X. Guan, P. Wu, X. Wang, L. Zhou, Y. Tong, R. Ren, K.S. Leung, E.H. Lau, J.Y. Wong, and X. Xing. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus–Infected Pneumonia. *New England Journal of Medicine* 382: 1199-1207 (2020).
17. U. Aqeel, M. Daud Ali, Z. Iqbal, and M.A. Mirza. Knowledge, attitudes, and practices toward coronavirus disease-19 infection among residents of Delhi NCR, India: a cross-sectional survey based study. *Asian Journal of Pharmaceutical and Clinical Research* 110-116 (2020).
18. A. Karim, M. Akter, A.T. Mazid, O.S. Pulock, T.T. Aziz, S. Hayee, N. Tamanna, G.S. Chuwdhury, A. Haque, F. Yeasmin, and M.A. Mitu. Knowledge and attitude towards COVID-19 in Bangladesh: Population-level estimation and a comparison of data obtained by phone and online survey methods. *Nature*. 5: 536-544 (2020).
19. J.M. Clements. Knowledge and Behaviors Toward COVID-19 Among US Residents During the Early Days of the Pandemic: Cross-Sectional Online Questionnaire. *JMIR Public Health and Surveillance* 6: e19161 (2020).
20. A. Nakhostin-Ansari, F. Aghajani, M.S. Khonji, R. Aghajani, P. Pirayandeh, R. Allahbeigi, and H. Hosami Rudsari. Did Iranians respect health measures during Nowruz holidays? A study on Iranians' knowledge, attitude and practice toward COVID-19. *Journal of Preventive Medicine and Hygiene* 61: E501-E507 (2020).
21. C.L. Wong, J. Chen, K.M. Chow, B.M.H. Law, D.N.S. Chan, W.K.W. So, A.W. Leung, and C.W. Chan. Knowledge, Attitudes and Practices Towards COVID-19 Amongst Ethnic Minorities in Hong Kong. *International Journal of Environmental Research and Public Health* 17: 7878 (2020).

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Research Article

Isolation and Detection of Bacterial Strains from Cosmetics Products available in Pakistan

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Abstract: Cosmetics products are the most essential and frequently used components in our daily life. Besides improving human health, they provide healthy lifestyles and boost our self-esteem. Globally cosmetics market is projected to be 287 billion USD in 2021 to 415 billion USD in 2022. This research study aims at the isolation, identification, and characterization of bacterial strains isolated from cosmetics. Six bacterial colonies were isolated by inoculating different cosmetics products on tryptic soya agar media. All the strains showed optimum growth at 37 °C. All strains were assessed through biochemical tests by using different media such as MacConkey agar, SIM, and Simmons citrate agar and were further proceeded for nucleotide sequencing through Sanger sequencing. Different bacterial strains were revealed in cosmetics products including *Sphingomonas paucimobilis, Cytobacillus oceanisediminis, Robertmurraya andreesnii, Cytobacillus firmus, Falsibacillus pallidus*, and *Acinetobacter junii.* Most of these strains were found to be pathogenic however, *Sphingomonas* has the potential for bioremediation and can be utilized for degrading toxic compounds to make the environment better. Similarly, *Cytobacillus* is found to be involved in biomineralization and also aids in fermentation. Our results have shown that there is a dire need to assure strict safety regulations regarding cosmetics. Improper manufacturing practices can lead to the contamination of cosmetics which could lead to severe consequences of deteriorating the quality of health. Further studies are needed to explore the potential of these isolates so that they can be utilized to improve our health as well as the environment.

Keywords: Cosmetics, Contamination, Molecular Identification, Biochemical Tests.

1. INTRODUCTION

Personal care products are frequently utilized by people in daily life and getting popular across the globe due to extensive use. They are purchased without any hurdle as they do not lie under the same regulation as those of medicines. The word "Cosmetic" is derived from the Greek word "*Kosmetike tekhne"* meaning "Technique of dressing and decoration". Cosmetics are defined as the substances which are applied to the external surface of the human body to alter the appearance to look attractive, improve the texture of the skin, keep the body clean, and smell good, and for skin protection. There are seven main categories of cosmetics which include oral care products, skin care products, body care products, products of hair care, sun care, fragrance products i.e., perfumes, and decorative cosmetic products [1].

Cosmetics are comprised of a combination of chemical ingredients derived from natural as well as chemical sources. The vital ingredients of cosmetics include water, emulsifiers, preservatives, thickeners, pigments, glitters, fragrances, etc. However, these ingredients act as a medium for the transport of pathogens in the daily lives of people because mostly these components encourage the growth of microbes. Microorganisms can survive at a suitable temperature, pH, moisture, and metabolites [2]. Almost all cosmetics products fulfill these requirements and harbor the growth of microbes. Most cosmetics contain growth stimulators, organic as well as inorganic components, and are stored in

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a humid atmosphere which stimulates the growth of microorganisms. The microbial contamination of these personal care products deteriorates their quality ultimately affecting human health and resulting in severe consequences [3]

Cosmetics products become prone to contamination either during manufacturing in a container or during use by the consumer. In the first case, the manufacturer of the product must adopt all the safety protocols to avoid contamination so that the best quality of the product must be ensured. While in the second scenario, the user must keep the products in a safe place to avoid health issues [4]. The most important concern about cosmetics is that they are not labeled with their manufacturing and expiring date due to which chemicals used for preservation degrade at their specific time. Ultimately, contamination will occur and people keep on using such contaminated products without being in their knowledge. The microbial contamination of cosmetics results in the production of such toxins which cause severe irritation and allergy on the skin [5].

Microbial contamination of cosmetics products is a global health issue and causes nuisance among consumers, manufacturing industries, and clinicians. The cosmetics become prone to contamination due to impurity of raw material, due to use in a contaminated atmosphere, or poor personal hygiene [6]. Microbial contamination of cosmetics products is a global health issue and causes nuisance among consumers, manufacturing industries, and clinicians. The cosmetics become prone to contamination due to impurity of raw material, contaminated atmospheres, or poor personal hygiene [7]. The microbes not only modify the physical features of products like color, viscosity, flavor, and scent but also degrade the crucial components of products which results in severe consequences. The microbial interference may produce certain toxic compounds and metabolites which cause a severe allergic reaction to the skin [8].

The risks associated with contaminated products can have a significant impact on human health ranging from mild to severe diseases [9]. Pathogenic microbes have been isolated from cosmetics products which include *Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter,* and *Klebsiella pneumonia*, etc. which cause a range of diseases from severe skin allergies to respiratory infections along with bacteremia and urinary tract infections as well [10].

An accurate and early-stage diagnosis of a disease is crucial to avoid long-lasting effects and complications. The precise diagnosis of infection improves the effectiveness of the treatment required to alleviate that infection and prevents unnecessary practices and medication. The precise diagnosis of a disease prevents the outbreak of that disease and minimizes the development of antibiotic resistance [11]. Conventionally, bacterial infections are diagnosed with the help of culture methods, however, due to certain limitations like some bacteria being difficult to grow and their growth requirements being different and time-consuming procedures do not make it an ideal method for bacterial diagnostics. Over several decades, nucleic acid testing has revolutionized the diagnosis of disease and it is faster, more accurate, and more sensitive than the traditional culture methods [12]. The present study aimed to isolate and identify bacterial strains from cosmetics products available in Pakistan via biochemical testing and molecular characterization.

2. MATERIALS AND METHODS

Six branded products of cosmetics (lip-gloss, foundation, sunscreen, and eyeshadows) available in Pakistan were purchased. These were used because of their causal usage or daily usage. Different biochemical tests were performed which included SIM (Sulfur, Indole, Motility), Simmons Citrate Agar, and MacConkey Agar test. The molecular characterization was performed for the confirmation of isolated bacterial strains at the Molecular Systematic and Applied Ethnobotany Lab (MoSAEL), Department of Biotechnology, Quaid-i-Azam University, Islamabad.

2.1 Isolation of Bacterial Strains

The isolation of bacterial strains was done by providing them with suitable nutrients, temperature, and environment. Thus, different bacterial strains were obtained from different products of cosmetics. Different types of media were prepared including

TSA ((Tryptic soya agar), MacConkey Agar, SIM (Sulfur, indole, motility), Simmons citrate agar, and Urea base agar.

The serial dilution was performed for each sample. Each product of cosmetics was dissolved in autoclaved distilled water and DMSO (dimethyl sulfoxide) and then placed on the TSA media plates for the emergence of bacterial colonies. While the direct cosmetic samples (without dilution) were also taken with the help of sterilized inoculation loop and placed on TSA media at 37 °C. The emergence of bacterial colonies was sub-cultured and maintained. Morphologically different and pure colonies were selected for investigation.

2.2 Molecular Identification of isolated bacterial strains

The molecular characterization was done in the following steps as depicted below diagrammatically in Figure 1. To extract DNA from bacterial colonies, the simple plain boiling method was used for a higher yield of DNA. For this purpose, a single colony from overnight grown bacteria was picked with a micro-pipette and dissolved in 1ml distilled water in an Eppendorf tube. Then each Eppendorf tube was kept at 95 °C in a water bath for 10 minutes.

After boiling in the water bath, each tube was centrifuged at 1000 rpm for 5 minutes. The supernatant having the bacterial DNA was separated and stored at -20 °C and the pellet was discarded.

2.2.1 Polymerase Chain Reaction

All the components of the reaction mixture were added in an optimized quantity making the total volume of 20 µl in the PCR tube. The PCR reaction mixture was comprised of Green Master Mix (Thermo Scientific). Primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3) and reverse primer 1492R (5'-GGTTACCTTTTTTACGACTT-3') of 16S rRNA gene were added. Also, the template DNA and nuclease-free water were added to make the total volume of 20 µl.

2.2.2 Gel Electrophoresis and Purification of RCR Products

PCR amplified products were loaded on 1.5 % agarose gel, prepared by 1X TAE buffer along with 1kb ladder, and run for 30 min at 90 V. After that gel was visualized on UV. The required amplified bands were cut and subjected to Thermo Scientific GeneJET Gel Purification Kit for the purification of PCR products.

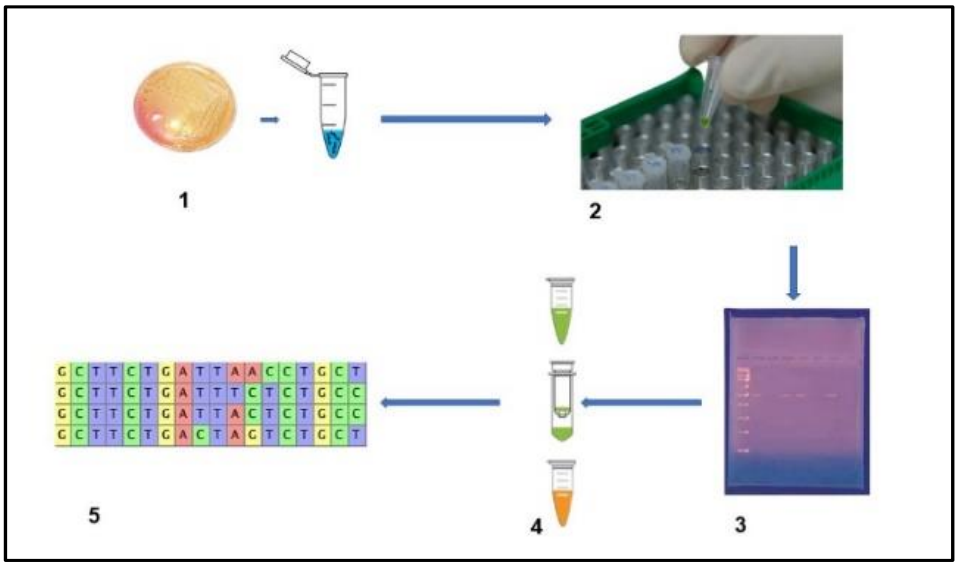

Fig. 1. The stepwise procedure of molecular characterization technique; 1. DNA extraction of pure bacterial isolates using the plain boiling method. 2. Polymerase chain reaction (PCR) using universal primers for the amplification of the 16S rRNA gene. 3. Agarose gel electrophoresis for the confirmation of amplified product. 4. Purification of amplified product from agarose gel electrophoresis. 5. Sanger sequencing and post-sequence analysis using bioinformatics tools.

According to the given protocol: the gel slice was first cut then binding buffer was added in the ratio of 1:1 and placed on the hot plate at the temperature of 65 °C for 10 minutes to melt the gel completely. Inverted mixing was done to homogenize the mixture. The mixture was vortexed briefly. The mixture was then transferred to the GeneJET purification column and centrifuged for 30-60 seconds. Flow through was discarded. Wash Buffer (700µl) was added to the GeneJET purification column and then centrifuged for 30-60 seconds. The flow-through was discarded and the purification column was placed back into the collection tube. The empty GeneJET purification column was again centrifuged for an additional 1 minute to completely remove the residue material. The GeneJET purification column was transferred to a sterile microcentrifuge tube with a 1.5 ml capacity. Elution buffer (30 µl) was added to the center of the GeneJET purification column membrane and centrifuged for 1 minute. The GeneJET purification column was then discarded and purified DNA was stored.

2.2.3 Sequencing of 16S rRNA Gene

After the elution of PCR products, they were processed for sequencing to Macrogen. The sequencing was carried out using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3) by the Sanger Sequencing Method.

2.3 Phylogenetic Analysis

After 16S rRNA gene sequencing, the sequences were compared to the reference sequence retrieved from the NCBI (National Center for Biotechnology Information) for phylogenetic analysis. BLAST tool was used for the comparison of the similarity index. The most similar sequences having the highest similarity index were selected and aligned using the software MEGA X. The phylogenetic tree was constructed using the same software by using the maximum likelihood method at a 1000 bootstrap value. The maximum likelihood method predicted the evolutionary relationship of the strains to the closely related strains.

3. RESULTS

In this study, we revealed the diversity of bacteria isolated from six cosmetic products of various brands. A total of n=6 bacterial colonies were obtained through culturing on TSA media after 24 hours of incubation at 37 °C as shown in Figure 2 which were identified based on biochemical as well as molecular characterization. The tested products were taken from famous brands. The names of brands are not mentioned in the study due to commercialization issues.

3.1 Biochemical Tests

Various biochemical tests were performed to identify bacterial isolates which included SIM (Sulfur, Indole, and Motility), MacConkey Agar, and Simmons citrate Agar.

3.1.1 Sulfur test

A sulfur test was done to identify isolates that were gram-negative enteric bacillus based on the production of hydrogen sulfide gas which caused the formation of black precipitates. Four isolates showed positive Sulfur test as represented in (Figure 3A) while all other isolates were negative.

3.1.1.1 Indole test:

This test aimed at the differentiation of gram-negative bacteria based on the production of hydrolysis of tryptophan. Indole-positive bacteria produced pink to red color rings on the top surface of the media as shown in (Figure 3B) while no color change was observed for indole-negative bacteria. Five bacteria gave indole positive tests while the rest of them gave negative tests.

3.1.2 Motility test

The motility test identified the strains which were motile and could move from those which were non-motile. The motile isolates produced turbidity and cloudiness in the medium while non-motile isolated grew along the stab line only and did not produce any turbidity as represented in (Figure 3C).

3.1.3 Simmons Citrate Test

Citrate-positive bacteria changed the color of the media from green to bright blue while citrate-negative bacteria did not change the media color.

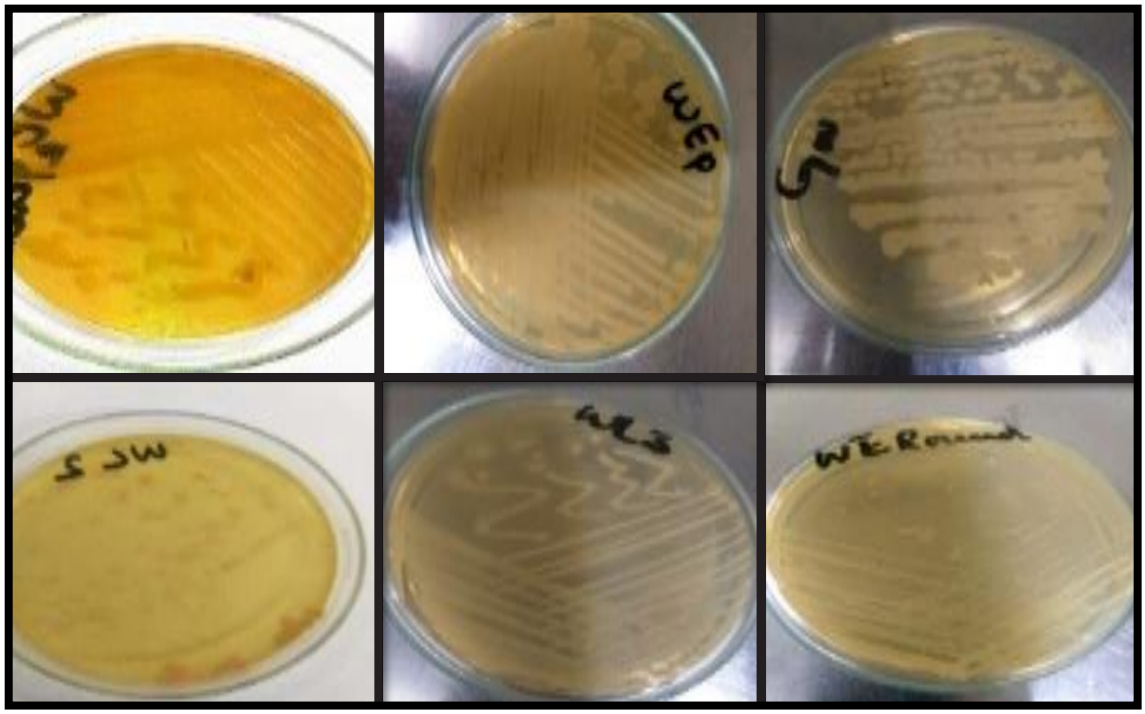

Fig. 2. Represents the bacterial colonies obtained after inoculation were mostly yellow, brown, and white in color and rest were transparent.

