Pakistan Academy of Sciences

Research Article

Biorisk Management and Antibiotic Susceptibility Pattern of Biofilm Producing *Pseudomonas aeruginosa* Isolated from Broiler Chicken: A Public Health Concern

Shahab Mehmood[1], Kashif Ali[1*], Asma Bashir[1], Neha Farid[1], Kiran Fatima[1], and Sheeba Naz[2]

[1]Department of Biosciences, Faculty of Life Sciences,
Shaheed Zulfikar Ali Bhutto Institute of Science and Technology, Karachi, Pakistan
[2]Dow College of Biotechnology, Dow University of Health Sciences, Karachi, Pakistan

Abstract: Control of biosecurity and biosecurity within poultry consists of a set of practical measures meant to prevent and control the spread of disease between people and animals. Infections, caused mainly by zoonotic agents, occur frequently due to the lack of safety monitoring regulations, as well as the inappropriate use of antimicrobial products, leading to the emergence of antimicrobial-resistant microorganisms. *Pseudomonas aeruginosa*, often known as the MDR pathogen has evolved resistance to multiple antibiotics. Because of its propensity to build biofilms in meat and other food products, *P. aeruginosa* is even more resilient to the phenomenon of drug resistance which is a major public health issue. Standard microbiological and biochemical tests were used to isolate and identify *P. aeruginosa* from a total of 100 meat samples (20 from each district from broiler chicken meat) gathered from various butcher shops and supermarkets. The Kirby Bauer method was used to identify antibiotic resistance, while the microtiter plate test was used to monitor biofilm formation. It was found that *P. aeruginosa* was identified from 22 % of the broiler chicken meat samples and showed resistance to Cloxacillin, teicoplanin, ciprofloxacin, imipenem, and meropenem, followed by linezolid, streptomycin, amikacin, compound sulphonamide, aztreonam and cefepime which showed intermediate resistance. Multiple Antibiotic Resistance Index (MARI) was calculated as 0.45 for a total of 11 antibiotics. Also, all 22 MDR isolates of *P. aeruginosa* tested positive for the presence of the biofilm. In conclusion, it was determined that chicken meat was contaminated with *Pseudomonas aeruginosa,* and these strains that produce biofilms are more resistant to antibiotics. Thus, there is a serious threat to public health from biofilm-forming isolates found in broiler chickens.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Multi-Drug Resistance, Laboratory Infections, Biorisk Management, Antibiotics

1. INTRODUCTION

Aside from being one of the most innovative animal businesses and quickest manufacturers of meat worldwide, poultry has made important contributions to the country's agricultural economy. Protein from poultry products is necessary for human health [1]. Proper management and cleanliness on the farm have a significant impact on the quality of the chickens produced [2]. It is generally accepted that a chicken production system's main priority should be ensuring the highest possible levels of biosecurity and farm hygiene. Better flock health, lower medical costs, fewer losses, and greater profits are all possible thanks to effective biorisk management [3]. The FAO believes that the best strategy to prevent the spread of infectious illnesses is through the strict implementation of biosecurity measures [4]. High-risk areas for the transmission of illness could be mapped using biorisk management tools. This has crucial implications for the prevention and control of epidemics and facilitates their monitoring [5].

For the maintenance and improved health status and standards of livestock, reducing the spread of infectious diseases is imperative. Strict biorisk management policies will help to reach that goal. Over-prescribing antibiotics, which leads to antibiotic resistance in infectious organisms, is a prevalent practice that arises from the need to lower the prevalence of infectious agents [6]. Misusing biocidal agents in livestock farming also promotes the spread of bacteria that are resistant to antibiotics [7, 8]. Antibiotic overuse can be prevented through the implementation of stringent biosafety and biosecurity measures, which will also preserve the efficacy of traditional antimicrobials used to treat acute and chronic diseases [9]. The biorisk management measures for animal farms are specifically prepared to prevent the introduction and spread of diseases and considered as key-factors for increased farm productivity [10].