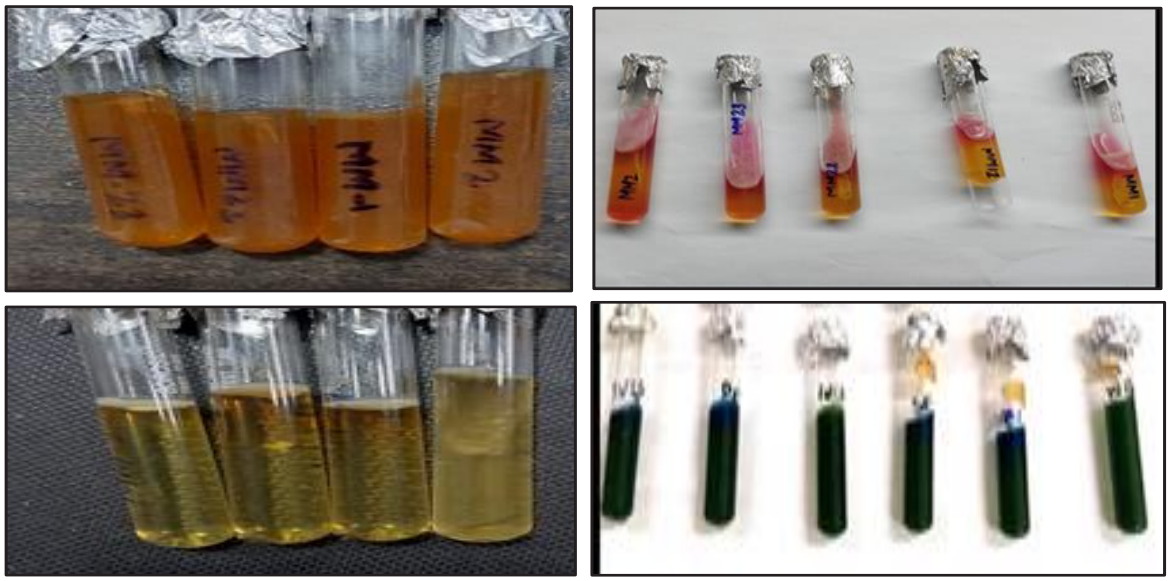

Fig. 3. SIM test and Simmons Citrate Test Results. A) Sulfur test: *A. junii, S. paucimobilis, C. firmus, R. andreesnii* gave sulfur positive test. B) Indole test: *C. oceanisediminis, S. paucimobilis, F. pallidus, R. andreesnii, C. firmus* gave indole positive test. C) Motility test: *C. firmus, A. junii, R. andreesnii* and *S. paucimobilis* were motile strains. D) Simmons Citrate Test: *C. oceanisediminis, F. pallidus, C. firmus, A. junii, R. andreesnii* and *S. paucimobili*s gave citrate positive tests.

Four isolates were found to utilize citrate as an energy source while the rest of the isolates were unable to do so as shown in Figure 3D.

3.1.4 MacConkey Agar Test

MacConkey agar test was used for the differentiation of gram-negative bacteria based on their ability to ferment lactose. Gram-positive bacteria did not grow on MacConkey agar Figure 4.

3.2 Molecular Identification

After the completion of PCR, the PCR products were visualized on 1.5 % agarose Gel as shown in (Figure 5).

3.2.1 Sequencing Analysis

After sequencing, the BLAST was used for comparing the sequences with reference sequences. The most similar sequences were retrieved. Sequencing results indicated one strain of *C. oceanisediminis* and another identified as *Sphingomonas paucimobilis*. There were also other strains including *C. firmus, R. andreesnii, C. pallidus*, and *A. junii*.

3.2.2 Construction of Phylogenetic Tree

Mega X was used to analyze the sequence of nucleotides of the bacteria isolated from cosmetics products in comparison with the reference sequence of nucleotides of bacteria from all over the world. The maximum likelihood method was used to evaluate the evolutionary history of strain. Phylogenetic analysis showed that isolate QAUT7F strain showed the most similarity to the *Cytobacillus firmus* strains found in Japan, QAU103 strain showed great similarity to *Sphingomonas paucimobilis* strains found in the USA while QAUT12R was found to be greatly related to the *Robertmurraya andreesnii* isolated from USA, Germany, and Belgium as shown in Figure 6. QAUT10F and QAUT11F show similarity to the *A. junii* strain and *F. pallidus* strain respectively, both the isolates have shown an evolutionary relationship with China (Figure 7). Figure 8 shows that QAU 112 isolate was significantly similar to the *C. oceanisediminis* strain of China.

4. DISCUSSION

Cosmetic products are inhabitable companions of everyone in daily life which enhances the elegance of the personality. The use of cosmetics has become indispensable as they are not merely used to improve appearance but also to keep the body in a healthy state. Cosmetics also act as an important vehicle for the transmission of pathogens to humans due to which concern about their use and safety is rising with time. They harbor a wide variety of microbes due to contamination from various sources which cause mild to severe allergic reactions leading to complicated life-threatening infections [13].

Current research studies have revealed bacterial diversity in cosmetics in terms of pathogenic as well as beneficial impacts. *Sphingomonas paucimobilis* is one of the bacteria isolated from cosmetics in my research work. It is a gram-negative, aerobic, and opportunistic pathogen and causes infections in immunocompromised individuals [14]. It causes soft tissue infection characterized by fluid and puss exudates from deep tissues. It is also reported to cause symptoms of pneumonia resulting in severe

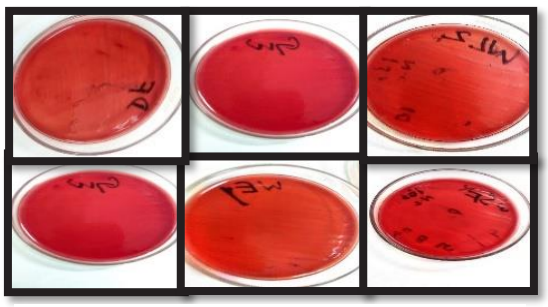

Fig. 4. Results of MacConkey Agar test which showed that almost all bacteria grew transparent as non-lactose fermenting while two strains were pink and lactose fermenting.

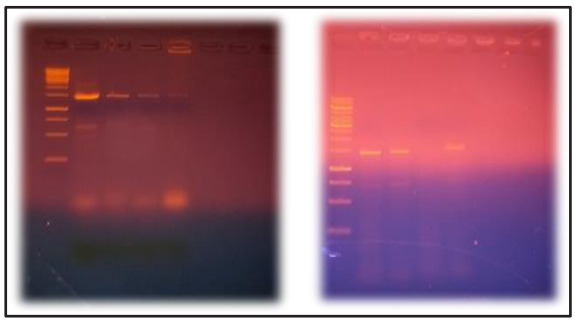

Fig. 5. Represents the PCR products of 1250bp compared with ladder of 1kb size.

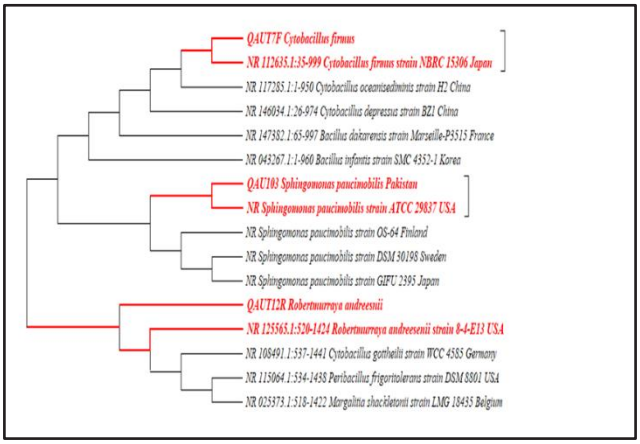

Fig. 6. Representation of phylogenetic tree of isolates constructed through maximum likelihood method (from n=14). The reference sequences were obtained from GenBank by using BLAST. QAUT7F which was identified as *C. firmus*, QAU103 was identified as *S. paucimobilis,* QAU12R identified as *R. andreesnii.*

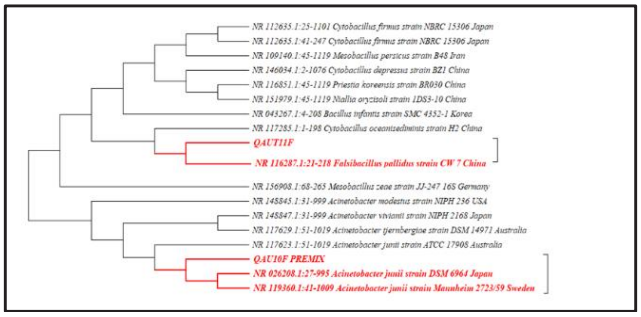

Fig. 7. Representation of phylogenetic tree of isolates constructed through maximum likelihood method (from n=16). The reference sequences were obtained from GenBank by using BLAST. QAU10F PREMIX and QAUT11F were identified as *A. junii* and *F. pallidus* respectively.

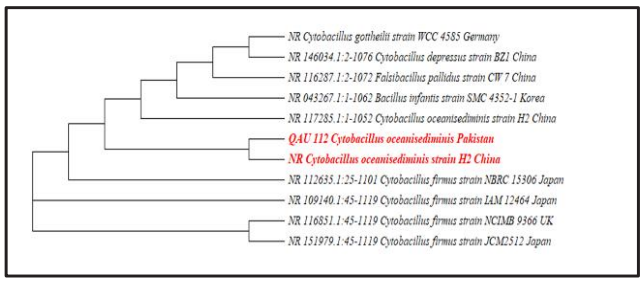

Fig. 8. Representation of Phylogenetic tree of isolate QAU112 (identified as *C. oceanisedminis*) constructed through the maximum likelihood method (from n=10). The reference sequences were obtained from GenBank by using BLAST.

difficulty in breathing [15]. It is not just significant in clinical terms but it has significant importance in the environment as it is the fundamental agent used in bioremediation. This bacterium can degrade aromatic compounds and it is an effective and eco-friendly tool for the degradation of pollutants and carcinogenic compounds from the soil. These bacteria can be used for *in-situ* bioremediation after enhancing the production of biomass in bioreactors. Further work and research are needed to explore and uncover the capacity of this bacteria to perform bioremediation and make our environment free of harmful substances. Recombinant DNA technology can be used to identify the genes responsible for bioremediation and recombinant strains can be developed to remediate the environment [14].

Another bacterial strain isolated from cosmetics in my research work is *C. oceanisediminis*. It is a gram-positive, rod-shaped, and aerobic bacteria found in marine sediments. It is not known whether it is pathogenic or not pathogenic. It helps in fermentation through the production of acetate, lactate, and ethanol [16]. They play an important role in biomineralization. It is a novel strain reported to be isolated from marine systems. Further research is needed to explore its potential and its effects on humans and animals [17].

A. junii has been isolated from cosmetic products. It is a gram-negative bacteria and coccobacillus in morphology. It is a well-known infectious agent and can colonize the skin, gastrointestinal tract, and respiratory pathways. It has been reported to cause a severe form of pneumonia associated with ventilators [18]. It is a notorious pathogen and also a causative agent of blood infection as well as urinary tract infection. It is one of the pathogens involved in eye infections and damages the eye epithelium which leads to corneal ulcers. A corneal ulcer is one of the most common diseases caused due to use of contaminated mascara and eyeliner [19].

R. andreesnii strain is another bacterium isolated in my research work. It is a gram-positive, motile, and rod-shaped bacterium. It is an opportunistic bacterium and causes gastrointestinal infections. It is reported to cause endocarditis, blood infection, pneumonia, and skin infection. It causes morbidity in immunocompromised patients and people having diabetes or serious injuries [20]. *C. firmus* has been isolated from one of the cosmetics samples. It is a gram-positive, aerobic bacterium. It is not known to have pathogenic effects on humans and does not cause any significant diseases in humans [21]. It has significant importance in promoting plant growth by interfering with and inhibiting nematode and cysts growth [22]. It is also used for the preparation of animal feed as probiotic supplements which have played a significant role in improving animal health [23].

5. CONCLUSION

Different bacteria have been isolated from cosmetics products which include *S. paucimobilis, C. oceanisediminis, F. pallidus, A. junii*, *C. firmus*, and *R. andreesnii*. Some of these bacteria are pathogenic and can cause numerous kinds of infectious diseases. Whereas beneficial bacterial components have been derived from these bacteria which have the potential to give maximum benefits to us and the environment as well. Cosmetics companies should develop a monitoring system for checking the quality of products and to assure product safety. A sterile environment should be used to manufacture products. Personnel should implement standard hygiene practices. The raw material used for products should be labeled and verified by the quality assurance officer. The closed system should be preferred for manufacturing products. The finished cosmetics products should be approved by quality control management before releasing in the market. Consumers should also follow the safety protocols during the use of products as well. They should check the manufacturing and expiry date before buying products and high-quality brands should be used instead of relying on cheap and lower-quality products. Bacterial count should be zero in these products because at a certain stage, they may cause serious harm to human health. However, some strains are still unknown whether they are pathogenic or not and there is a dire need to explore and check the metabolic profile of these bacteria so that they can be employed for therapeutic purposes.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. REFERENCES

1. H.W. Kim, Y.S. Seok, T.J. Cho, and M.S. Rhee. Risk factors influencing contamination of customized cosmetics made on-the-spot: Evidence from the national pilot project for public health. *Scientific Reports* 10: 1-9 (2020).
2. A.K. Mohiuddin. Cosmetics in use: A pharmacological review. *Journal of Dermatology & Cosmetology* 3: 50-67 (2019).
3. J. Ding, B. Wu, and L. Chen. Application of Marine Microbial Natural Products in Cosmetics. *Frontiers in Microbiology* 13 (2022).
4. A.A. Jairoun, S.S. Al-Hemyari, M. Shahwan, and S.E.H. Zyoud. An investigation into incidences of microbial contamination in cosmeceuticals in the UAE: Imbalances between preservation and microbial contamination. *Cosmetics* 7: 92 (2020).
5. I.M. Michalek, S.M. John, and F.L. Caetano dos Santos. Microbiological contamination of cosmetic products–observations from Europe, 2005–2018. *Journal of the European Academy of Dermatology and Venereology* 33: 2151-2157 (2019).
6. I.H. Jung, J.H. Kim, Y.J. Yoo, B.Y. Park, E.S. Choi, and H. Noh. A pilot study of occupational exposure to pathogenic microorganisms through lip cosmetics among dental hygienists. *Journal of Occupational Health* 61: 297-304 (2019).
7. T.M. Janetos, L. Akintilo, and S. Xu. Overview of high-risk Food and Drug Administration recalls for cosmetics and personal care products from 2002 to 2016. *Journal of Cosmetic Dermatology* 18: 1361-1365 (2019).
8. A. Bashir, and P. Lambert. Microbiological study of used cosmetic products: Highlighting possible impact on consumer health. *Journal of Applied Microbiology* 128: 598-605 (2020).
9. M.J. Zirwas. Contact dermatitis to cosmetics. *Clinical Reviews in Allergy & Immunology* 56: 119-128 (2019).
10. A.D. Khan, and M.N. Alam. Cosmetics and their associated adverse effects: A review. *Journal of Applied Pharmaceutical Sciences and Research* 1-6 (2019).
11. L. Fernández, M.D. Cima-Cabal, A.C. Duarte, A. Rodriguez, P. García, and M.D.M García-Suárez. Developing diagnostic and therapeutic approaches to bacterial infections for a new era: implications of globalization. *Antibiotics* 9: 916 (2020).
12. J.S. Coleman, and C.A. Gaydos. Molecular diagnosis of bacterial vaginosis: an update. *Journal of Clinical Microbiology* 56: e00342-18 (2018).
13. J.B. Sharmeen, F.M. Mahomoodally, G. Zengin, and F. Maggi. Essential oils as natural sources of fragrance compounds for cosmetics and cosmeceuticals. *Molecules* 26: 666 (2021).
14. R.S. Jaafar. The potential role of sphingomonas paucimobilis in bioremediation of soils contaminated with hydrocarbon and heavy metal: Bioremediation using Sphingomonas paucimobilis. *Malaysian Journal Of Science* 48-58 (2019).
15. M.A. Goulart, M. Predic, and C.D. Neilsen. Sphingomonas paucimobilis infection among a patient with a history of injection drug use: An opportunity for improvement of medical chart documentation. *Infection Control & Hospital Epidemiology* 42: 1152-1153 (2021).
16. M. Yadav, T. Kumar, A. Kanakan, R. Maurya, R. Pandey, and N.S. Chauhan. Isolation and characterization of human intestinal bacteria Cytobacillus oceanisediminis NB2 for probiotic potential. *Frontiers in Microbiology* 13 (2022).
17. K. Tarasov, M. Zarubin, A. Yakhnenko, A. Gangapshev, and E. Kravchenko. Isolation of new methylotrophic species of Cytobacillus from deep underground hot spring of Baksan Neutrino Observatory. In *Bioinformatics of Genome Regulation and Structure/Systems Biology:* (BGRS/SB-2022) p. 539-539 (2022).
18. K.J. Towner. Biology of Acinetobacter spp. In *Acinetobacter* CRC Press p. 13-36 (2020).
19. M. Sardarinia, S. Rabani, G. Aghai, and L. Ghiasian. Acinetobacter Corneal Ulcer, an Ocular Involvement by an Uncommon Organism; a Case Report and a Review of Literature *Research Square* (2020).
20. M. Esmkhani, and S. Shams. Cutaneous infection due to Bacillus cereus: A case report. *BMC Infectious Diseases* 22: 1-4 (2022).
21. S. Mingmongkolchai, and W. Panbangred. Bacillus probiotics: an alternative to antibiotics for livestock production. *Journal of Applied Microbiology* 124: 1334-1346 (2018).
22. M. Huang, A. Bulut, B. Shrestha, C. Matera, F. M. Grundler, and A. S. S. Schleker. Bacillus firmus I-1582 promotes plant growth and impairs infection and development of the cyst nematode Heterodera schachtii over two generations. *Scientific Reports* 11: 1-15 (2021).
23. A. Javaid, M. Yasinzai, and K.S. Kiyani. Enhanced Phytase Production from Indigenous Bacillus subtilis KT004404 by Response Surface Methodology to be Used as Poultry Feed Supplement. *Pakistan Journal of Zoology* 1-12 (2022).

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Research Article

Lumpy Skin Disease: An Emerging Threat to Livestock in Tehsil Bara, Pakistan

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Abstract: Lumpy skin disease (LSD) is a highly contagious and significant economic disease of cattle caused by a virus belonging to the family *Poxviridae* and genus *Capripoxvirus*. The present study aimed to determine the severity of the lumpy skin disease outbreak and associated losses in tehsil Bara, Pakistan. The data was collected through a questionnaire from farmers who have cows in their homes; the reported data varied in five different areas of tehsil Bara, collected from June 1, 2022, to August 31, 2022. The total number of reported cases from five selected areas were 2021, of which 168 were healthy and 1853 were infected. Out of the total infected 766 were recovered and 922 were in the recovery stage. Out of five selected areas, the highest infection rate of the disease followed by the highest mortality rate and lowest recovery cases were 466 (96.28 %), 59 (12.66 %), and 173 (37.12 %) respectively, recorded from Shlobar Quam. The lowest mortality rate was 8 (4.65 %) recorded from Nala Sourdandh and the highest recovery rate was 113 (53.30 %) recorded from Bar Qambar Khel. The disease is characterized by widespread nodules on the skin and causes decreased milk production and lack of appetite, and animals show pharyngeal and nasal secretions, accompanied by secondary infection. It is transmitted by the transportation of illegally bought and sold animals across borders to a new area and spread by insect vectors, including biting flies, mosquitoes, and ticks. Antibiotics, antihistamines, analgesic-antipyretics, immunity boosters, and the management of wounds are the general lines of treatment. The current study recommends the multi-task role of government, and the private sector, as well as the isolation of infected animals, burial of dead bodies, annual vaccination, and the prevention of illegal transportation across the border.

Keywords: Lumpy skin disease, Cattle, Tehsil Bara, Mortality

1. INTRODUCTION

Lumpy skin disease (LSD) is an extremely contagious and economically important cattle disease caused by a double-stranded DNA virus belonging to the genus *Capripoxvirus* and family *Poxviridae* [1, 2]. With high morbidity and low mortality, it causes the livestock industry to suffer significant financial losses [3, 4]. When a virus enters the body of a cattle, it can incubate there for 4 to 12 days, while incubation has typically been recorded to last seven days and may potentially extend up to 28 days [5, 6]. The disease infection is characterized by numerous lumps and nodules on the skin and other body parts, which cause skin damage that cannot be reversed [7]. It causes loss of hunger, reduced production of milk, and weight gain [8]. The infected animal shows lacrimation, pharyngeal and nasal secretion, conjunctivitis, and infertility [9]. According to the World Organization for Animal Health (WOAH) standards, the disease has been observed to worsen over time due to secondary bacterial infections, leading to additional complications like mastitis and myiasis or even

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animal death. For this reason, the disease has been labelled as a new disease. [10, 11].

It is a transboundary disease that spreads by the movement of animals from one location to another. Illegal livestock trade across borders further increases the risk of the disease spreading to new areas. When an infection outbreak occurs in an area, insects like mosquitoes (*Culex mirificens* and *Aedes natrionus*), ticks (*Riphicephalus appendiculatus* and *Amblyomma hebraeum*), and biting flies (*Stomoxys calcitrans* and *Biomyia fasciata*) are the principal vectors that spread the disease [12-15]. Moreover, it spreads among the herd by direct contact between animals or through contact with contaminated saliva, blood, nasal secretions, sperm, and milk [3, 16-17]. The standard course of treatment for sickness includes the use of antibiotics, antihistaminics, analgesic-antipyretics, immunity boosters, and wound care [18-20]. Lumpy skin disease can be avoided by adopting guidelines among farmers, limiting animal movement from one place to another, quarantining sick animals, keeping diseased animals separate from the rest of the herd, and avoiding sharing drinking or feeding troughs. All animals should receive a annual vaccination against lumpy skin disease, especially in the early spring, as vaccines are the frontline protector [21]. It is safe to vaccinate pregnant cows. There are commercially available live and attenuated LSD vaccines. In Pakistan, Jamshoro Sindh reported the first incidence of this disease in November 2022. The disease spread throughout Punjab and has already crossed the border into the Khyber Pakhtunkhwa region through Dera Ismail Khan.

2. MATERIALS AND METHODS

2.1 Study Design

This study was conducted in five selected areas of tehsil Bara district Khyber, Khyber Pakhtunkhwa, Pakistan; Akakhel, Speen Qaber, Shlobar Quam, Bar Qamber Khel, and Nala Sourdandh, from June 1, 2022, to August 31, 2022. To conduct this study, the data was collected through questionnaires and interviews with local cow owners.

2.2 Survey through Questionnaire

Three sections of a questionnaire have been created to collect data in the chosen area. Each section was further formulated to gather particular data. The questionnaire has already been verified and clarified as necessary. The first section of the survey asked questions on the Lumpy skin disease epidemic and mortality in five designated areas. Farmers who kept cattle in their houses were asked to fill out a questionnaire to gather the information for section one. The questions in section 2 pertain to the general knowledge and viewpoint of the community regarding the prevalent Lumpy skin disease in five selected regions. Data for section two was gathered from community members, social activists, and farmers who possess cattle. The third section describes the Lumpy skin disease condition in detail, including its signs and symptoms, transmission, detection, and treatment, as well as precautions, risk factors, and economic impact. The information for section three was gathered via interviews, a questionnaire, and input from veterinary experts, livestock veterinarians, researchers, veterinary assistants, and technicians. The gathered information has been compiled and evaluated.

2.3 Data Analysis

Questionnaires from reported cases (Total 2021) from five selected areas of tehsil Bara, district Khyber in Khyber Pakhtunkhwa were filled out and included in the study. Out of the total cases, 1853 were reported to be infected and 168 to be healthy. The percentage of the study data was arranged and analyzed through SPSS software.

3. RESULTS

The total recorded cases of cattle across all five areas of tehsil Bara, district Khyber, including those that were infected, normal, died, recovered, and in the recovery stage, are displayed in Table 1. There were a total of 580 cattle cases reported in the Speen Qabar area, of which 523 (90.17 %) were infected and 57 (9.58 %) were normal. Out of the total infected in the region of Speen Qabar, 32 (6.11 %) died, 209 (39.96 %) recovered, and 243 (51.48 %) were still in the recovery stage. A total of 496 cases of cows were reported in the Akakhel area, of which 472 (95.16 %) were infected and 24 (4.88 %) were normal cases. Among the total number of infected, 46 (9.74 %) died, 183 (38.77 %) recovered, and 243 (51.48 %) were still

in the recovery stage. In the Shlobar Quam area, the total reported cases of cows were 484 of which the infected were 466 (96.28 %) and 18 (3.71 %) were normal. Out of the total infected, the mortality rate was 59 (12.66 %), the recovered were 173 (37.12 %), and in the recovery stage 234 (50.21 %). The total number of cases reported in Bar Qambar Khel were 255, of which 220 (86.27 %) were infected and 35 (13.72 %) were normal. Out of the total infected, the mortality rate was 12 (5.67 %), recovered were 113 (53.30 %), and 87 (41.04 %) were reported to be in the recovery stage. The total recorded cases in the Nala Sourdandh area were 206, of which 172 (83.50 %) were infected and 34 (16.50 %) were normal. The mortality rate of the total infected was 8 (4.6 %), 88 (51.16 %) were recovered, and 76 (44.18 %) were in the recovery stage.

The highest infection rate recorded was 466 (96.28 %), followed by a mortality rate of 59 (12.66 %), and the lowest recovery rate recorded was 173 (37.12 %) in the area of Shlobar Quam. The lowest mortality rate reported from Nala Sourdandh was 8 (4.65 %), followed by Bar Qambar Khel at 113 (53.30 %), and the highest recovery rate from Bar Qambar Khel was 113 (53.30 %).

The general responses from the community in the tehsil Bara, district Khyber, expressed as a proportion of yes and no, are presented in Table 2. For the parameter listed in the questionnaire, the word “Yes” was used for a positive response, and the word “No” for a negative response. Prior to the disease outbreak, 100 % of respondents said that their cattle were not vaccinated for lumpy skin disease. This indicates that cows in particular places have not received the lumpy skin disease vaccine. Only 9 % of farmers segregated the sick animals, while 91 % of farmers did not separate the infected cows from the healthy ones. The majority of individuals lifted the dead cow bodies into the open area on the ground rather than burying them underneath. According to the respondents, 70 % agreed that the meat of infected animals is eatable, and 75 % of the total respondents said that milk is usable. Moreover, 60 % of the farmers wanted to treat the infected animals, while 40 % of the respondents did not want to treat the infected animals.

Out of the total respondents, 5 % believed the prevalence of lumpy skin disease among humans and buffalo, while 95 % of respondents believed that neither humans nor buffalo can catch the disease. The majority of respondents (85 %) held the superstitious belief that the disease can be transmitted through the consumption of meat and milk. Treatment of the disease using technical approaches was only preferred by 10 % of the respondents, with 90 % preferring the traditional approach. Interestingly, 88 % of respondents responded with the lack of government funds for the eradication of the disease. Following an outbreak, vaccines have been made available in government hospitals for cow immunization. The efficacy of local vaccines was reported to be zero percent, while branded and multinational vaccines were found to have an efficacy rate of 95 % against Lumpy skin disease.

The clinical manifestations of Lumpy skin disease in tehsil Bara, district Khyber are presented in (Table 3). The incubation period of the Lumpy skin virus ranges from 4 to 12 days. The disease is characterized by the appearance of widespread nodules on the skin and swelling of superficial lymph nodes. Necrotic and ulcerative lesions may develop, which later convert into fibrotic lesions. In severe cases, ulcerative lesions may also appear in the mucous membranes of the mouth, esophagus, larynx, and trachea. Decreases in milk production in affected cows were reported. Other clinical manifestations include inflammation of the eye membrane, excessive salivation, nasal secretion, and rhinitis. The body temperature ranges from 103.40 ºF to 106.00 ºF. Secondary bacterial infections and infestation by maggots are common. The disease also causes severe emaciation and abnormal thinness of the skin, with cows assuming a recombinant position. Reluctance to move and depression was also observed. The recovery time reported in this study varied from one week up to 2 months, while the reported death rate varied: 55 % for heifers and 75 % for calves and pregnant cows.

The reported sources of transmission of the Lumpy skin disease virus in tehsil Bara, district Khyber are presented in Table 4. The disease is primarily transmitted through the illegal buying and selling of animals across borders, as well as the transportation of infected animals from one place to another. Vector-borne transmission is also reported, with insects such as mosquitoes, flies, and ticks, which play a significant role in spreading the disease

from infected to healthy animals. Other sources of transmission include direct contact, sharing of feed and water, the use of semen from infected breeding bulls, and blood contact. Wildlife and birds do not appear to play a role in the transmission of the virus. However, for the first time, crows have been reported to transmit the disease by snatching skin nodules from infected cows.

The reported tests and methods for the diagnosis of Lumpy skin disease in tehsil Bara, district Khyber are presented in Table 5. Complete blood profile (CBP) and blood serum chemistry analysis are effective tools for determining the infection status of animals. Preparation of histopathological slides is the best approach to estimate tissue lesions in infected parts of the skin, mouth, esophagus, and lungs of animals, as well as lesions observed in the postmortem. Advanced diagnostic techniques such as immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA), PCR, RT-qPCR, and transmission and scanning electron microscopy can be used to identify the Lumpy skin disease virus.

The reported drugs and medicines for the treatment of Lumpy skin disease virus in tehsil Bara district Khyber are displayed in Table 6. In this study, no proper antiviral drugs or medicines have been reported against the Lumpy skin virus. For combating secondary infections of the skin and lungs, broad-spectrum antibiotics like sulfonamides (SN) and others have been reported. Dexamethasone has been reported for anti-inflammatory purposes, but its efficacy report shows a suppressive effect on the immune system. It is better to use other anti-inflammatory medicines available in the market. For insect repellent, antiseptic ointment has been suggested. Aspirin has been reported for antipyretic purposes to relieve temperature, and diclofenac gel has been reported for analgesic purposes to relieve pain. Gel was externally used on skin lesions. Multi-vitamin injections like AmiVicom, Metabolase Forte, and Minerals tablet 'White Gold' have been recommended for immunity boosting. Management of wounds with proper care and nursing has been reported to prevent better Lumpy skin disease. In this study, ethno-veterinary medicine, including oils of medicinal plants, especially turpentine oil, has been reported.

The reported control as well as a risk factor for Lumpy skin disease in tehsil Bara, district Khyber, are shown in Table 7. To control Lumpy skin disease, a prohibition on the importation and transportation of animals and their products from one place to another has been reported. It is reported to be important to ban unauthorized transboundary animal movements, separate sick animals from the other members of the herd, and avoid sharing feeding and drinking troughs. To control the disease, monitor the legalized trade, grazing, nomadic life, testing of imported animals, and quarantine. Vaccines have been reported as the most effective tool to control the disease. To control the disease, vaccines have been recommended for all cows of all ages (including pregnant cows) annually in early spring. We have observed live, attenuated vaccines commercially available against Lumpy skin disease. In this study, the use of disposable syringes and hygienic tools for cow surgery has been reported. Disease transmission has been reported through insect vectors (biting flies, mosquitoes, and ticks). It must be controlled and prevented in disease-outbreak areas. In the summer season, the insect population was greater in comparison to other months, so the disease was at its peak in the month of monsoon. It is reported that high temperatures and high humidity in the environment are favorable for vector populations, so avoid standing water to minimize insect populations. To control disease, farming practices such as avoiding contact with neighboring herds, stopping purchases from untrustworthy sources, using local and healthy bulls for breeding, raising awareness among farmers, and obtaining technical and expert support in relevant fields have been reported.

The total estimated loss in Pakistani rupees (PKRs) of dead cattle in the selected areas of tehsil Bara, district Khyber, are shown in Table 8. The total number of animals died in Speen Qaber, Akakhel, Shlobar Quam, Bar Qamber Khel, and Nala Sourdandh were 32, 46, 59, 12, and 8 respectively. The average price per cow in Speen Qaber and Akakhel is seventy-five thousand Pakistani rupees (PKR. 75000.00), in Shlobar Quam is seventy-two thousand Pakistani rupees (PKR.72000.00), in Bar Qamber Khel is seventy-three thousand Pakistani rupees (PKR. 73000.00), and in Nala Sourdandh

Table 1. Total number of reported cattle, infected, normal, died, recovered and in the recovery stage of Lumpy skin disease in the selected area of Tehsil Bara, District Khyber.

S. No.	Selected Areas	Total inspected cattle	Infected	Normal	Out of total infected Cattle		
					Died	Recovered	In recovery stage
1	Speen Qaber	580	523	57	32	209	282
	% age of cases		(90.17 %)	(9.82 %)	(6.11 %)	(39.96 %)	(53.91 %)
2	Akakhel	496	472	24	46	183	243
	% age of cases		(95.16 %)	(4.88 %)	(9.75 %)	(38.77 %)	(51.48 %)
3	Shlobar Quam	484	466	18	59	173	234
	% age of cases		(96.28 %)	(3.71 %)	(12.66 %)	(37.12 %)	(50.21 %)
4	Bar Qamber Khel	255	220	35	12	113	87
	% age of cases		(86.27 %)	(13.72 %)	(5.30 %)	(53.30 %)	(41.04 %)
5	Nala Sourdandh	206	172	34	8	88	76
	% age of cases		(83.50 %)	(16.50 %)	(4.65 %)	(51.16 %)	(44.18 %)

Table 2. General opinions from community respondents (% age of Yes and No) regarding the outbreak of Lumpy skin disease in Tehsil Bara, District Khyber.

S. No.	Parameter	Reported Respondent (% age) in Yes and No	
		Yes	No
1	Pre-vaccination/ before outbreak	0	100
2	Isolation of infected cattle	09	91
3	Burial of dead bodies	01	99
4	Openly lifted dead cow bodies	99	01
5	Meat is eatable	70	30
6	Milk is useable	75	25
7	Treatment of cattle	60	40
8	Human got disease	05	95
9	Buffalo got diseased	05	95
10	People superstition opinion of disease transmission through meat and milk	85	15
12	Technical approach for treatment of disease	10	90
13	Government funds available	12	88
14	Vaccines available after outbreak	100	00
15	Efficacy of local vaccines	00	100
16	Efficacy of branded and Multi-national vaccines	95	05

Table 3. Reported signs and symptoms of Lumpy skin disease in Tehsil Bara, District Khyber.

Organs	Reported Signs and Symptoms
Skin	On skin wide spread lump/nodules were appeared and swelling of superficial lymph nodes were observed. Later on necrotic and ulcerative lesions converted into fibrotic. In severe cases, the ulcerative lesions may develop in mucous membranes of mouth, esophagus, larynx and trachea.
Milk	Milk production was decreased
Eye	Inflammation of eye membrane and lacrimation was observed.
Pharyngeal Secretion	Excessive salivation was observed.
Fever	Temperature was recorded from 103.40 to 106.00 ºF body temperature.
Nasal	Increased nasal secretion and rhinitis was observed.
Secondary infection	Secondary bacterial infection and fly worm infestation (Maggots) was observed in some cases.
Incubation	Incubation period of Lumpy skin virus was observed from 4-12 days
Movement	Reluctance movement and some depression of cattle was observed.
Emaciation	Severe emaciation and abnormal thinness of skin was observed in this disease and even cow went into recombinant position.
Feeding	At last in morbidity position, they stop feeding and lack of appetite was observed.
Death	Death rate was reported variant, heifer death rate was 55 %, while calf and pregnant cow death rate was 75 %.
Recovery time	In this study, recovery time was reported variant (from one weak up to 2 months)

Table 4. Reported sources of transmission of Lumpy skin disease virus in Tehsil Bara, District Khyber.

Source/Agent	Reported Source of Transmission
Trans Boundary Disease	The disease was transmitted through illegal buying-selling across borders and through transportation of animals from one place to another place was reported.
Vector Borne	Insect vectors like mosquitoes, flies, and ticks have been observed to transmit disease from infected cows to healthy one.
Other Sources	Sources like direct contact, shared feed and water, semen of breeding bull and blood contact has been observed in transmission of this disease.
Bird	In bird only crow has been reported to transmit disease by snatching the skin nodules from infected cow.

Table 5. Reported tools and tests for virus diagnosis of Lumpy skin disease in Tehsil Bara, District Khyber.

Test	Reported Diagnosis
Blood Samples	Complete blood profile (CBP) and blood serum chemistry analysis are effective tools for determining the infection status of animals.
Histopathological Slides	Preparation of histopathological slides is the best approach to estimate tissue lesions in infected parts of the skin, mouth, esophagus, and lungs of animals, as well as lesions observed in postmortem.
Advanced Virus Identification	Advanced tests like Immunofluorescence Assay, Enzyme-Linked Immunosorbent Assay (ELISA) PCR, RT-qPCR, and transmission and scanning electron microscopy has been reported for virus identification.

Table 6. Reported drugs and medicines for the treatment of Lumpy skin disease in Tehsil Bara, District Khyber.

Drugs/Medicines	Reported Effect of Medicines
Antiviral Drugs	No proper antiviral drugs or medicines has been reported against lumpy skin disease virus.
Antibiotic	Broad-spectrum antibiotics like sulphonamide and others were reported effective against bacterial secondary of skin and lungs infection.
Dexamethasone	It was used as an anti-inflammatory medicine, but its efficacy report showing suppressive effect on the immunity.
Antiseptic Ointment	It was reported as the repellent of insects.
Aspirin	It was reported for antipyretics purpose to relieve temperature.
Diclofenac gel	It was used for analgesic purpose to relieve pain. Gel was externally used.
Multi-Vitamins Injection and Minerals	Injections like *AmiVicom* and *Metabolase Forte*, and tablets of mineral 'White Gold' are available in market used for immunity boosting.
Management of Wounds	Management of wounds with proper care and nursing was reported best approach to cure this disease.
Ethno-veterinary Medicine	Variant traditional treatments like using oil of medicinal plants especially turpentine oil was reported.

Table 7. Control and risk factor of Lumpy skin disease in Tehsil Bara, District Khyber.

Control Parameter	Effective Measurement
Restriction of Animal Movement	To control the disease, restriction of cattle importation and transportation and their products from one place to another and banned of unauthorized transboundary animal movements, separating sick animals from rest of the herd and avoid shared feeding and drinking troughs have been reported.
Monitoring of Animals	To control disease monitor legalized trade, grazing, nomadic life and testing of imported animals and quarantine.
Vaccination	To control disease, vaccination was reported as most effective tool and vaccines have been recommended for all cows of all ages (including pregnant cows) annually in early spring. We have observed live, attenuated vaccines commercially available "LSD Vaccines" against Lumpy skin disease in the market.
Disposable Syringe	For surgery use of disposable syringe and discarded used ones was reported.
Vector	Disease transmission has been reported through insect vectors (mosquitoes, ticks and biting flies). To prevent disease insects must be controlled in outbreak areas.
Isolation	For disease control isolation of infected cows from healthy one was strongly suggested.
Seasons	This disease was reported in summer season in which insect population are high. It is reported that weather of high temperature and humidity are suitable for insect growth, and avoid standing water in such weather to minimize insect population.
Farming Practices	To control disease create awareness among farmers, to avoid contact of neighbors herd, and do not purchase from untrusted sources and get support of technical and expert in relevant field, use scientific medical approach and avoid traditional approach of treatment.

Table 8. Total reported estimated death loss in Pakistani rupees (PKRs.) of cattle due to Lumpy skin disease in selected area of Tehsil Bara, District Khyber.