Pseudomonas aeruginosa is an opportunistic pathogen, with the ability to produce biofilm, that causes severe problems in chicken farms [11], while *Pseudomonas spp.* have historically been the most common pathogens found on chicken carcasses in industrial poultry processing facilities [12]. *Pseudomonas aeruginosa*, which causes serious respiratory infections [13] & cystic lung fibrosis [14] is widely believed to originate from chicken-based goods sold in grocery shops, especially among the immunocompromised. In the agriculture sector of Pakistan, and particularly in poultry farms and retail shops, there is a serious scarcity of understanding and application of biorisk management measures and protocols. This study is designed to establish a baseline regarding the presence of infectious agents, like *P. aeruginosa*, as a contaminant in poultry products. The research was also meant to aid in the development of an antibiogram of isolated strains to highlight the impact of over-use of antibiotics in farm settings and to identify the severity of the risk to which the producers and consumers of poultry products are exposed.

2. MATERIALS AND METHODS

2.1. Study Area and Sample Collection

The research was carried out in Karachi, Pakistan. Five meat markets from five districts in Karachi, Pakistan, namely Karachi East, Karachi West, Karachi Central, Karachi South, and District Malir, were randomly featured. In total, 100 samples of broiler chicken meat, 20 from each area, were randomly purchased from the meat shops in meat markets, and subsequently pooled. Briefly, five chicken meat pieces were taken randomly from each market, and samples of chicken meat were collected with sterile swabs, separately packaged, and sent within 2 to 4h to the research laboratory of the Department of Biosciences, Faculty of Life Sciences, SZABIST. In this laboratory, all microbiological investigations were undertaken.

2.2. *Pseudomonas aeruginosa* Isolation and Identification

Using a homogenizer, samples of broiler chicken meat were placed in 1ml Eppendorf tube containing one ml phosphate-buffered saline (PBS) and a 5-mm steel bead (Qiagen #69989) for 5 minutes at 30 Hz (Retsch; MM400). The meat homogenates were then plated on Cetrimide Agar and then allowed to incubate overnight at 37 °C. Biochemical tests confirming the presence of *Pseudomonas aeruginosa* colonies were found positive for indole and negative for Simmons citrate. Twenty-two out of a hundred samples from all the districts were found to be positive for *Pseudomonas aeruginosa*, and isolates with visible colony morphologies were chosen for further examination.

2.3. Biochemical Identification of *Pseudomonas aeruginosa* isolates:

On cetrimide agar plates, the putatively *Pseudomonas aeruginosa* colonies were streaked for biochemical confirmation. For additional phenotypic screening using the Quick Test Strip (QTS)-24 kit, conventional biochemical assays such as Gram staining, indole, catalase, citrate, oxidase, and motility were done on chosen pure *Pseudomonas aeruginosa* colonies.

2.4. Bacterial Growth and Media Conditions

For long-term preservation, bacterial isolates were kept in 20 % glycerol at -80 °C. From frozen stocks, isolates were inoculated onto Tryptic soy agar (TSA; Becton Dickinson) and incubated at 37 °C for 18 h. A single colony was inoculated into 5 mL

of LB broth and cultivated overnight at 37 °C with 200 rpm of shaking. All subsequent tests utilized overnight cultures.

2.5. Phenotypic Antibiotic Resistance Pattern

To determine antibiotic resistance among *Pseudomonas aeruginosa* strains, the Kirby Bauer disc diffusion method was used in accordance with CLSI recommendations. To determine which antibiotics would be effective against each potential positive isolate, a panel of 11 antibiotics was used. For the test, we took one colony of the *Pseudomonas aeruginosa* isolates from plates of cultures grown overnight established on Cetrimide Agar (Oxoid, Basingstoke, United Kingdom) and transferred it to plates of nutrient agar, where it was incubated at 37 °C for 24 h. *Pseudomonas aeruginosa* colonies were then emulsified into sterile saline until the turbidity reached 0.5 McFarland standard which is correspondent to 10^8 CFU/ml. Mueller Hinton (MH) agar plates (Oxoid, Basingstoke, United Kingdom) were prepared by spreading suspensions with sterile cotton swabs, and antibiotic discs were placed aseptically on the surface of the MH agar with sterile forceps; the plates were then incubated at 37 °C for 24 h. The widths of the inhibitory zones were measured and recorded; the values were then interpreted following CLSI guidelines.

2.6. Multiple Antimicrobial Resistance Indices (MARI)

The multidrug resistance level was enumerated using the multiple antibiotic resistance indices (MARI) as per the formula defined by the previous author [15].

Where a = total number of antibiotics to which an isolate shows resistance and b = total number of antibiotics to which the isolate was exposed.

2.7. Detection of Biofilm Production

Microtiter plate test was used to determine the biofilm-forming phenotype (MPA). There was a 100-fold dilution of the fresh cultures into tryptic soy broth. After that, the 96-well (flat bottom) plate was loaded with 250 μL aliquots of isolates and left to incubate at 37 °C overnight. Agitating and shaking the wells during washing helped get rid of any bacteria that had not properly detached. Because biofilms are so easily fixed by heat, the plates were heat dried to achieve this. Afterward, a crystal violet (0.1 %) stain in 250 μL was applied and left to set for 20 minutes. Once again, washed and dried the wells before treating them with 250 μL of 50 % acetone. The un-inoculated wells still filled with sterilized tryptic soy broth served as negative controls. The biofilms' OD values were evaluated at 594 nm using an ELISA reader from (BioRad, USA). In addition, a threshold value (ODc) was calculated as follows:

According to Saxena *et al*. a lack of opacity was observed in non-biofilm producers (OD<ODc), a lack of opacity was observed in weak producers (ODc<OD<2×ODc), a lack of opacity was observed in moderate producers (2×ODc<OD<4×ODc), and a lack of opacity was observed in strong producers of biofilm (4×ODc<OD).

3. RESULTS AND DISCUSSION

3.1. Bacterial Identification, Biochemical, and Growth Characterization

A total of 22 morphologically distinct colonies of non-lactose-fermenting bacteria were identified as *Pseudomonas aeruginosa* as shown in Table 1. The remaining 78 strains were found to be of *Serratia fonticola, Pseudomonas gessardii, Pseudomonas mucidolens, Lysinibacillus fusiformis, Pseudomonas stutzeri, Bacillus aryabhattai, Pseudomonas viridiflava,* and *Bacillus megaterium.*

3.2. Prevalence of *Pseudomonas aeruginosa* and the Multidrug Resistance Profile in Broiler Chicken Meat

The overall prevalence of *Pseudomonas aeruginosa* positive samples after isolation was 22 %. MDR profile revealed that the strains were highly resistant to 5 out of 11 antibiotics as shown in Figure 1. Table 2 presents the antimicrobial resistance profile of the isolates to the antibiotics and multiple antibiotic resistance Index (MARI) estimated as 0.45 for all of the MDR isolates (ranging from 0.2 to 0.5). Cloxacillin, Teicoplanin, Ciprofloxacin, Imipenem, and Meropenem showed 100 % resistance, followed by Linezolid, Streptomycin, Amikamicin, Aztreonam, Compound Sulphonamide, and
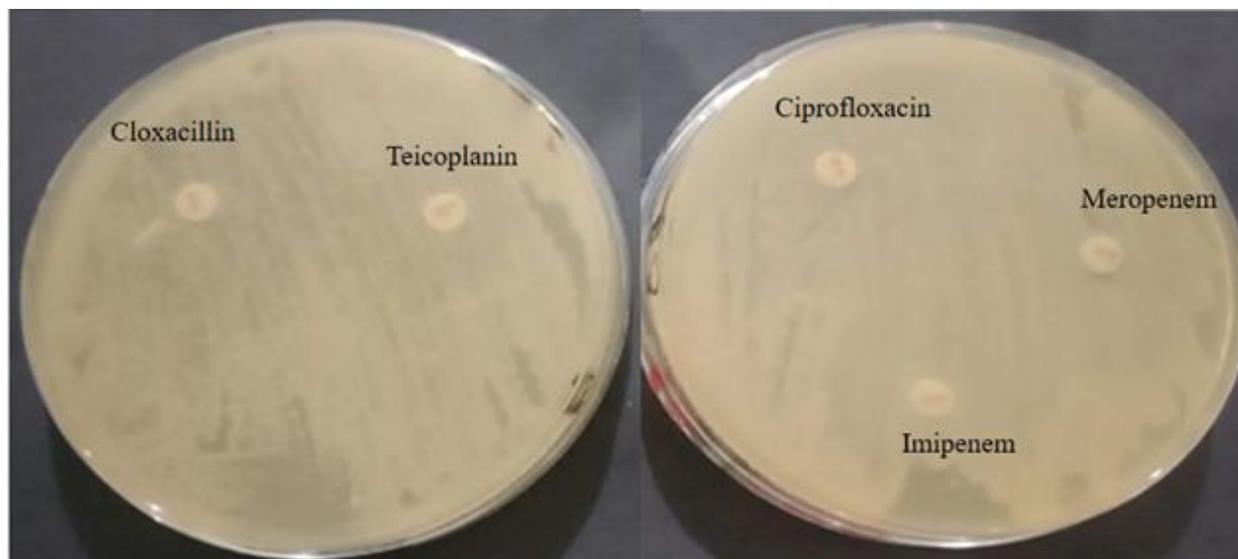
Fig. 1. Antimicrobial activity of *Pseudomonas aeruginosa* against antibiotics (Cloxacillin, Teicoplanin, Ciprofloxacin, Imipenem, and Meropenem) showing complete resistance.