S. No.	Areas	Total Number of Died Cows	Average Price per Cow	Total Price (PKRs.)
1	**Speen Qaber**	32	75000	2400000
2	**Akakhel**	46	75000	3450000
3	**Shlobar Quam**	59	72000	4280000
4	**Bar Qamber Khel**	12	73000	876000
5	**Nala Sourdands**	8	76000	608000
	Gross total loss (PKRs.)			PKRs. 10738000 **10.741 Million**

is seventy-six thousand Pakistani rupees (PKR. 76000.00). The gross total loss in tehsil Bara district Khyber due to the death of cattle excluding the treatments and care costs is more than ten million Pakistani rupees (PKR. 10738000.00 equal to 10.738 Million).

4. DISCUSSION

Lumpy skin disease (LSD) is an extremely contagious, eruptive, and economically important cattle disease. The morbidity rate ranges between 10 and 20 %, with mortality rates ranging between 1 and 5 % deemed acceptable [8]. In Pakistan, the first LSD case was identified in November 2022, in the Sindh district of Jamshoro. The disease was spread from Sindh to Punjab and parallel reached through the border of Dera Ismail Khan to Khyber Pakhtunkhwa province of Pakistan. In the district of Khyber, it was first observed in April 2022. The incidence of the disease has been spreading since early summer 2022 in district Khyber due to the illegal transportation of animals from India to Pakistan (District Khyber) on occasion of Eid al-Adha. Every year, Pakistani people sacrifice a huge number of animals on the occasion of the religious festival of Eid al-Adha. According to expert opinion, cattle numbers have increased tenfold in the district of Khyber due to their scarification on this holy occasion. It is a trans boundary disease, which can be introduced to a new area through the transportation of animals from one place to another and the illegal buying and selling of livestock across borders [22]. The transported animal (cow) brings the Lumpy skin disease virus to district Khyber.

The study was conducted in five selected areas when the disease was in the outbreak and ongoing in tehsil Bara. The total number of reported cases of dead cows in selected areas of Speen Qaber, Akakhel, Shlobar Quam, Bar Qamber Khel, and Nala Sourdandh were 32, 46, 59, 12, and 8 respectively. Among these areas, the highest infection (96.28 %), followed by the highest mortality of 59 (12.66 %), and the lowest recovery (37.1 %) have been reported from the Shlobar Quam area of Lumpy skin disease. As indicated by the results of this study, a high infection rate was followed by a high mortality rate. We can speculate that the highest mortality rate was caused by a lack of vaccination, openly lifted dead cow bodies and a lack of proper wound treatment and management. Before the outbreak of the disease vaccines were not available in these areas, while later on during the outbreak, vaccines have been available at Veterinary hospitals with the collaboration of the Government of Pakistan. The efficacy of local vaccines has been reported at zero percent, while the efficacy of branded and multinational vaccines has shown 95 % results. In this study, the reported mortality rate ranged from 4.65 % to 12.66 %. However, in literature, morbidity ranges between 5 % and 45 % (sometimes up to 100 %), and the mortality rate is generally less than 10 % (sometimes up to 40 %) [23]. In Greece, outbreak morbidity and mortality rates were reported to be 8.7 % and 0.4 % [17] and in Turkey to be 12.3 % and 6.4 % respectively [24]. The lowest mortality has been observed in Nala Sourdandh at 4.65 %, followed by Bar Qambar Khel at 5.30 %. In Bar Qamber Khel, people also benefited from the existence of a Civil Veterinary hospital, and this area had the highest recovery rate

of cattle from Lumpy skin disease.

In the current study, wide-spread lumps and nodules appeared on the skin, and swelling of superficial lymph nodes was observed after an incubation period of 4–12 days, which was also reported in previous studies [8, 25, 26]. The status of this disease ranges from acute to chronic in which necrotic and ulcerative skin lesions convert into fibrotic lesions. It was observed that in extreme cases, ulcerative lesions may form on other body parts, like the mucous membranes of the mouth, esophagus, larynx, and trachea [27, 28]. The lesion worsens with time and converts to a secondary bacterial infection and fly worm infestation (maggots) occurred [29]. According to Tuppurainen *et al*. the Lumpy skin disease causes decreased milk production, conjunctivitis that produces lacrimation (tear flow), pharyngeal secretion (excessive salivation), nasal discharge, loss of appetite, and fever (raised from 103.40 °F to 106.0 °F), which may last for 6 to 72 h or more and, in exceptional cases, up to 10 days [8]. According to the findings of this study, calves and pregnant cows experienced more typical and severe lesions 24 to 48 hours earlier than their adult counterparts [30]. Cows and bulls may experience temporary or permanent infertility problems [8]. Animal shows reluctance movement and some depression. In this study, significant emaciation (weakness) and abnormal skin thinness (caused by disease and inadequate nutrition) have been documented. Finally, the animal stops eating, loses interest in food, and even adopts a reclining posture. According to the findings of this study, the Lumpy skin disease in district Khyber resulted in the deaths of 55 % of heifers, while the death rate of calves and pregnant cows was 75 %. The pregnant cows and calves died at higher rates due to their weaker immunity compared to their adult counterparts.

The precise animal species affected by the Lumpy skin virus, as well as the specific clinical disease symptoms they display, have yet to be established in the literature. However, it is known that the disease is not zoonotic in nature [22], and transmission of the virus through the consumption of meat and milk is not a concern. While many individuals consume milk from infected animals, they tend to avoid consuming the meat due to superstitious beliefs that it may cause infection. Conflicting reports exist in the literature regarding the infection of Asian water buffaloes with Lumpy skin disease; this study has only observed cattle to be affected, with no reports of the disease in buffalo [31]. Non-ruminant species are not susceptible to the virus [31], and wildlife does not appear to play a role in the epidemiology of the disease [32]. Transmission of the disease primarily occurs via insect vectors such as mosquitoes, flies, and ticks, with disease incidence peaking during the rainy season and summer months [32]. Other sources of transmission, such as direct contact, water, and feed, have also been reported in this study [33].

Studies have indicated that Lumpy skin disease (LSD) can be initially suspected based on case history and clinical presentation [34]. However, laboratory tests are necessary to confirm the diagnosis of the disease. Blood samples can be obtained from cows via jugular vein puncture in cases where the disease is epidemic. Samples should be taken on the 1st and 2nd days post-appearance of skin nodules, as well as between the 4th and 14th days [35]. Multiple samples should be collected for accurate diagnosis, including nodular lesions on the skin, scabs, crusts on the external coat, blood (7 to 21 days post-infection), ocular and nasal discharges, semen, and blood at least 7 days after infection [36-38]. For histopathological analysis, skin nodules from ulcerative lesions in affected animals should be surgically removed after local anesthesia and within the first week of clinical symptoms. Virus isolation can be performed on a biopsy sample collected during the post-mortem examination of skin nodules, lung lesions, or lymph nodes [25, 39].

Lumpy skin disease is a viral infection for which no effective antiviral drugs have been reported to date for a complete cure [22]. Nevertheless, field observations indicate that farmers need not panic as the disease is not fatal and can be treated successfully if proper care is taken at the right time. The disease is caused by a virus, and therefore, treatment is aimed at managing symptoms and enhancing the animal's immune response. After an incubation period of 4-12 days following primary viral infection, widespread nodules appear on the skin, which may worsen over time and become complicated by secondary infections [26]. Antibiotics, anti-histaminic, analgesic-antipyretics,

antiparasitic drugs, immunity boosters, and wound management are among the general groups of drugs recommended for treating the disease. Secondary bacterial infections of the skin, mouth, and lungs are common, and broad-spectrum antibiotics like sulphonamide are effective against them [39]. However, prolonged recovery may be required, especially if the animal's defense mechanism is weakened [18-20]. Recovery times reported in this study varied from one week to three months, depending on factors like age, breed, immunological response, and the production period of the animal [40]. Diclofenac gel is a good option for pain relief, while antiseptic ointment with fly-repellent properties is useful for managing wounds [8]. Dexamethasone is not recommended due to its immune suppressive effects, which can exacerbate the disease. Instead, multi-vitamin injections like Amivicom and Metabolase Forte, and mineral tablets like 'White Gold' are useful for boosting immunity [41, 42]. Traditional treatments using oil of medicinal plants like turpentine oil are also common in ethno-veterinary medicine. Proper care and nursing of the animal during the disease course are crucial for successful treatment.

The present study indicates that the Lumpy skin disease virus (LSDV) affects cattle of different breeds and is capable of surviving for extended periods both on and off animal hosts. LSDV is highly resilient to various chemical and physical agents and can survive for up to six months in shaded animal pens under favorable environmental conditions [43]. Additionally, LSDV can persist for approximately 33 days in necrotic skins and up to 18 days in lesions on air-dried hides at room temperature, as well as in moist environments, which offer protection against the sun rays [44]. The virus has been found in various bodily fluids, including nasal, lachrymal, saliva, sperm, pharyngeal secretions, and milk, where it can remain infectious for up to 22 days [6]. Environmental factors, such as high temperature, standing water, dunghills, grasslands, and high humidity, contribute to an increase in the vector population and therefore LSDV cases also increase [45]. In addition, contact with nearby herds, acquisition of animals from untrustworthy sources, use of a local breeding bull, and lack of regular observation are contributing factors to the spread of LSDV [46]. To prevent LSD, it is crucial to restrict the importation and transportation of animals and their products from one place to another. Animal monitoring, such as grazing, trade, nomadic and transhumance farming, isolation of sick animals from the rest of the herd, regular testing, legal and unauthorized transboundary animal movements, quarantine, and separation of feeding troughs, can be helpful parameters for the prevention of this disease [47]. Vaccination is an important measure for disease prevention, and every animal should be vaccinated at least once a year, ideally in early spring. Animals over six months of age, including pregnant cows, should be vaccinated against LSD annually in early spring [21]. Commercially available live, attenuated vaccines are effective against LSD. During a disease outbreak, it is crucial to use one needle per animal to prevent the spread of the virus from sick to healthy animals. [6, 21, 43-47].

The economic and social impact of lumpy skin disease (LSD) on society and the economy is considerable, as recognized by the World Organization for Animal Health, which classifies it as a notifiable disease [42]. In particular, farmers may face major economic losses due to decreased milk production (ranging from 10 % to 85 %), infertility, abortion, inferior hide quality, and secondary infections. Mortality rates from LSD are generally low (ranging from 1 % to 5 %), but the direct economic burden on farmers can be significant, regardless of whether the infected animals are native or exotic [19]. In addition, there may be indirect economic consequences, such as restrictions on animal trade, immunization and treatment costs, quarantine expenses, and the need to maintain herd biosecurity [47]. These losses can result in significant financial loss for industries related to livestock and its byproducts, as well as for poor farmers who rely on livestock for their livelihoods. *Capripoxviruses*, which cause LSD, can also cause sheep-pox and goat-pox, which are economically significant diseases [47]. Moreover, given their ability to spread across regions, they pose a significant impediment to global trade and may even serve as an economic bioterrorism agent [3]. In the current study, conducted in tehsil Bara, it was found that the gross total loss due to LSD in three months, considering the death of untreated animals, was more than 10 million Pakistani rupees (PKR. 10.738 million).

5. CONCLUSION

Cattle and buffalo play a vital role in the global economy as they provide significant economic benefits. Lumpy skin disease (LSD) is an emerging disease that has a detrimental effect on livestock populations, which may lead to a decrease in livestock and product exports. Accurate diagnosis and timely treatment can significantly aid in the cure of the disease. Vaccination is currently considered the most effective tool in controlling the disease, but it should be combined with timely preventive measures and good management practices to prevent the disease from spreading to livestock. Since LSD is a transboundary disease, it is essential to quarantine, legalize, and restrict animal transportation across borders and their products, as it could negatively impact rural economies, if it spreads to new regions. Effective prevention measures include isolating infected animals and burying deceased ones. Previously, the disease was limited to African countries and a few other nations; however, it has gradually spread to Asian countries, including Pakistan. The government alone cannot combat this disease; it requires support from society and the private sector. To combat the disease, trained veterinarians and expert health workers are needed to diagnose and treat the disease in the field. Therefore, all stakeholders, including government, non-governmental organizations, and private and independent sectors, should work together to combat this harmful Lumpy skin disease.

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7. CONFLICT OF INTEREST

The authors confirmed that they have no conflict of interest.

8. REFERENCES

1. E.R. Tulman, C. Afonso, Z. Lu, L. Zsak, G. Kutish, and D. Rock. Genome of lumpy skin disease virus. *Journal of virology* 75(15): 7122-30 (2001).
2. R. Buller, B. Arif, D. Black, K. Dumbell, J. Esposito, E. Lefkowitz, G. McFadden, B. Moss, A. Mercer, R. Moyer, and M. Skinner. Family poxviridae. *In: Virus taxonomy: Classification and nomenclature of viruses. 8th report of the International Committee on Taxonomy of Viruses* pp. 117-33 (2005).
3. S. Abutarbush, M. Ababneh, I. Al Zoubi, O. Al Sheyab, M. Al Zoubi, M. Alekish, and RJ. Al Gharabat. Lumpy Skin Disease in J ordan: Disease Emergence, Clinical Signs, Complications and Preliminary-associated Economic Losses. *Transboundary and emerging diseases* 62(5): 549-54 (2015).
4. M.A. Alkhamis, and K.V. Waal. Spatial and temporal epidemiology of lumpy skin disease in the Middle East. *Frontiers in veterinary science* 3(19): 2012-2015 (2016).
5. J.A. Woods. Lumpy skin disease virus. In: Virus infections of ruminants Amsterdam: Elsevier Science publishers BV. pp. 53-67 (1990)
6. B. Barnard, E. Munz, K. Dumbell, and L. Prozesky. Lumpy skin disease. *Infectious diseases of livestock with special reference to Southern Africa* 1: 604-12 (1994).
7. E. Tuppurainen, and C. Oura. Lumpy skin disease: an emerging threat to Europe, the Middle East and Asia. *Transboundary and emerging diseases* 59(1): 40-8 (2012).
8. F.G. Davies. Lumpy skin disease of cattle: a growing problem in Africa and the Near East. *World Animal Review* 68(3): 37-42 (1991).
9. S.E. Amin, N.U. Haq, S. Haider, M.F. Shaukat, A. Arif, A. Fareed, K. Arslan, M. Abid, and S. Qammer. Emergence and transboundry spread of lumpy skin disease in South Asian Countries: a review. *Journal of Xi'an Shiyou University, Natural Science* 18(9): 517-545 (2021).
10. E. Tuppurainen, E.H. Venter, J. Shisler, G. Gari, G. Mekonnen, N. Juleff, N. Lyons, K.D. Clercq, C. Upton, T. Bowden, and S. Babiuk. Capripoxvirus diseases: current status and opportunities for control. *Transboundary and emerging diseases* 64(3): 729-745 (2017).
11. B. Ali, and H. Obeid. Investigation of the first outbreaks of lumpy skin disease in the Sudan. *British Veterinary Journal* 133(2): 184-9 (1977).
12. J. Lubinga, E. Tuppurainen, W. Stoltsz, J. Ebersohn, and E. Venter. Detection of lumpy skin disease virus in saliva of ticks fed on lumpy skin disease virus-infected cattle. *Experimental and applied acarology* 61(1): 129-38 (2013).
13. J.C. Lubinga. The role of Rhipicephalus (Boophilus) decoloratus, Rhipicephalus appendiculatus and

Amblyomma hebraeum in the transmission of lumpy skin disease virus. Doctoral dissertation, University of Pretoria (2013).
14. E.S. Tuppurainen, J.C. Lubinga, WH. Stoltsz, M.Troskie, ST. Carpenter, JA. Coetzer, EH. Venter, and CA. Qura. Evidence of vertical transmission of lumpy skin disease virus in Rhipicephalus decoloratus ticks. *Ticks and tick-borne diseases* 4(4): 329-33 (2013).
15. H. Ali, A. Ali, M. Atta, and A. Cepica. Common, emerging, vector-borne and infrequent abortogenic virus infections of cattle. *Transboundary and emerging diseases* 59(1): 11-25 (2012).
16. K. Tasioudi, S. Antoniou, P. Iliadou, A. Sachpatzidis, E. Plevraki, E. Agianniotaki, E. Fouki, C. Mangana, O. Vougiouka, O. Chondrokouki, and C. Dile. Emergence of lumpy skin disease in Greece, 2015. *Transboundary and Emerging Disease*s 63(3): 260-5 (2016).
17. J. Woods. Lumpy skin disease: a review. Tropical animal health and production 20(1): 11-7 (1998).
18. H. Abdulqa, H. Rahman, H. Dyary, and H. Othman. Lumpy skin disease. *Reproductive Immunology: Open Access* 1 (2016).
19. G.H. Jameel. Determination of complications decrease the risk factor in Cattle infected by lumpy skin disease virus in diyala province, Iraq. *International Journal of Micro Biology, Genetics and Monocular Biology Research* 2: 1-9 (2016).
20. P. Kumar, R.R. Kumari, S. Devi, M.K. Tripathi, J. Singh, R. Kumar, and M. Kumar. Emergence and transboundary spread of lumpy skin disease in South Asia. *FM. Abdallah, HM. El Damaty, GF. Kotb* 150-58 (2018).
21. J. Coetzer, and E. Tuppurainen. Lumpy skin disease. *Infectious diseases of livestock* 2: 1268-76 (2004).
22. M. Şevik, and M. Doğan. Epidemiological and molecular studies on lumpy skin disease outbreaks in Turkey during 2014–2015. *Transboundary and emerging diseases* 64(4): 1268-79 (2017).
23. E. Tuppurainen, T. Alexandrov, and D. Beltrán-Alcrudo. Lumpy skin disease-a manual for veterinarians. *FAO Animal Production and Health Manual* 20: 1-60 (2017).
24. B.S. Bernardo, I.R. Haga, N. Wijesiriwardana, P.C. Hawes, J. Simpson, L.R. Morrison, N. MacIntyre, E. Brocchi, E. Atkinson, A. Haegeman, and K.D. Clercq. Lumpy skin disease is characterized by severe multifocal dermatitis with necrotizing fibrinoid vasculitis following experimental infection. *Veterinary pathology* 57(3): 388-96 (2020).
25. H. Vorster, and H. Mapham. Pathology of lumpy skin disease. *International Journal of Livestock Production* 1: 16-21 (2008).
26. P.S. Yousefi, K. Mardani, B. Dalir-Naghadeh, and G.J. Amin. Epidemiological study of lumpy skin disease outbreaks in North-western Iran. *Transboundary and emerging diseases* 64(6): 1782-1789 (2017).
27. C.M. Kahn, and S. Line. The Merck veterinary manual. Merck Whitehouse Station, NJ, EUA (2010).
28. M.H. Tageldin, D.B. Wallace, G.H. Gerdes, J.F. Putterill, R.R. Greyling, M.N. Phosiwa, R.M. Al Busaidy, and S.I. Ismaaily. Lumpy skin disease of cattle: an emerging problem in the Sultanate of Oman. *Tropical animal health and production* 46(1): 241-6 (2014).
29. Y.J. Shen, E. Shephard, N. Douglass, N. Johnston, C. Adams, C. Williamson, and A.L. Williamson. A novel candidate HIV vaccine vector based on the replication deficient Capripoxvirus, Lumpy skin disease virus (LSDV). *Virology journal* 8(1): 1-12 (2011).
30. E. Mulatu, and A. Feyisa. Review: Lumpy skin disease. *Journal of Veterinary Science and Technology* 9(535): 1-8 (2018).
31. EA. Panel. Scientific opinion on lumpy skin disease. *European Food Safety Authority Journal* 13(1): 3986-73 (2015).
32. P. Mashamba. Molecular characterisation of lumpy skin disease virus in Mahikeng local municipality. *Doctoral dissertation, North-West University, South Africa* (2020).
33. A.N. Neamat-Allah. Immunological, hematological, biochemical, and histopathological studies on cows naturally infected with lumpy skin disease. *Veterinary world* 8(9): 1131 (2015).
34. S. Kasem, M. Saleh, I. Qasim, O. Hashim, A. Alkarar, A.A. Obeida, A. Gaafer, R. AL-Sahaf, A. Al-Doweriej, and F. Bayoumi. Outbreak investigation and molecular diagnosis of Lumpy skin disease among livestock in Saudi Arabia 2016. *Transboundary and emerging diseases* 65(2): e494-e500 (2018).
35. J. Banchroft, A. Stevens, and D. Turner. Theory and practice of histological techniques. *Elsevier health sciences* (1996).
36. E.S. Tuppurainen, E. Venter, and J. Coetzer. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort Journal of*

Veterinary Research 72(2): 153-64 (2005).
37. A. OIE. Manual of diagnostic tests and vaccines for terrestrial animals. *Office international des epizooties, Paris, France* 1092-106 (2008).
38. K. Weiss. Lumpy skin disease virus. Cytomegaloviruses Rinderpest Virus Lumpy Skin Disease Virus. *Springer* 111-31 (1968).
39. A.F. Feyisa. A case report on clinical management of lumpy skin disease in bull. *Journal of Veterinary Science & Technology* 9(3): 538 (2018).
40. D. Biswas, S. Saha, S. Sayeed. Outbreak of lumpy skin disease of cattle in south-west part of Bangladesh and its clinical management. *Veterinary Sciences: Research and Reviews* 6(2): 100-8 (2020).
41. F.A. Murphy, E.P.J. Gibbs, M.C. Horzinek, and M.J. Studdert. Veterinary virology, Elsevier (1999).
42. M. Das, M.S.R. Chowdhury, S. Akter, A.K. Mondal, M. Uddin, M. Rahman. An updated review on lumpy skin disease. Perspective of Southeast Asian countries. *Journal of Advanced Biotechnology and Experimental Therapeutics* 4(3): 322-33 (2021).
43. R. Kitching, P. Bhat, D. Black. The characterization of African strains of capripoxvirus. *Epidemiology & Infection* 102(2): 335-43 (1989).
44. G. Gari, A.W. Szkuta, V. Grosbois, P. Jacquiet, and F. Roger. Risk factors associated with observed clinical lumpy skin disease in Ethiopia. *Epidemiology & Infection* 138(11): 1657-66 (2010).
45. J. Casal, A. Allepuz, A. Miteva, L. Pite, B. Tabakovsky, D. Terzievski, T. Alexandrov, and D.B. Alcrudo. Economic cost of lumpy skin disease outbreaks in three Balkan countries. Albania, Bulgaria and the Former Yugoslav Republic of Macedonia (2016-2017). *Transboundary and emerging diseases* 65(6): 1680-8 (2018).
46. F. Namazi, and A. Khodakaram Tafti. Lumpy skin disease, an emerging transboundary viral disease: A review. *Veterinary Medicine and Science* 7(3): 888-96 (2021).
47. S. Babiuk, T. Bowden, G. Parkyn, B. Dalman, L. Manning, J. Neufeld, C.E. Hyatt, J. Copps, D.B. Boyle. Quantification of lumpy skin disease virus following experimental infection in cattle. *Transboundary and Emerging diseases* 55(7): 299-307 (2008).

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Short Communication

Bacteriophage-based Vaccine: A New Dawn for Vaccine Design and Development

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Abstract: The COVID-19 epidemic has strained healthcare systems, causing stress among personnel and facing significant economic and social issues. COVID-19 patients have significant symptoms, necessitating prompt treatment. It is a global urgency to develop effective vaccinations against COVID-19. Quick immunization of the whole world population against an ever-changing, extremely deadly virus is alarming, and various vaccine techniques are being researched. Bacteriophages are helpful in the treatment of multidrug-resistant bacterial infections. But, their clinical efficacy may go far beyond. One of the most significant bioproducts in medicine is thought to be vaccines. Vaccines for a variety of diseases have been made. However, certain vaccinations have disadvantages, such as high prices and immunological responses. In this regard, the use of bacteriophages has been suggested as an exciting alternative for making more inexpensive vaccines. Bacteriophage-displayed vaccines are based on the antigens being expressed on the phage surface. This tactic uses the inherent advantages of these particles, including their high stability, inexpensive production, and adjuvant capacity. Phage-displayed, phages DNA and hybrid phage-DNA vaccines are the three phage-based vaccines that are currently offered. The traditional method for finding novel barrier protection epitopes, antigens, and mimotopes is phage display. In this frame of reference, phage particles serve as a versatile, effective, and promising strategy for making vaccine delivery systems that are more effective and should be widely applied in the future. The phage-vaccine technique can potentially address the growing demand for innovative vaccinations against emerging diseases. This short communication addresses bacteriophage uses in vaccine development and discusses recent developments in bacteriophage-based vaccinations. It also focuses on and describes bacteriophages as a novel vaccine candidate for COVID-19.

Keywords: Antigen delivery, Bacteriophage, COVID-19, Vaccine.

1. INTRODUCTION

Bacteriophages (phages) are natural bacteria predators that identify, target, and kill a bacterial host while causing no harm to normal flora and human cells. Bacteriophages, bacteria's natural predators, are valuable in modern biotechnology. They've been proposed as antibiotic substitutes for various antibiotic-resistant bacterial species. Additionally, phages have applications such as DNA and proteins vaccine delivery systems, harmful bacterial strain identification agents, and protein and antibody display systems [1]. Phages are broadly used in biotechnology and have helped to illuminate essential molecular biology concepts. Phage biology offers many new Genetic technologies, medical diagnostics, and synthetic biology methods [2].

Bacteriophages are used as vaccine delivery systems [1]. Because of their excellent stability under harsh environmental circumstances, simplicity, low-cost, large-scale production, and robust adjuvant powers, phages are well-suited for vaccine formulation. Phage vaccines have a high safety level and good immunostimulatory effects because bacteriophages have a rich legacy of association with the mammalian body. The advent of bacteriophage display technology is a watershed moment in the evolution of phage-based

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vaccinations [3].

Vaccine development is one of the tremendous benefits of phage display, which has recently received considerable attention [3]. Vaccine development represents one of the essential impacts in the medical field, as it has saved countless animal and human lives [4]. Numerous research groups worldwide are currently focusing on developing medical vaccines that are more effective, safe, and less expensive. They possess a prolonged immune response [5]. Multiple vaccination programs, such as live attenuated, inactivated, and synthetic, have been developed and successfully tested for preventive purposes. Traditional vaccinations include destabilized or inactivated microorganisms [6]. Despite significant improvements in conventional vaccinations, there have been reports of challenges in microbes, low efficacy, and possible risks from virulent conversion or transmission to immunocompromised people [5].

Bacteriophage-based vaccines are thought to be a viable alternative to traditional vaccines. The intrinsic properties of bacteriophages increase the lifetime and immunogenicity of the expressed antigen [7]. Modern molecular tools, which allow for bacteriophage genome manipulation via phage display technology, facilitate the synthesis of phage-based vaccines. The current use of bacteriophages in vaccine direction has created an entirely new market opportunity [8]. Recombinant bacteriophage technology is one proposed method for overcoming the drawbacks faced by present vaccinations. Moreover, the phage-vaccine technique has the potential to meet the growing demand for novel vaccines against emerging diseases [9].

This brief communication article explains recent advances in synthesizing bacteriophage-based vaccines, particularly emphasizing the current phage COVID-19 vaccine strategy. In addition, the advantages of phage-based vaccines. Finally, critical issues and prospects for phage-based vaccinations are addressed.

2. BACTERIOPHAGE-BASED VACCINE DEVELOPMENT

The development of vaccines represents an array of significant medical and scientific breakthroughs, protecting numerous animals and humans [4]. Many research organizations worldwide are now concentrating on making a vaccine that is more efficient, cheaper, and safe for clinical use and has a more extended immune response [5].

To overcome the limitations of traditional vaccines, phage-based immunization is a promising alternative. This method uses bacteriophage properties to extend the shelf life and high stability of expressed antigens [10]. This method uses phage ability to promote both humoral and cell-mediated immunity [8, 11].

Bacteriophages are helpful in the treatment of multidrug-resistant bacterial illnesses. But, their clinical utility may go far beyond. Some researchers are enthusiastic about putting phages to work since the COVID-19 global epidemic presented a new and demanding challenge [12]. Bacteriophages have several characteristics that make them appealing for vaccine development. Phages are extremely simple and inexpensive to make vaccines on a large scale. They are also exceptionally stable in challenging, hostile environments, necessitating no sophisticated refrigeration for shipping and storage. Bacteriophages have also been found to stimulate both the innate and adaptive body's immune system. Bacteriophages are safe and have no severe side effects [13]. A turning point in applying bacteriophages in vaccine production occurred in 1985 with the invention of phage display technology. During phage display, a nucleotide sequence encoding a necessary amino acid or protein is inserted into the phage's coat protein gene. The protein or amino acid is then shown on the phage's surface. The surface of phages can be coated with foreign antigens to stimulate an immune response in vaccination applications. Using phage display technology, phages may be detached and put back together like jigsaw puzzle pieces. Whatever we layer on their surface will cause our immune systems to respond [14].

The most important technical advancement in molecular biology in recent years may be bacteriophage displays, which are effective research tools. This strategy is based on the bacteriophage surface's fusion amino acid proteins depiction. The virus genome is simultaneously encoded in DNA and wrapped around the fusion-displaying

mechanism. Phage display offers, at the very least, different approaches and strategies for developing vaccines [15].

Escherichia coli, a typical and unharmful bacterium in the human digestive system, is infected by phage T4. The finding made by Rao of Catholic University has opened the door for the phage to be used as a vehicle for the delivery of novel vaccines and treatments to patients. To accomplish this, he and his team wrap therapeutic Genetic code in proteins and attach them to the capsid's top shell. In Rao's work, chimera phages for vaccine and gene-targeted treatments are produced using the genome-editing tool CRISPR and bacteriophage display technology [16]. Professor Rao's T4 bacteriophage research has allowed his team to study the bacteriophages' therapeutic potential, including the invention of a dual anthrax-plague vaccination that has proven efficient in protecting mice models from pathogenic bacteria [17]. Rao's team was also involved with another T4 bacteriophage application. They give excellent proof studies for developing next-generation influenza vaccines employing bacteriophage T4 virus-like particle (VLP) platforms [18].

By including protective antigen expression mimicking epitopes or cassettes in the phage genomes, phage DNA vaccines are produced. Due to the protection provided by the coat protein, bacteriophage DNA vaccines are safer for injection, preservation, and transportation than conventional DNA vaccinations. This allows for the oral delivery of phage DNA vaccines. Nonetheless, phage-displayed vaccines are more widespread and popular than phage DNA vaccines. Phage-displayed vaccines are recombinant phages expressing immunostimulatory peptides or proteins on their surface through transcriptional fusing or by entrapping antigens with previously expressed antigen-binding peptides [19].

3. BACTERIOPHAGE-BASED VACCINE AND COVID-19

Rao and his colleagues took on a new challenge with the T4 phage platform and the novel idea of incorporating any microbe into a vaccine. Their efforts resulted in the development of a phage vaccine candidate that inhibited SARS-CoV-2 infections in cell cultures and led to elevated antibody levels in inoculated animals [16]. The work caught the attention of Adaptive Phage Therapeutics, a biotech start-up investigating the clinical uses of phages against multi-drug resistant pathogens. The focus shifted from using bacteriophages to treat multiple drug-resistant infections to making a COVID-19 vaccination using bacteriophages. The US defense department financed Adaptive Phage Therapeutics and the Catholic University Rao group to make the vaccine. If the Phase-one trials are effective, the T4 vaccine will enter humans Phase 3 clinical trials [20].

4. CLINICAL TRIALS OF A BACTERIOPHAGE-BASED VACCINE

In a clinical phase I/II trial (NCT04839146), healthy volunteers were tested with ABNCoV2, a vaccine based on virus-like particles (VLPs) of Phage AP205 designed with recombinant receptor domain (RBD) of SARS-CoV-2 produced in S2 Drosophila cells [21]. This study was completed on February 25, 2022, but no research findings have been published on clinicaltrials.gov. In addition, an open-label phase two trial with ABNCoV2 (NCT 05077267;EUCTR2021-001393-31) is underway in Germany. A phase three trial (NCT05329220) assessed the safety, tolerability, and Immunogenicity of ABNCoV2 in adults who had previously received SARS-CoV-2 vaccination [22].

Human aspartate-hydroxylase (ASPH) has been linked to several types of cancer and has been found to have good vaccine tolerance, provoking an immune response. SNS-301 is a bacteriophage lambda with an ASPH fragment coated inframe with the coat protein gpD. The safety, immunogenicity, and preliminary clinical efficacy of the phage-based vaccine SNS-301 were evaluated in a clinical trial (clinicalTrials.gov, identifiers NCT04217720, and NCT04034225). Individuals with persistent myelomonocytic leukemia and high-risk myelodysplastic syndromes were given SNS-301 in conjunction with pembrolizumab. According to the findings, the combination of SNS-301 and pembrolizumab was well tolerated by sick people, resulting in disease normalization and tumor response. Because these are preliminary findings, more research into the effectiveness of the combination treatment is required [23].

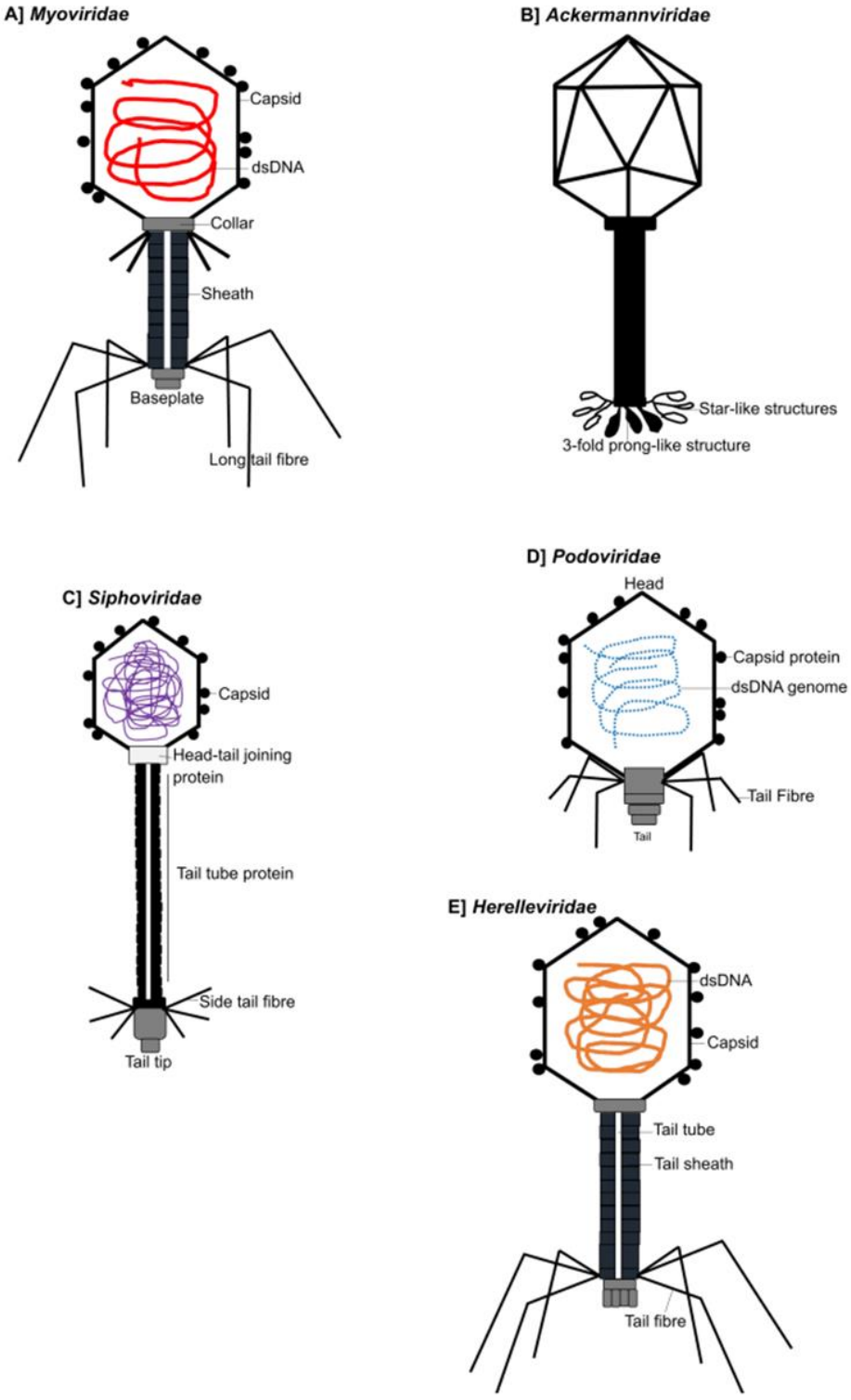

Fig. 1. A schematic representation of various morphologies of dsDNA bacteriophages. These are tailed phages belonging to the Caudovirales order.

5. PHAGE DISPLAYED AND COVID-19 PROTEIN TARGETS

A bacteriophage library can contain millions of diverse and distinct displayed peptide ligands. Using the ligand-receptor interactions that underpin phage display and an affinity selection-based method called "biopanning," epitope mapping and antigen display on the surface of bacteriophages has been effectively accomplished. This versatile approach has been considerably refined over the last three decades, resulting in various multimodal peptide display platforms. These recently developed diagnostic methods have been used in hazardous bacteria and viruses. Researchers are looking for neutralizing antibodies in the current COVID-19 pandemic to find possible treatment targets for the new SARS-CoV-2. This strategy contributes to studies on host-pathogen interactions and novel methods to discover coronavirus medication [24].

Knowing how coronaviruses interface with human receptor molecules has been made possible by extensively using bacteriophage display technology to aid in investigating targeted therapies via epitope mapping. While symptomatic treatment is provided to SARS-CoV-2 pandemic victims, there are currently no established methods for avoiding COVID-19 infection. By providing a library of inhibiting peptides and neutralizing antibodies, phage display could aid in our understanding of the infectious biology, pathogenesis, and blocking pathways of the very pathogenic, presently circulating SARS-CoV-2 [24].

Shortly, Phage display advancement will drive innovations to build practical techniques to uncover the processes associated with infections, cellular interaction, and the development of SARS-CoV-2 and other virus post-exposure therapies [24].

6. APPLICATIONS OF BACTERIOPHAGE-BASED VACCINES

Phages have a variety of unique characteristics that can be used to produce a potent inflammatory immune response against cancer or viral infection. Phage vaccines activate stimulatory pathways and present antigens to the immune system, like other nanotechnologies. Beyond vaccination, the medical industry is researching the use of nanotechnology for things like disease characterization, targeted drug delivery, and tissue regeneration. Mainly phages have been used in various applications, from cancer immunotherapies to biosensors [14]. Phage-based vaccines can be used therapeutically to treat non-infectious diseases as well as preventatively to fight microbial and parasitic infections. Phage-based vaccines are therapeutically applied using immunotherapy, which relies on the body's natural ability to fight disease. This way, phage-inspired vaccines can treat diseases like drug addiction, cancer, and neurodegenerative disorders. Phages may be a novel means of delivering genes or drugs because the mounting evidence suggests they can interact with and release their cargo inside mammalian cells. Phages are prokaryotic antagonists, so their capacity to deliver eukaryotic cargo naturally is severely constrained. The effectiveness of these viral particles at delivering preventative, diagnostic, and therapeutic cargoes into eukaryotic hosts can be significantly enhanced by phage surface engineering [3].