Table 1. Sampling data of meat samples collected from different districts of Karachi

Districts of Karachi	Total Sample Collected	*Pseudomonas aeruginosa*	Positive Percentage
East	20	5	25 %
West	20	2	10 %
South	20	4	20 %
Korangi	20	6	30 %
Central	20	4	20 %

Table 2. Prevalence of AMR pathogens in livestock-sourcefood products

Antibiotics	Zone of Inhibition (mm)
Cefepime (Fep)	18
Compound Sulphonamide	15
Cloxacillin	6
Imipenem	4
Teicoplanin	6
Ciprofloxacin	6
Aztreonam	16
Meropenem	7
Linezolid	11
Amikamicin	14
Streptomycin	11

*Multiple Antibiotic Resistance Index (MARI) for the above antibiotics is found to be **0.45**. Zone of Inhibition (ZOI): 0-10 mm: Resistant; 10.1-16 mm: Intermediate; 17+ mm: Sensitive.*

Cefipime which showed intermediate resistance.

4. DISCUSSION

In this study, we compared the antibiotic resistance profile of *P. aeruginosa* that was isolated from poultry sources from 5 different districts of Karachi. There were a total of 100 samples were collected, and there were 22 % of the isolates showed the presence of *P. aeruginosa*. After antibiotic resistance profiling we found that strains exhibited high levels of resistance to five of the eleven antibiotics tested. MDR isolates were calculated to have a (MARI) of 0.45 overall (ranging from 0.2 to 0.5) All strains showed resistance to Cloxacillin, Teicoplanin, Imipenem, Meropenem and Ciprofloxacin but showed intermediate resistance towards Linezolid, Streptomycin, Amikamicin, Compound Sulphonamide, Aztreonam and Cefipime.

Antibiotic uses excessively in poultry to accelerate the development of broiler chicken is causing the spread of MDR *P. aeurginosa*, a harmful form of bacterial resistance, as evidenced by significantly greater numbers of MDR isolates. Isolates exhibit resistance to types of antibiotics, which contributes to their progression toward MDR status. The study's authors conclude that the widespread prevalence of multidrug-resistant *P. aeruginosa* strains in chickens poses serious risks to human health. Preventative actions that can be taken to reduce the transmission of MDR *P. aeruginosa* from chicken to humans include the proper management of raw products and the boiling of meat to sterilize it. These precautionary actions can be implemented to lessen the likelihood that multidrug-resistant *P. aeruginosa* will be transferred from chickens to people. The presence of biofilm promotes the growth of bacterial communities that are resistant to biocides and antibiotics [16]. Antibiotic resistance was caused by multiple factors, including biofilm formation, metabolic processes within in the biofilm, efflux pumps, and even outer membrane structures that prevented antimicrobials from penetrating the biofilm. Changes in bacterial phenotypic, gene expression, antibiotic resistance, and metabolic activity that result in the generation of virulence-associated proteins are all hallmarks of biofilm-forming microorganisms [17]. Significant economic losses as a result of deterioration, illness epidemics, and even deaths are caused by biofilm-producing bacteria's impact on the cattle and food industries [18]. *Pseudomonas*, along with *Clostridium, Campylobacter, Bacillus, Salmonella, Staphylococcus, Acinetobacter, Listeria, Acinetobacter, Klebsiella, Enterococcus, E. coli*, & *Aeromonas spp*. are a major health risk in chicken farms [19]. Maintenance and raising standards for livestock health require a reduction in the transmission of infectious diseases among livestock. High biosecurity standards are used to attain this goal, along with several preventive strategies that work to regress the prevalence of infectious pathogens and, as a result, decrease the requirement for the overprescription of antibiotics [20]. High biosecurity measures will reduce antibiotic use and preserve the efficacy of traditional antimicrobials for acute or chronic illnesses [18]. Similarly, the improper use of antimicrobials in farm animals contributes to the evolution of antimicrobial resistance [21]. Controlling the invasion of pathogens and their subsequent spread is the primary goal of biosecurity. They are also "important factors" in lowering infection rates, boosting agricultural output, and reducing antibiotic consumption. Experts have also proposed regular animal testing for different illnesses, accompanied by quarantine of diseased animals [22].