7. LIMITATIONS AND FUTURE PROSPECTS

Although bacteriophages have been used as vaccines, much research still needs to be done before they can be used clinically. The US FDA authorized the first phage therapy human clinical trial. A phages combination is used in this phage therapy study to cure a resilient *Staphylococcus aureus* infection. By using phages, this procedure enables the design of more fruitful clinical studies for vaccine development [13].

Although bacteriophage-based vaccines have been the subject of many preclinical studies, both *in vivo* and *in vitro,* demonstrating their potential for preventing infectious diseases, none of these vaccines have yet been approved for use in clinical settings. Numerous potentially fruitful preclinical studies aren't documented and never brought to market [13]. Regarding the activation of adaptive immune responses by phage-based vaccines, there is still considerable measure that we do not fully understand. It also shows how aspects of phage preparation, such as the amount of endotoxin bound to phages, can affect both quantitative and qualitative elements of immunogenicity [25].

It is essential to point out that the rising cost of

such clinical evaluations limits the use of phage-based products. In addition, the wide range of phage-based vaccines has the potential to assist in developing combinatorial vaccination strategies, which may prove to be more successful than those currently in use. Priority should be given to the design of clinical trials to expedite the approval process for these bioproducts. This is necessary because of the numerous applications for phage-based bioproducts and the effectiveness of phage-based vaccines that are currently under development [8]. Because there is no possibility of genetic transfer, vaccines based on phages established through *in vitro* display rather than the insertion of outside DNA into the phage genome can be categorized as natural bioproducts. These aspects are necessary to speed up commercializing vaccines based on phages [13]. Phage-based vaccines are an intriguing possibility for vaccine development because they have several benefits that cannot be found in traditional vaccine delivery systems. More study is needed to comprehend the immunological mechanism underlying phage vaccines to develop more highly specialized antigen delivery systems [13].

8. CONCLUSION

The SARS-CoV-2-related COVID-19 pandemic is wreaking havoc on public health, education, travel, and economic situations worldwide. How should we prepare for future outbreaks? Phages can contribute to the pandemic. The current situation highlights the critical significance of a novel therapeutic vaccination and diagnostic measures to combat COVID-19. A universal vaccine design platform capable of rapidly creating multiplex vaccine candidates is required to control future pandemics. As a result, a global effort is needed to make an effective vaccination widely available. This demands the participation of skilled scientists and financing to produce a vaccine that will help reduce future pandemics.

The benefits of bacteriophage-based vaccines overhead other vaccine technical advancements. Phage-based vaccinations get the potential to give considerable benefits by launching a novel strategy that allows the vaccine to change in response to coronavirus alterations quickly. Furthermore, phage-based vaccines are self-adjuvanted, automatically stimulating and boosting immune response while displaying multiple antigens. Phage therapy in humans is well recognized and has a satisfactory safety profile. The key benefits of phage-based vaccine:

1) Phage has an excellent safety profile.
2) Rapid adaptation of new vaccines to potential coronavirus mutations.
3) Manufacturing costs less than alternative vaccine approaches.
4) Immune response enhancement through self-adjuvant. https://aphage.com/science/vaccines/.
5) Phages stimulate innate and adaptive immune systems, making them particularly interesting for vaccines and immunotherapies.

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11. CONFLICT OF INTEREST

No conflict of interest.

12. REFERENCES

1. B. Loh, V.S. Gondil, P. Manohar, F.M. Khan, H. Yang, and S. Leptihn. Encapsulation and delivery of therapeutic phages. *Applied and Environmental Microbiology* 87: e01979-20 (2021).
2. N. Brown, and C. Cox. Bacteriophage use in molecular biology and biotechnology. In: *Bacteriophages: Biology, Technology, Therapy.* D.R. Harper, S.T. Abedon, B.H. Burrowes, & M.L. McConville (Ed.) Springer, Cham. p. 465-506 (2021).
3. L. Aghebati-Maleki, B. Bakhshinejad, B. Baradaran, M. Motallebnezhad, A. Aghebati-Maleki, H. Nickho, M. Yousefi, and J. Majidi. Phage display as a promising approach for vaccine development. *Journal of Biomedical Science* 23: 1-18 (2016).
4. B. Greenwood. The contribution of vaccination to

global health: past, present and future. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369: 20130433 (2014).

5. S. Jorge, and O.A. Dellagostin. The development of veterinary vaccines: a review of traditional methods and modern biotechnology approaches. *Biotechnology Research and Innovation* 1: 6-13 (2017).
6. S. Rahman, M. Din, S. Khan, L. Yar, F. Zia, M. Idrees, and M. Ali. COVID-19 Vaccines: Pakistan's Perspective. *Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences* 58: 11-17 (2021).
7. J. Nicastro, K. Sheldon, and R.A. Slavcev. Bacteriophage lambda display systems: developments and applications. *Applied Microbiology and Biotechnology* 98: 2853-2866 (2014).
8. S. Adhya, C.R. Merril, and B. Biswas. Therapeutic and prophylactic applications of bacteriophage components in modern medicine. *Cold Spring Harbor Perspectives in Medicine* 4: a012518 (2014).
9. J. Morales, J.J. Martínez, K. Manoutcharian, M. Hernández, A. Fleury, G. Gevorkian, G. Acero, A. Blancas, A. Toledo, and J. Cervantes. Inexpensive anti-cysticercosis vaccine: S3Pvac expressed in heat inactivated M13 filamentous phage proves effective against naturally acquired Taenia solium porcine cysticercosis. *Vaccine* 26: 23 2899-2905 (2008).
10. J. Nicastro, K. Sheldon, and R.A. Slavcev Bacteriophage lambda display systems: developments and applications. *Applied Microbiology and Biotechnology* 98: 2853-2866 (2014).
11. T.I. Samoylova, M.D. Norris, A.M. Samoylov, A.M. Cochran, K.G. Wolfe, V.A. Petrenko, and N.R. Cox. Infective and inactivated filamentous phage as carriers for immunogenic peptides. *Journal of Virological Methods* 183: 63-68 (2012).
12. J. Zhu, S. Jain, J. Sha, H. Batra, N. Ananthaswamy, P.B. Kilgore, E.K. Hendrix, Y.M. Hosakote, X. Wu, and J.P. Olano. A bacteriophage-based, highly efficacious, needle-and adjuvant-free, mucosal COVID-19 vaccine. *MBio* 13: e01822-22 (2022).
13. A.G. Mora, J.H. Pérez, H.M. Iqbal, M.R. Palomares and J. Benavides. Bacteriophage-based vaccines: A potent approach for antigen delivery. *Vaccines* 8: 504 (2020).
14. K.L. Hess, and C.M. Jewell. Phage display as a tool for vaccine and immunotherapy development. *Bioengineering and Translational Medicine* 5: e10142 (2020).
15. K. Manoutcharian. Bacteriophages as tools for vaccine and drug development. *Expert Review of Vaccines* 4: 5-7 (2005).
16. J. Zhu, N. Ananthaswamy, S. Jain, H. Batra, W.C. Tang, D.A. Lewry, M.L. Richards, S.A. David, P.B. Kilgore, and J. Sha. A universal bacteriophage T4 nanoparticle platform to design multiplex SARS-CoV-2 vaccine candidates by CRISPR engineering. *Science Advances* 7: eabh1547 (2021).
17. P. Tao, M. Mahalingam, J. Zhu, M. Moayeri, J. Sha, W.S. Lawrence, S.H. Leppla, A.K. Chopra, and V.B. Rao. A bacteriophage T4 nanoparticle-based dual vaccine against anthrax and plague. *MBio* 9: e01926-18 (2018).
18. M. Li, P. Guo, C. Chen, H. Feng, W. Zhang, C. Gu, G. Wen, V.B. Rao, and P. Tao. Bacteriophage T4 Vaccine Platform for Next-Generation Influenza Vaccine Development. *Frontiers in Immunology* 4256 (2021).
19. Q. Bao, X. Li, G. Han, Y. Zhu, C. Mao, and M. Yang. Phage-based vaccines. *Advanced Drug Delivery Reviews* 145: 40-56 (2019).
20. E.N. Woods. Viral Sensation. *CatholicU Magazine, Fall 2021* (2021). https://communications.catholic.edu/magazine/fall-2021/viral-sensation.html
21. C. Fougeroux, L. Goksøyr, M. Idorn, V. Soroka, S.K. Myeni, R. Dagil, C.M. Janitzek, M. Søgaard, K.L. Aves, and E.W. Horsted. Capsid-like particles decorated with the SARS-CoV-2 receptor-binding domain elicit strong virus neutralization activity. *Nature Communications* 12: 324 (2021).
22. M. Palma. Aspects of Phage-Based Vaccines for Protein and Epitope Immunization. Vaccines 11: 436 (2023).
23. A. Algazi, W. Smith, T. Panella, D. Shin, M.L. Fjaellskog, J. Celebi, A. Drumheller, J. Campbell, R. Pierce, and M. Guarino. 349 Early safety and efficacy of a phase 1/2 open-label, multi-center trial of SNS-301 added to pembrolizumab in patients with advanced squamous cell carcinoma of the head and neck (SCCHN). *Journal for ImmunoTherapy of Cancer* 8: 349 (2020).
24. T. Anand, N. Virmani, B.C. Bera, R.K. Vaid, M. Vashisth, P. Bardajatya, A. Kumar, and B.N. Tripathi. Phage display technique as a tool for diagnosis and antibody selection for coronaviruses. *Current Microbiology* 78: 1124-1134 (2021).
25. C.R. de Vries, Q. Chen, S. Demirdjian, G. Kaber, A. Khosravi, D. Liu, J.D. Van Belleghem, and P.L. Bollyky. Phages in vaccine design and immunity; mechanisms and mysteries. *Current Opinion in Biotechnology* 68: 160-165 (2021).

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Conference Proceedings

Proceedings of the ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness

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OVERVIEW

An EPIDEMIC is a disease that affects many people within a community, population, or region. ENDEMIC is something that belongs to a particular people or country. A PANDEMIC is an epidemic that's spread over multiple countries or continents. Epidemics and pandemics are some of the leading threats to global health security. They not only affect people's health and well-being, but they can also have a massive impact on livelihoods and entire societies too. Pandemics can cause sudden, widespread morbidity and mortality as well as social, political, and economic disruption. The world has endured several notable pandemics, including the Black Death, Spanish flu, and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS). Most new pandemics have originated through the "zoonotic" transmission of pathogens from animals to humans, and the next pandemic is likely to be a zoonosis as well. Zoonoses enter human populations from both domesticated animals (such as farmed swine or poultry) and wildlife. Many historically significant zoonoses were introduced through increased human-animal interaction following domestication, and potentially high-risk zoonoses (including avian influenzas) continue to emerge from livestock production systems. Some pathogens (including Ebola) have emerged from wildlife reservoirs and entered human populations through the hunting and consumption of wild species (such as bushmeat), the wild animal trade, and other contacts with wildlife. Spending and costs specifically associated with pandemic preparedness and response efforts are poorly tracked. There is no widely accepted, consistent methodology for estimating the economic impacts of pandemics.

To highlight strategies to combat pandemics, a three-day ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness was jointly organized by the Pakistan Academy of Sciences (PAS), Alliance of International Science Organization (ANSO), and Monbukagakusho-MEXT Alumni Association of Pakistan (MAAP) organized a three-day ANSO-PAS-MAAP Conference on "Epidemic and Pandemic Preparedness" from December 5 - 7, 2022 in the Pakistan Academy of Sciences, Islamabad. In total, 31 lectures were delivered at the ANSO-PAS-MAAP Conference by leading experts in five technical sessions focused on the surveillance and preparedness against global Pandemics and epidemics. Of these, seven (07) lectures were presented by international speakers and twenty-six (26) lectures were presented by Pakistani speakers. The resource persons were leading foreign experts from different countries i.e., China, New Zealand, Italy, USA, and Pakistan. While 32 posters were presented on various themes of pandemics and epidemics in the Poster competition, in which young scientists from across the country participated. Over 400 academicians, scientists, researchers, and postgraduate students from Pakistan and abroad have registered to participate in the deliberations of the conference through physical and/or virtual (online) presence. In addition to the technical sessions, four group works were conducted to formulate recommendations. Recommendations of the conference will be shared with national and international bodies and research institutions dealing with the development of vaccines for the control of potential epidemic and pandemic-related issues worldwide.

1. DAY 1 (5 DECEMBER 2022)

1.1 Inaugural Session

The event was inaugurated by Prof. Dr. Mukhtar Ahmed, Chairman, Higher Education Commission. Dr. Mukhtar appreciated the efforts of PAS, ANSO, and MAAP for jointly organizing this useful event for the health and safety of mankind from epidemics and pandemics. He motivated the young scientists and students for their role in diagnosis, control, therapeutic and vaccine strategies for different viruses. He mentioned that HEC has been preempting to have the concept of smart universities to have higher education online. Prof. Dr. CAO Jinghua, Executive Director, ANSO addressed the gathering online on behalf of Prof. Dr. Chun Li Bai, President of ANSO. He appreciated the efforts of the Pakistan Academy of Sciences and COVID-related management in the Country for spreading awareness about the adverse impacts of epidemics and pandemics.

Earlier, Prof. Dr. Tasawar Hayat, Sec. General, PAS, welcomed the Chief Guest, distinguished scientists, speakers, and participants. He shed light on the role of the Academy in facilitating Pakistani academicians, scientists, and researchers for innovative R&D in various scientific disciplines for implications in the domain of health, energy, and technology. Prof. Dr. Zabta Khan Shinwari (President MAAP; Distinguished National Professor; Prof. Emeritus, QAU) presented the keynote address and thanked PAS, ANSO, and HEC for collaborating in the organization of this event and highlighted the improvement in pandemic control-related strategies, risk management and policies against future possible outbreaks. He had a draft strategy to combat future pandemics.

In his inaugural address, Prof. Dr. Khalid M. Khan (President, PAS) congratulated the collaborating institutions and organizing team for their efforts in organizing the event. He talked about the spread and impacts of some devastating pandemics in past (Hong Kong flu, Swine flu, and COVID-19 outbreaks) on mankind. Dr. Muhammad Ali (PI, ANSO Project; Assistant Professor QAU) talked about zoonotic viruses that are responsible for pandemics and endemics, which affect almost every field of life throughout the world. He specifically targeted viruses associated with Bats and that Bats are the main reservoir of viruses. He presented his valuable project and the inevitable struggle for identification and studying these bats and their microbiome all over Pakistan. The inaugural ceremony was concluded with a vote of thanks by the organizers. A group photo was taken after the inaugural session with the Chief Guest (Figure 1).

1.2 Technical Session I: Strengthening Diagnostic and Pandemic Preparedness

After the inaugural session, Technical Session I on Strengthening Diagnostic and Pandemic Preparedness was chaired by Maj. Gen. Dr. Aamer Ikram (Executive Director, NIH, Islamabad) and co-chaired by Dr. Quaid Saeed (CEO, IHRA). The session started with the presentation of a leading foreign expert from China, Prof. Dr. Di Liu from the Wuhan institute of virology, Chinese Academy of Sciences on the topic 'Oxford-Nanopore Technology Rapidly Determination of SARS CoV-2 Genome and TRACE-seq Meta-Transcriptome Detection Method' and Dr. Muhammad Qasim Director Research at Te Rangawairua o Paratene Ngata Research Centre, New Zealand shared his views on Importance of diagnostics in epidemics preparedness and response: Lessons from COVID-19 pandemic. They explained that emerging and re-emerging infectious diseases continuously threaten humanity and timely diagnostics and detection are foundational and starting points for a successful outbreak containment strategy. The development of diagnostic tests, validation, and implementation at a community level is still lacking for those pathogens which are listed by WHO as most likely to cause a future epidemic.

The national experts of this session were Dr. Saeed Khan (Professor of Pathology at DOW Institute of Health Sciences, Karachi), Dr. Hasnain Javed (Lab Head and Focal person of Provincial Public Health Reference Lab Punjab), Dr. Ibrar Ahmad (Head of Alpha Genomics Private Limited, Islamabad, Pakistan), and Dr. Massab Umair (National Institute of Health, Islamabad, Pakistan), shared their experiences and views about strengthening diagnostics and pandemic preparedness. They emphasized on the

Fig. 1. Participants of the Inaugural Session of a three-day ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness with the Chief Guest Prof. Dr. Mukhtar Ahmed (Chairman, Higher Education Commission), Prof. Dr. Khalid M. Khan (President, PAS), Prof. Dr. Tasawar Hayat (Secretary General PAS), Prof. Dr. Zabta Khan Shinwari (Chief Organizer, Fellow PAS & President MAAP), Dr. Quaid Saeed (CEO, IHRA) and Dr. Muhammad Ali (PI ANSO Project) organized by the Pakistan Academy of Sciences (PAS), Alliance of International Science Organization (ANSO), and Monbukagakusho-MEXT Alumni Association of Pakistan (MAAP).

availability of timely and accurate diagnostics to combat the spread of pandemics or outbreaks. It is important to equip with the necessary technological advancements to detect and effectively manage the spread of such diseases in the future. It is now thought to be essential to overcome future threats to evaluate the public health systems with creative, coordinated, and collaborative actions across humans, animals, and the environment (One Health).

However, the preparations made by public health laboratories including well-resourced, diagnostic labs, skilled staff, and mobile labs made it possible to overcome the problems. Without these resources, the country would quickly collapse due to immense healthcare and economic burden and there would have been no way to contain outbreaks from then onwards.

1.3 Technical Session II: Molecular Pathology and Efforts for Controlling Pandemics

Technical Session II was chaired by Dr. Osman Yusuf (Chief Consultant, Allergy and Asthma Institute, Islamabad) and co-chaired by Dr. Javed Muhammad (Assistant Prof. University of Haripur). The leading topic of this session was "Molecular Pathology and efforts for controlling Pandemics". The session started with the presentation of Prof. Dr. Elisabetta Affabris (University Roma Tre, Rome, Italy), a Professor of Virology. She presented her extensive research study about the human immune system specifically how interferons react and produce viral infection. She explained the role of Type I interferons, the first line of defense in microbial infections, and their critical role in blocking early virus replication, spread, and tropism as well as promoting the adaptive immune response. She elaborated on the role of innate and adaptive immunity during acute viral infection and the production of different interferons. She proposed that the immune evasion of the IFNs system could be a therapeutic tool to reduce the virulence of the disease.

Dr. Ali Talha Khalil (Assistant Professor and Consultant Molecular Biologist at (LRH-MTI), Peshawar) expressed his thoughts about learning from pandemics, it is now generally accepted that the local or global response to public health emergencies is complex and requires a sector-wide coordinated response. However, in preventing pandemics a variety of factors are required. Short-term intervention can help mitigate the immediate threats to life and the economy. Zoonosis and the

spread of zoonotic pathogens to humans are the two main causes of pandemics, requiring global efforts to maintain the stability and integrity of the environment. Future pandemic risk can be controlled by low infectious agent transmission and exposure. It is now thought to be essential to overcome future threats to evaluate the public health system with creative, coordinated, and collaborative actions across human, animal, and environmental (One-Health).

In this session, the next speaker was Dr. Yasir Waheed (Director, ORIC at Shaheed Benazir Bhutto Hospital Islamabad, Pakistan). He elucidated the importance of Global and Local Efforts to control Hepatitis Epidemics. He enlightened the annual death related to hepatitis globally and talked about the world's first hepatitis day celebrated in 2008. He highlighted the strategies like; Find the Missing Millions of the World Health Organization (WHO) for the detection and elimination of Hepatitis by 2030, using five different impact targets including, HBV vaccination, HBV mother-to-child transmission, blood safety, harm reduction, and HBV/HCV diagnosis and treatment. He further talked about progress in 2022, that has been made to achieve targets for blood donation screening, treatment, reduction in drug pricing, and decreasing incidence of HBV and HCV. He also discussed that Pakistan has the 2nd highest burden of HCV in the world. A hepatitis control program was initiated which needs good investment to control the hepatitis epidemic.

Another admirable speaker was Dr. Muhammad Rafiq a professor of Mathematics at the University of Central Punjab, Lahore. He delivered his research study on Mathematical Modeling for "Transmission Dynamics of Infectious Diseases: A COVID-19 Perspective". In his talk, he discussed the importance of a Mathematical model for the analysis and control of infectious diseases in a population. He further elaborated a mathematical model that included some important theories that are built and tested, and some quantitative speculations were made, that lead to a better strategy to overcome the transmission of infectious diseases.

Dr. Saima Saleem, Associate Professor (Tenured) at Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi. She presented a topic about Leadership and Communication for Epidemic and Pandemic Preparedness: An Awareness Program for Researchers on SARS-CoV-2. In her talk she discussed a training program designed for the education of leaders, focusing on behaviors, and nurturing their workforce to prepare, protect, and promote the frontline research scientists and healthcare workers in the biological research laboratories during the pandemic of SARS-CoV-2. A workshop was conducted at The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Pakistan in collaboration with Safer Behaviors (SB) in Atlanta Georgia, USA. She said that the training program aimed to evaluate the behavioral-based biosafety culture and provide biosafety training according to international standards in the biosafety parameters were engineering control, personal protective equipment (PPE), standard operating procedures (SOP), and administrative control. Moreover, she mentioned the biosecurity aspects which were monitored as physical, personal, material, and information security protocols. In the last, she concluded that leadership is not an authoritative title but a philosophy that demonstrates the highest and lowest levels within an organization.

Mr. Zakir Hussain, Agriculture Officer, IPM, Department of Agriculture Gilgit Baltistan. He elucidated Armyworm (*Mythimna unipuncta*) outbreak scenario in horticultural crops under the impact of climate change in Gilgit-Baltistan, Pakistan. He mentioned the major crops that grow in the region and mentioned its constrained production due to the negative impacts of climate change and pest population. He further highlighted that due to climate changes in GB over from last 4-5 years, in the early days of April 2022, the outbreak of *Mythimna unipuncta* has been observed in all agricultural fields of Gilgit which gradually flared up throughout GB till the end of May 2022. He suggested that farmer awareness of climate change and climatic factors influencing pest prevalence within the GB is highly needed to be prioritized. He also proposed that risk assessment maps are needed to be prepared and improved by collaborating with the different stakeholders and farmers to manage insect pests under changing climatic situations.

At the end of technical session 2, the audience were invited for discussion regarding the topics discussed in today's session, where they highlighted different issues that were effectively addressed by the experts present at the conference.

2. DAY 2 (6 DECEMBER 2022)

The second day of the conference started by welcoming the participants and speakers of the day by Prof. Dr. Zabta Khan Shinwari, he also gave an overview of the first day of the conference to the participants (physical and online).

2.1 Technical Session III: One Health, Zoonotic, and Animal Diseases

Technical Session III on "One Health, Zoonotic and Animal Diseases" was Chaired by Prof. Dr. Muhammad Mukhtar (Vice Chancellor, National Skills University Islamabad), and co-chaired by Dr. Ali Talha Khalil (Assistant Professor, LRH Peshawar). Prof. Dr. David Hayman (Professor of infectious disease, Massey University, New Zealand) was the first speaker of the session. His topic of the presentation was "One health approach and pandemic disease prevention". He discussed the objectives of "one health summary" presented by WHO along with different health, agriculture, and food organizations to identify the source of the SARS-CoV-2 (Severe Acute Respiratory Syndrome-Coronavirus 2) including the route of introduction to the human population. His study aimed to prevent both reinfections with the virus in humans, and identify potential zoonotic reservoirs to reduce future risks of zoonotic disease. He discussed the unprecedented and unsustainable level of anthropogenic environmental changes including agriculture intensification, climate change, and wildlife trade likely increasing pandemic risk. One health high-level expert panel (OHHLEP) guidance on pandemic risk through policy-relevant, scientific assessment of health at the human and animal ecosystem, and research gaps. Long-term strategic approaches to reduce zoonotic risk. He also discussed floods in Pakistan that covered 1/3 of the country. His concluding remarks were a rational approach for global pandemic policies that reduce the drivers of pandemics along with current efforts by intergovernmental science-policy platforms on biodiversity and ecosystem services to implement health approaches.

Dr. Zengyun Hu, Professor at Xinjiang Institute of Ecology and Geography (XIEG), Chinese Academy of Sciences (CAS) topic of discussion was "Future features of COVID-19 with the SARS-CoV-2 omicron in Pakistan". He discussed the rapid transmission of the SARS-CoV-2 variant to a record-breaking case incident rate around the world. He discussed the importance of future features of the omicron variant in Pakistan. He explained the SECIR disease dynamical model and autoregressive integrated moving average (ARIMA) to investigate the future variation of COVID-19. He concluded his presentation by saying these two models are important to reduce the spread of COVID-19 in Pakistan.

Dr. Muhammad Usman, Assistant Professor of Microbiology, GCU Faisalabad presented his work entitled "Coexistence of blaNDM and mcr-1 producing *Escherichia coli* isolated from human, poultry and environment water from Pakistan- A one health concern". He explained the emergence and spread of New Delhi metallo-β-lactamase (NDM) and mcr-1-producing *E. coli* as a serious threat around the globe, particularly in developing countries like Pakistan. His study aimed to determine the prevalence of both blaNDM and mcr-1-producing *E. coli* in poultry cloacal swabs, environmental water, and human samples. The highest number of positive cases were found in poultry samples, in which 22 *E. coli* were positive for mcr-1 and 17 E. coli were NDM producers. Dissemination of blaNDM mcr-1-producing *E. coli* from clinical, poultry, and environmental water is a matter of great concern for both livestock and public health.

Dr. Azam Jan Afridi (Assistant Professor in Biology at GDC Darra Adam Khel, TSD Kohat) topic of his presentation was "Lumpy skin disease in Tehsil Bara District Khyber; An emerging threat to livestock in Pakistan". He explained transmission and infection caused by Lumpy skin disease. The objective of his study was to check the ongoing outbreak of Lumpy skin disease and its effects in Tehsil Bara. The data was collected through a questionnaire method from cattle and dairy farmers from five selected areas in Tehsil Bara. The highest cases were found in the Shlobar area where the

infection rate was 96.28 % and the mortality rate was 12.66 %. The lowest cases were reported in Nala Sour Dandh where the infection rate was 53.30 % and the mortality rate was 4.65 %. He recommended the multi-task role of government, and private sector, as well as the isolation of infected animals and prevention of illegal transportation across the border.

Dr. Yasir Mehmood Yousafzai (Associate Professor and Director, Public Health Reference Laboratory, Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar) enlightened "The vital role of Khyber Medical University in pandemic preparedness and response during Covid-19". He explained how this medical university volunteered to play a front-line role in the prevention, detection, and control of the pandemic. This engagement aimed to provide significant assistance to the department of health, Khyber Pakhtunkhwa (KP) while continuing to teach safely. During the COVID Pandemic, PCR testing capacity was rapidly enhanced increasing up to 6000 tests/day with a total of 1.6 million PCR tests so far. Twenty-eight (28) PCR labs were established within the first six months of the pandemic response. Telemedicine services provided valuable support to the community when hospital OPDs were shut down. A drive-through vaccination center was established.

2.2 Poster Session

The poster competition was held, in which young scientists from across the country participated. Out of Eighty-two (82) posters received, Thirty-two posters were short-listed. A three-member evaluation committee was formulated comprising of Prof. Dr. Masoom Yasinzai (Former Rector, IIU, Islamabad), Maj Gen. Dr. Aamer Ikram, S.I.(M) (Executive Director, NIH, Islamabad), and Prof. Dr. Mushtaq Ahmad, Fellow PAS and Chairman Department of Plant Sciences, Quaid-i-Azam University, Islamabad). The following six (06) posters were selected for prizes as indicated by their names shown in Table 1. Glimpses from the poster session are shown in Figure 2.

2.3 Technical Session IV: Vaccines and Public Health

Prof. Dr. Saeed Khan (Professor, Dow University of Health Sciences; President, PBSA) along with Dr. Tariq Khan (Lecturer, University of Malakand) moderated the Technical Session on Vaccines and Public Health. The session started with the intriguing title, Monkeypox: poxviruses are back? Prof. Maria R. Capobianchi (Saint Camillus International University of Health Sciences, Rome, Italy) presented major findings on the respective topic. The disease killed between 15 and 30 % of individuals in the 20th century in Africa and has started to spread again even in individuals not traveling from Africa. The growing global pandemic was deemed a Public Health Emergency of International Concern on July 23, 2022, by WHO Director-General Tedros A. Ghebreyesus. By the middle of October, 111 different nations had recorded more than 60,000 cases. Monkeypox belongs to the Orthopox Virus, which is an enveloped, Double-stranded DNA virus, prevalent in Equatorial Africa, where hundreds of cases of human infections are reported annually. The mode of transmission for this virus is usually from wild animals, like rodents to humans. However, in the present outbreak, mode of transmission from human to human is involved too, including homosexuals, and are considered the main risk factor of monkeypox outbreak. Further, she emphasized on the fact that understanding the viral mode of transmission and its epidemiological patterns can help in preventing further disease spread and a better approach towards epidemic preparedness.

Afterward, another interesting topic, Roadmap for vaccine development and its indigenization in Pakistan was presented by Dr, Liaqat Ali Khan (Assistant Professor, Department of Biological Sciences, National University of Medical Sciences, Rawalpindi). He discussed the importance of vaccines that vaccines have significantly lessened the burden of many infectious diseases, supporting human development and well-being on a global scale. He highlighted the fact that vaccines are expected to save the lives of 25 million individuals in the upcoming decade and remain the foundation of public health initiatives. It is estimated that six of every ten infectious diseases are zoonotic, this way, vaccines are not limited to humans but to animals as well. He further discussed the developmental stages and myths of vaccines and stressed on the fact that the lack of new and improved vaccinations to fight against infectious diseases, particularly in under-resourced countries remains a problem. The

Table 1. Result of the poster competition

S. No.	TITLE	PRESENTER AND AFFILIATION	Evaluation Criteria	Position/ Ranking in Competition
1.	Macrophage targeting with the novel carbopol based miltefosine-loaded transfersomal gel for the treatment of cutaneous leishmaniasis: in vitro and in vivo analyses	**Sibgha Batool** Ph.D. Scholar Nanomedicine Research Group, Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan		First Position
	In vivo immunostimulatory effect of Syringic Acid on cyclophosphamide induced immunosuppression	**Khoula Sharif Mughal** Ph.D. Scholar Department of Pharmacy COMSATS University Islamabad, Abbottabad Campus	• Layout & Graphics Quality	
2.	Origin, Pathogenesis, Diagnosis and Treatment Options for SARS-CoV-2: A Review	**Humna Sajjad** M.Phil. Scholar Department of Biotechnology, Quaid-i-Azam University, Islamabad	• Contents / Text Quality • Presentation Skills	
	Serotyping and Characterization of Non-Structural Genes of Dengue Virus Circulating in Pakistan.	**Muhammad Bilal Khan** Laboratory Technologist Department of Biological Sciences, National University of Medical Sciences (NUMS), Rawalpindi	• Quality of Results & Conclusions	2nd Position
3.	The potential of plant biotechnology in preparing us for pandemics; a case for plant in vitro cultures and plant-based products	**Reema Iqbal** M.Phil. Scholar Agriculture university Peshawar		3rd Position
	Control of Charcoal rot Disease in Mung bean by using Plant Growth Promoting Rhizobacteria	**Amjid Khan** Ph.D. Scholar Department of Plant Sciences, Quaid-i-Azam University, Islamabad		

progression of the necessary scientific and technical skill set, adequate funding, and governance of a framework that supports research and innovation are direly needed. In the end, he highlighted a point that Pakistan is very far from the world of vaccine development and production. Considering the fact that Pakistan's population is continuously expanding and the dangers of newly developing infections and bioterrorism are a major health concern, a constant effort is needed from all stakeholders to excel in this country's capabilities in the respective field.

Later, Dawood Ghafoor (Wuhan Institute of Virology, CAS) presented a novel methodology to design a multi-epitopes subunit vaccine against the Hantaan virus. The Bunyaviridae family of viruses,

Fig. 2. Glimpses from the poster session. A three-member committee comprising of Prof. Dr. Masoom Yasinzai (Former Rector, IIU, Islamabad), Maj Gen. Dr. Aamer Ikram, S.I.(M) (Executive Director, NIH, Islamabad), and Prof. Dr. Mushtaq Ahmad, Fellow PAS and Chairman, Plant Science Department, QAU evaluated the posters.

which includes the Hantaan virus, is an emerging group of viruses that cause hemorrhagic fevers. The virus is widespread around the world, and as of right now, no effective antiviral medication or vaccine has been created to guard against viral infections. Dawood Ghafoor discussed a potential approach in which B and T-cell epitopes for the Hantaan virus's enveloped protein and poly-protein were predicted using a variety of computational tools. A prospective multi-epitope subunit vaccine was created by connecting B and T-cell epitopes with their respective linkers after the individual epitopes were modeled for docking with their respective HLAs. Furthermore, 3-D modeling and docking analysis with TLR-4 was done and a number of physiochemical characters were assessed. Later, the docked complexes of vaccine-TLR-4 were evaluated for residual interactions, and immune simulations were performed by the C-IMMSIM server. In the end, he predicted the natural immune response by immune simulation analysis and evaluated the expression analysis through in-silico cloning using *E. coli*. The overall values suggested that the vaccine is efficient and causes high immunogenicity. In the end, he concluded that this was a computational study and further *in-vitro* studies are required to confirm the effectiveness of this vaccine.

Subsequently, Dr. Niamatullah Kakar (Assistant Professor, Department of Natural and Basic Sciences, University of Turbat (Kech), Pakistan) presented on a topic, Epidemiological, clinical, and laboratory findings of COVID-19 positive patients in a hospital in Quetta, Pakistan. He highlighted the demographic, clinical, and laboratory data of COVID-19-positive patients. He conducted his study from September 2020 to March 2021 at Sheikh Khalifa Bin Zaid Hospital and Fatima Jinnah Institute of Chest Disease. 199 patients out of which 127 were males and 72 were females tested positive for COVID-19 and were included in the study. He further discussed the disease outcomes with respect to age, gender, ethnic background, and employment status. He concluded that a significant number of cases were

reported from the age group 51-75 Years and the mortality rate was higher (>22 %) in older age and was recognized in males than females. Similarly, based on ethnic background and region most of the patients were from KPK and Baluchistan. Furthermore, with respect to employment status, non-health workers were more prone to COVID-19 infection. Among the hospitalized patients diagnosed with comorbidities, 45 (22.61 %) were diabetic, 24 (2.01 %) with respiratory disease, and 4 (2.01 %) with chronic kidney conditions. Patients who tested positive had a significantly higher white blood cell, neutrophil, and lower red blood cell count. They also had higher C-reactive protein, D-dimer, urea, creatinine, glucose fasting, and lactate dehydrogenase levels in serum. In a nutshell, he presented different indicators that play an important role in the intensity of the disease. These findings will help to fight against the disease with better diagnosis and treatment.

After that, Engr. Adnan Bashir (Chief Executive Officer, Health Information Systems Program) gave his wonderful presentation on a very peculiar topic, Dynamics of Health Information Systems (HIS) in Pakistan; How to Best Utilize What We Have. He highlighted the point that modern health information systems provide an integrated framework for managing many responsibilities in the healthcare system. The COVID-19 pandemic presented how fragile our health system was. Later on, he pinpointed the importance of public health and its role in evaluating program impact, monitoring progress, and determining populations to target for intervention and barriers to care. He then discussed major information systems and their role in outbreak detection and response with preparedness. In the end, he discussed the fragmented structure of the Health Information System (HIS) in Pakistan and the dire need for a centralized health information system to make sure that health-related data are produced and integrated at the provincial and national levels. This system can be achieved in Pakistan to combat the upcoming pandemics and outbreaks.

In the end, Dr. Tahir Usman (Assistant Professor, Abdul Wali Khan University Mardan, KP) presented the topic, Knowledge, Attitude, and Practice Regarding COVID-19 Pandemic in Pakistan. He studied the psychosocial impact of COVID-19 on the general population. He used knowledge, attitude, and perceptions (KAPs) as an indicator to analyze the psychology of a certain person. A cross-sectional study was conducted from September 1, 2021, to December 31, 2021, in Pakistan. The questionnaire included socio-demographic information, awareness, attitudes toward the disease, safety precautions, and opinions about it. According to the analysis, there was a strong association between the knowledge score and gender, marital status, and education. 94.5 % (n=700) of the participants believed that elder people were more prone to COVID-19 infection. Moreover, the majority of the participants showed their concern for their family members regarding COVID-19 and it can be controlled if the standard SOPs are followed. In the end, he discussed that the general population has good awareness and perceptions about COVID-19 and that effective health education programs can further improve the gap between KAP and COVID-19.

The session was concluded by moderators of the session, Dr. Saeed khan and Dr. Tariq Khan. In addition, souvenirs were given to the speakers and moderators and the session ended with a big round of applause.

3. DAY 3 (7 DECEMBER 2022)

The Third day of the conference started by welcoming the participants and speaker of the day by Dr. Muhammad Ali (PI, ANSO Project), he also gave an overview of the second day of the conference to the participants (physical and online). After the welcome address, he invited the Chairs of the session and technical session three which was based on Antimicrobial and Nano Biology was initiated.

3.1 Technical Session V: Antimicrobials and Nanobiology

The final technical session on Antimicrobials and Nanobiology was chaired by Prof. Dr. M. Aslam Baig (Distinguished National Professor, National Center for Physics; Professor Emeritus, QAU) and Prof. Dr. Mushtaq Ahmad (Chairman, Department of Plant Sciences, QAU) along with Dr. Muhammad Ovais as moderator. They welcomed the speakers and participants.

Dr. Afreenish Amir (Medical Microbiologist, Technical Officer AMR, Project Director National Fungal Disease Surveillance, National Institute of Health, Islamabad, Pakistan) was the first speaker of the day. She talked about "antifungal agents; mechanism of actions, resistance, and newer agents". Fungal infection is an emerging threat to public health that is being neglected. Antifungal resistance is increasing because of little antifungal production and also because everyone is using antibiotics and antifungals without prescriptions. She discussed some new antifungal agents that are in clinical trials with enhanced activity and fewer side effects. She talked about stewardship that can help to save the existing antifungal agents, besides it, there was a strong emphasis on one health approach for future therapies and interventions.