The primary risk factors for contamination of the environment in poultry farming with pathogenic bacteria that produce biofilms are contact with chicken feed, plants, pipelines, air, utensils, contact surfaces, and equipment. Furthermore, there are numerous points of entry for contamination of chicken products like meat and eggs along the food chain, from production to processing to distribution to retail to handling to preparation.

5. CONCLUSION

In conclusion, the findings of this study emphasize the importance of biorisk management and antibiotic susceptibility pattern of *Pseudomonas aeruginosa* biofilm-forming bacteria found in broiler chicken. The presence of antibiotic-resistant strains of this species highlights the need for further research to understand the mechanisms of resistance and for the development of procedures to slow the spread of bacteria resistant to many drugs. These strategies should include measures such as the implementation of appropriate hygiene and sanitation practices,

the careful use of antibiotics, and the adoption of antibiotic stewardship programs. To safeguard public health by lowering the risk of zoonotic illnesses, it is particularly critical to monitor the overall antimicrobial susceptibility of *P. aeruginosa* strains from animals, notably poultry.

6. CONFLICT OF INTEREST

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. REFERENCES

1. Y.A. Attia, M.T. Rahman, M.J. Hossain, Basiouni, A.F. Khafaga, A.A. Shehata and H.M. Hafez. Poultry production and sustainability in developing countries under the COVID-19 crisis: Lessons learned. *Animals* 12(5): 644 (2022).
2. J.M. Diaz Carrasco, L.M. Redondo, N.A. Casanova, and M.E. Fernandez Miyakawa. The role of farm environment and management in shaping the gut microbiota of poultry. *In Gut Microbiota, Immunity, and Health in Production Animals* 193-224 (2022).
3. G.B. Goualie, S.Bakayoko, and K.J. Coulibaly. Practices of biosecurity measures and their consequences on poultry farms in Abidjan district. *Food and Environment Safety Journal* 19(1) (2020).
4. FAO. FAO Viet Nam urges improved application of biosecurity along poultry production chain. http://www.fao.org/vietnam/news/detailevents/en/c/1098535 (2020).
5. N.T.K. Cuc, N.C. Dinh, N.T.L Quyen, and H.M. Tuan. Biosecurity level practices in pig and poultry production in Vietnam. *Advances in Animal and Veterinary Sciences* 8(10): 1068-1074 (2020).
6. K. Kirtonia, M. Salauddin, K.K. Bharadwaj, S. Pati, A. Dey, M.A. Shariati, V.K. Tilak, E. Kuznetsova, and T. Sarkar. Bacteriocin: A new strategic antibiofilm agent in food industries. *Biocatalysis and Agricultural Biotechnology* 36: 102141 (2021).
7. I. Magouras, L.P. Carmo, K.D. Stärk, and G. Schüpbach-Regula. Antimicrobial usage andresistance in livestock: Where should we focus? *Front. Vet. Sci.* 4:148 (2017).
8. N.T. Nhung, N. Chansiripornchai, and J.J. Carrique-Mas. Antimicrobial resistance in bacterial poultry pathogens: A review. *Frontiers in Veterinary Sciences* 4:126 (2017).