The next Speaker, Dr. Fazal Mehmood Khan (Post-doctoral Fellow, Institute for Advanced Study, Shenzhen University China) discussed "LysAB54, an Acinetobacter baumannii Bacteriophage Endolysin with potent Antibacterial Activity Against a broad range of Gram-Negative Bacteria". A. baumannii and other gram-negative bacteria have emerged and also antimicrobial resistance is increasing. It takes years to develop a new antibiotic, synthetic phages can be a better option to cope with antibiotic resistance. They have a wider host spectrum. He presented his research work on endolysin therapeutic agents. He also provided information on the limitation of the use of endolysin as it lost activity in the serum, limit to use on the skin surface but can be used as a disinfectant in hospital wards. He invited collaborations from different institutes around the world.

Dr. Tariq khan (Lecturer, Department of Biotechnology, University of Malakand) presented a topic on "Nano-biotechnology against a resistant pathogen; a way forward in preventing pandemic". He said that antimicrobial resistance may be the next pandemic. Antimicrobial resistance may be due to treatment failure, approval of new antibiotics, and lack of awareness. He stated nano-biotechnology as a novel strategy to overcome antimicrobial resistance. He concluded his talk by saying that antibiotic–nanomaterial conjugate can be a good way forward. Low molecular weight nanomaterial based on antibiotics could be very effective against multi-drug resistance.

Dr. Ikram Ullah (Assistant Professor, Department of Biotechnology and Genetic Engineering, University of Hazara, Pakistan) topic of the presentation was "Biogenic silver nanoparticles (AgNPs) stimulates J774 macrophage cells to induce the production of nitric oxide (NO) and reactive oxygen species (ROS) that have leishmanicidal effects". *Leishmania* is the 3rd most important vector-borne disease that may lead to a pandemic. Currently available treatments have side effects so there is a need for alternative treatments. He presented nanobiotechnology as a non-toxic alternative therapy and of low cost. Further, *in-vivo* studies are required to use nanoparticles as anti-leishmanial agents.

Dr. Fakhar-ud-Din (Assistant Professor, Department of Pharmacy, Faculty of Biological Sciences Quaid-i-Azam University, Islamabad) talked about "Nanomedicines for tumor targeting" as cancer is increasing rapidly and current treatments are unable to cope with a problem that may be due drug delivery issues. Site Specific drug delivery is necessary because conventional drug delivery systems have many problems associated with them. He presented a smart drug delivery system that may be able to cope with all these problems.

Dr. Zul Kamal (Department of Pharmacy, Shaheed Benazir Bhutto University, Sheringal) talked about "surface functionalization/coating of nanoparticles with red blood cell membrane for own demand antibiotic delivery". He gave an idea about a cell-based drug delivery system. He worked on erythrocytes as the cell to load his drugs that can be used against microbial resistance. He talked about the problems associated with the nanoparticles as these can be easily picked by the immune system so there is a need to prepare drug delivery on demand to reduce toxicity and make it more receptor specific.

3.2 Concluding Session

H. E. Mr. Agha Hassan Baloch, Federal Minister for Science and Technology chaired the Concluding Ceremony of the International 3-day ANSO-PAS-MAAP Conference on "Epidemic and Pandemic preparedness" organized by the Pakistan Academy of Sciences (PAS), in collaboration with the Alliance of International Science Organization

(ANSO), and Monbukagakusho-MEXT Alumni Association of Pakistan (MAAP) held on December 07, 2022, in the Pakistan Academy of Sciences. H. E. Mr. Agha Hassan Baloch congratulated the Pakistan Academy of Sciences for organizing the International conference on such an important topic, he emphasized that Pakistan must have an integrated health system to cope with future epidemics. As exemplified by the ongoing coronavirus disease (COVID-19) pandemic major infectious diseases and epidemics have devastating impacts on human lives, destroying long-term social and economic development. Global health crises threaten to overwhelm already overstretched health systems, disrupt global supply chains, and cause unequal devastation of the livelihoods of people, including women and children, and the economies of the poorest and most vulnerable countries.

Federal Minister for Science and Technology stated that Covid-19 is a human tragedy. But it has also created a generational opportunity. An opportunity to build back a more equal and sustainable world. The response to the pandemic and to the widespread discontent that preceded it must be based on a new social contract and a new global deal that create equal opportunities for all and respect the rights and freedoms of all. This is an urgent need to have a robust health system, that reaches those who are vulnerable or in vulnerable situations. There is a great need of raising awareness, the exchange of information, scientific knowledge and best practices, quality education, and advocacy programs on epidemics at the local, national, regional, and global levels as effective's measures to prevent and respond to epidemics. He further added I am confident that the participants will utilize the knowledge and skills gained through this event in their research so that the objectives of the conference can be fully realized. His Excellency distributed shields among the Winners of the poster competition held during the conference at the Pakistan Academy of Sciences.

On behalf of the Pakistan Academy of Sciences, and the Organizing Committee of the ANSO- PAS- MAAP Conference on "Epidemic and Pandemic Preparedness", Prof. Dr. Tasawar Hayat, Secretary General PAS thanked the Honourable Chief Guest of today's session, His Excellency, Agha Hasan Baloch Sahib (Federal Minister for Science & Technology) for sparing time from his very busy schedules to grace the occasion with his participation to conclude the event. His physical presence in this session indeed shows his keen interest in the scientific activities of this country, and it is also a source of motivation, inspiration, and encouragement for us all and especially the young scientists and researchers who are physically and virtually attending this conference.

Further, extended deepest gratitude to ANSO for financially supporting the event as well as MAAP for joining hands with the Pakistan Academy of Sciences in the organization of the Conference in Islamabad on topics of immense importance for scientists, researchers, medical specialists, and technologists who are engaged in developing innovative vaccines, medicines, and technologies to combat COVID-19 and alike pandemics and epidemics across the globe for the health and safety of the mankind. Very special thanks are also due to Prof. Dr. Khalid Khan, President PAS, and Prof. Dr. Zabta Khan Shinwari, Chief organizer of the Conference, Dr. Muhammad Ali (PI, ANSO Collaborative Project), and organizers for their efforts, valuable inputs, and dedication that we have been able to host this conference. A group photo was taken after the concluding session with the Chief Guest H. E. Mr. Agha Hassan Baloch, Federal Minister for Science and Technology (Figure 3).

The conference was well attended; in addition to the speakers, the participants for deliberations on topics of vital importance for scientists, researchers, medical specialists, and technologists who are engaged in developing novel vaccines, medicines, and technologies to fight pandemics and similar diseases around the world for the wellbeing and safety of humanity

Fig. 3. Participants of the Concluding Session of a three-day ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness with the Chief Guest H. E. Mr. Agha Hassan Baloch, Federal Minister for Science and Technology, Prof. Dr. Tasawar Hayat (Secretary General PAS), Prof. Dr. M. Aslam Baig (Distinguished National Professor, National Center for Physics; Professor Emeritus, QAU) and Dr. Muhammad Ali (PI ANSO Project) organized by the Pakistan Academy of Sciences (PAS), Alliance of International Science Organization (ANSO), and Monbukagakusho-MEXT Alumni Association of Pakistan (MAAP).

4. RECOMMENDATIONS

In the culmination of the ANSO-PAS-MAAP Conference, participants worked together to compile a list of recommendations that would further help in developing a better and more effective pandemic response. These recommendations are enlisted below;

- Should improve the information system to establish a global network of groups worldwide that identify any outbreak and develop new strategies and stop the spread of infection and identify and report novel pathogens. To make a systematic database that collects and shares authentic viral genetic information and can identify any new viral variant and took timely measures against infections.
- The public health communication system should be improved. It's been very challenging to stop misinformation about infectious diseases, partly because of a widespread lack of trust between communities, governments, and healthcare systems. Controlling the misinformation spread by religious scholars and proposing the strategy to bring them on the same platform without discrimination of science with religion. Standardized nomenclature system for viruses and variants to avoid infodemics.
- Adopt a serious plan for domestic and international pandemic preparedness, such as one that suggests building new infrastructure and making global and national investments to promote pandemic preparedness. Initiate a review of the responsibilities for pandemic preparedness and response among public health authorities at the tribal, local, federal, and state levels. Adopt national guidelines and standards for pandemic preparedness to strengthen health equity in healthcare systems.
- Before the next pandemic strikes, take quick action to identify the populations that are most susceptible to epidemic disease, attempt to reduce these gaps and strengthen the resilience of these communities. Make international efforts to develop national, global, and modern epidemic surveillance and forecasting capacity. Pandemic planning and preparedness activities are not only based on worst-case scenarios. They should be flexible and adaptable.
- Healthcare personnel should be prioritized, recruited, and trained. Because a healthy population will typically be more resistant to infection and should take public health

seriously. In healthcare settings, authorities should be empowered, by using examples of best practices from hospital and health center administrators.

- To develop strategies for implementing pandemic interventions, there should be effective communication among healthcare professionals, the public, and other stakeholders. Healthcare professionals should play a significant role in promoting the use of vaccines as a preventive step among the general public. Avoidance of false paradigms in health care regulation system.
- Increase the number of Mobile Healthcare Units with their effective use. Biotechnologists and Microbiologists should be authorized to supervise healthcare labs independently.
- Telemedicine investment to provide access to patient populations in underdeveloped areas. Hospital bed capacity and personnel equipment tracking systems that are centrally managed to promote resource sharing within and between healthcare systems at the local and regional levels.
- It is recommended that more training and educational activities (e.g., conferences, workshops, seminars, symposiums, etc.) should be available for healthcare workers. Authorities and medical professional associations address vaccination uptake, including a discussion of the leadership responsibilities of healthcare professionals and their moral/professional duty to accept vaccination and support public health preventative initiatives. Policymakers should be part of conferences. Awareness of the rural population should be sponsored.
- It is recommended to reduce population vulnerabilities to the Pandemic and Endemic by creating an essential medications list to set priorities for policy, investments, and regulatory reviews. Enlightening the transparency of global supply chains, notably by providing better information on the source, cost, and drug quality. The intercity movement of individuals must be regulated through proper QR codes.
- Most epidemics and pandemics are considered to be originated from wildlife/animals to human populations. We should protect nature to prevent future outbreaks by reducing population displacement. Start one health campaign worldwide to reduce deforestation, intensive land forming, wildlife trading and hunting, and better surveillance of zoonotic pathogens that can help to prevent future pandemics. Can produce genetically modified crops that have more yield of cellulose that wouldn't be needing of deforestation.
- Take new initiatives for animal care to prevent or minimize contact between animals and humans to prevent zoonotic infections. Start identification of species that are most likely to act as reservoirs, vectors, or intermediate hosts for transmission of infectious diseases and restrict their contact with humans.
- We should adopt a One-Health approach to protect the health of all living beings by bringing experts across fields together to solve problems threatening humans, animals, and the environment. Promote one-health research in Pakistan.
- Pandemics and widespread epidemics have the power to destroy economies, upend nations, and claim millions of lives. The WHO's Health Emergencies Program (WHE) should collaborate with Member States to assist nations in being ready for widespread outbreaks and pandemics. A new WHO Global Health Board is required to support WHO decision-making, especially on controversial matters.
- There should be strong leadership and government involvement in PPA (Pandemic plans and preparedness activities). To carry out PPA, effective political leadership that can coordinate with the ministries of health and other pertinent departments is required.
- Strengthening and boosting the distribution and manufacture of cost-effective vaccines. Least and middle-income countries have no capacity to manufacture their vaccines although need to ensure that there is sufficient production capacity in developing countries that can be quickly scaled up.
- Promoting local manufacture of protective equipment. Encouragement of local production of diagnostic kits and other pharmaceuticals. Ensure fair allocation and distribution of vaccines, diagnostic kits, and other pharmaceuticals during epidemics. All diagnostic kits should be registered by the Drug Regulatory Authority of Pakistan (DRAP).
- Establishment of collaboration between biopharma companies, governments, and non-

governmental organizations that can share information and timely coordinate to maximize pandemic preparedness.

- Advancement of policies regarding the supply and procurement of vaccines should be constantly visited and improved for rapid supply and availability of vaccines in the region of the outbreak. The concept of herd immunity should be introduced.
- Global Health Fund should be established as a first line of action for any outbreak which is primarily focused on investments for pandemic preparedness and improvement of the public healthcare system. Collective funding should be initiated that can be directly utilized to help countries under serious threat. There should be proper funding for Research and Development (R&D) of therapeutics drugs and vaccines. Also, such funds can be used for the timely distribution of vaccines and therapeutics to the infected population. Proper strategies should be introduced for the effective implementation of pandemic measures that include the use of vaccines, transport during the pandemic, and essential protection measures.
- People's mental states should be considered as well so psychological counseling to avoid the panic of the pandemic. Should Avoid social stigma and promote a positive outlook during the pandemic.
- We should have to build trust in healthcare workers, National health agencies, and Health institutions about giving vaccines to people so that they stay healthy. we can help prevent future pandemics by building trust and making sure the guidance from these sources is evidence-based and respected. Raise awareness by mobilizing local religious scholars and the local community for increasing vaccine acceptance.
- Should improve the industry and academia linkages (Universities and Health Industries, hospitals) in which HEC can play a major role. Biomedical teaching labs in universities can be converted to diagnostic labs in emergencies. It's high time to invest in biomedical labs in Academia.

5. MEDIA COVERAGE

ANSO-PAS-MAAP Conference on Epidemic and Pandemic Preparedness got huge media coverage. Some of the links are as follows;

1. https://www.technologytimes.pk/2022/12/05/pas-plans-to-formulate-pandemic-control-related-strategies/
2. https://www.pakistantoday.com.pk/2022/12/07/science-minister-emphasizes-on-integrated-health-system-in-country-to-cope-with-future-epidemics/
3. https://www.radio.gov.pk/07-12-2022/agha-hasan-emphasizes-on-integrated-health-system-in-country
4. https://www.app.com.pk/national/science-minister-emphasizes-on-integrated-health-system-in-country-to-cope-with-future-epidemics/
5. https://leadpakistan.com.pk/news/pakistan-academy-of-sciences-plans-to-formulate-strategies-to-control-the-pandemic/
6. https://www.nation.com.pk/08-Dec-2022/science-minister-emphasises-on-integrated-health-system-to-cope-with-epidemics
7. https://www.brecorder.com/news/40213409/minister-calls-for-integrated-health-system-to-deal-with-epidemics
8. https://www.app.com.pk/photos-section/federal-minister-for-science-and-technology-agha-hassan-baloch-distributes-shield-to-position-holder-poster-presenter-during-a-conference-on-epidemic-pandemic-preparedness-organized-by-pakistan-acad/
9. https://www.technologytimes.pk/2022/12/08/epidemic-and-pandemic-preparedness-conference-held-by-anso-pas-maap/
10. https://hamariweb.com/enews/agha-hasan-emphasizes-on-integrated-health-system-in-country_nid3561730.aspx
11. https://www.urdupoint.com/en/pakistan/science-minister-emphasizes-on-integrated-hea-1606202.html

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The manuscript may contain Abstract, Keywords, INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION (or RESULTS AND DISCUSSION), CONCLUSIONS, ACKNOWLEDGEMENTS, CONFLICT OF INTEREST and REFERENCES, *and any other information that the author(s) may consider necessary*.

Abstract (font size 10; max 250 words)**:** Must be self-explanatory**,** stating the rationale, objective(s), methodology, main results, and conclusions of the study. Abbreviations, if used, must be defined on the first mention in the Abstract as well as in the main text. Abstract of review articles may have a variable format.

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MATERIALS AND METHODS: Provide an adequate account of the procedures or experimental details, including statistical tests (if any), concisely but sufficient enough to replicate the study.

RESULTS: Be clear and concise with the help of appropriate Tables, Figures, and other illustrations. Data should not be repeated in Tables and Figures, but must be supported with statistics.

DISCUSSION: Provide interpretation of the RESULTS in the light of previous relevant studies, citing published references.

ACKNOWLEDGEMENTS: (font size 10)**:** In a brief statement, acknowledge the financial support and other assistance.

CONFLICT OF INTEREST: State if there is any conflict of interest.

REFERENCES (font size 10)**:** Cite references in the text **by number only** in **square brackets**, e.g. "Brown et al [2] reported ..." or "... as previously described [3, 6–8]", and list them in the REFERENCES section, in the order of citation in the text, Tables and Figures (not alphabetically). Only published (and accepted for publication) journal articles, books, and book chapters qualify for REFERENCES.

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a. Journal Articles *(Name of journals must be stated in full)*

1. I. Golding, J. Paulsson, S.M. Zawilski, and E.C. Cox. Real time kinetics of gene activity in individual bacteria. *Cell* 123: 1025–1036 (2005).
2. W. Bialek, and S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100: 258–263 (2008).
3. R.K. Robert, and C.R.L.Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1(6) (2009).
4. D. Fravel. Commercialization and implementation of biocontrol. *Annual Reviews of Phytopathology* 43: 337359 (2005).

b. Books

5. W.R. Luellen. Fine-Tuning Your Writing. *Wise Owl Publishing Company, Madison, WI, USA* (2001).
6. U. Alon, and D.N. Wegner (Ed.). An Introduction to Systems Biology: Design Principles of Biological Circuits. *Chapman & Hall/CRC, Boca Raton, FL, USA* (2006).

c. Book Chapters

7. M.S. Sarnthein, and J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: The Northern North Atlantic: A Changing Environment. P.R. Schafer, & W. Schluter (Ed.), *Springer, Berlin, Germany*, pp. 365–410 (2000).
8. J.E. Smolen, and L.A. Boxer. Functions of Europhiles. In: Hematology, 4th ed. W.J. Williams., E. Butler and M.A. Litchman (Ed.), *McGraw Hill, New York, USA*, pp. 103–101 (1991).

d. Reports

9. M.D. Sobsey, and F.K. Pfaender. Evaluation of the H2S method for Detection of Fecal Contamination of Drinking Water, Report WHO/SDE/WSH/02.08, *Water Sanitation and Health Programme, WHO, Geneva, Switzerland* (2002).

e. Online references

These should specify the full URL for reference and give the date on which it was consulted. Please check again to confirm that the work you are citing is still accessible:

10. L. Branston. SENSPOL: Sensors for Monitoring Water Pollution from Contaminated Land, Landfills and Sediment (2000). http://www.cranfield.ac.uk/biotech/senspol/ (accessed 22 July 2005)

Tables and Figures

Insert all tables as editable text, not as images. Number tables consecutively following their appearance in the text, Figures should appear in numerical order, be described in the body of the text, and be positioned close to where they are first cited. Each figure should have a caption that describes the illustration, and that can be understood independently of the main text (Caption Table 1. and Fig 1. font size 10; Bold; Captions should be in sentence case; left-aligned). All Figures should have sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher) to enhance the readability. Figures may be printed in two sizes: column width of 8.0 cm or page width of 16.5 cm; number them as **Fig. 1**, **Fig. 2**, … in the order of citation in the text. Parts in a figure can be identified by A, B, C, D, ... and cited as Figure 2A, Figure 2B, Figure 2C. Captions to Figures must be concise but self-explanatory. Laser printed line drawings are acceptable. Do not use lettering smaller than 9 points or unnecessarily large. Photographs must be of high quality. A scale bar should be provided on all photomicrographs.

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