9. D. Lahiri, M. Nag, H.I. Sheikh, T. Sarkar, H.A. Edinur, S. Pati, and R.R. Ray. Microbiologically-Synthesized Nanoparticles and Their Role in Silencing the Biofilm Signaling Cascade. *Frontiers in Microbiology* 12: 636588 (2021).
10. V.H. Oliveira, J.T. Sørensen, and P.T. Thomsen. Associations between biosecurity practices and bovine digital dermatitis in Danish dairy herds. *Journal of Dairy Science* 100: 8398–8408 (2017).
11. S.E. Walker, J.E Sander, J.L. Cline, and J.S Helton. Characterization of *Pseudomonas aeruginosa* isolates associated with mortality in broiler chicks. *Avian Diseases* 46 (4):1045-1050 (2002).
12. S.H. Chen, N. Fegan, C. Kocharunchitt, J.P. Bowman, and L.L. Duffy. Changes of the bacterial community diversity on chicken carcasses through an Australian poultry processing line. *Food Microbiology* 86:103350 (2020).
13. M.W. Azam, and A.U. Khan, Updates on the pathogenicity status of *Pseudomonas aeruginosa*. *Drug discovery today* 24(1): 350-359 (2019).
14. M. Rybtke, L.D. Hultqvist, M. Givskov, and T. Tolker-Nielsen. *P. aeruginosa* biofilm infections: Community structure, antimicrobial tolerance and immune response. *Journal of Molecular Biology* 427: 3628–3645 (2015).
15. O. Adebowale, M. Makanjuola, N. Bankole, M. Olasoju, A. Alamu, E. Kperegbeyi, O. Oladejo, O. Fasanmi, O. Adeyemo, and F.O. Fasina. Multi-drug resistant *Escherichia coli*, biosecurity and anti-microbial use in live bird markets, Abeokuta, Nigeria. *Antibiotics* 11(2): 253 (2022).
16. X. Liu, H. Wang, L. Li, C. Deng, Y. Chen, , H. Ding, and Z. Yu. Do microplastic biofilms promote the evolution and co-selection of antibiotic and metal resistance genes and their associations with bacterial communities under antibiotic and metal pressures? *Journal of Hazardous Materials* 424:127285 (2022).
17. R.V. Sionov, and D. Steinberg. Targeting the holy triangle of quorum sensing, biofilm formation, and antibiotic resistance in pathogenic bacteria. *Microorganisms* 10(6): p.1239 (2022).
18. E. Butucel, I. Balta, D. McCleery, F. Morariu, I. Pet, C.A. Popescu, L. Stef, and N. Corcionivoschi. Farm Biosecurity Measures and Interventions with an Impact on Bacterial Biofilms. *Agriculture* 12(8):1251 (2022).
19. S.H. Toushik, A. Roy, M. Alam, U.H. Rahman, N.K. Nath, S. Nahar, B. M.J. MatubberUddin, and P.K. Roy. Pernicious Attitude of Microbial Biofilms in Agri-Farm Industries: Acquisitions and Challenges of Existing Antibiofilm Approaches. *Microorganisms* 10(12): 2348 (2022).
20. A. Catalano, D. Iacopetta, J. Ceramella, D. Scumaci, F. Giuzio, C. Saturnino, S. Aquaro, C. Rosano and M.S. Sinicropi. Multidrug resistance (MDR): A widespread phenomenon in pharmacological therapies. *Molecules*, 27(3): 616 (2022).
21. A. Saman, M. Chaudhry, M. Ijaz, W. Shaukat, M.U. Zaheer, A. Mateus, and A. Rehman. Assessment of knowledge, perception, practices and drivers

of antimicrobial resistance and antimicrobial usage among veterinarians in Pakistan. *Preventive Veterinary Medicine* 212: 105836 (2023).

22. A.A. Saied, A.A. Metwally, M. Dhawan, O.P. Choudhary and H. Aiash. Strengthening vaccines and medicines manufacturing capabilities in Africa: challenges and perspectives. *EMBO Molecular Medicine* 14(8): e16287 (2022).