Prevalence of multiple drug-resisting (MDR) pathogenic bacteria isolated from a broiler farm at Anandapur, Paschim Medinipur

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ABSTRACT

The farming of chicken (broiler/poultry) is a magnificent resource globally playing a key role in supplying protein-rich foodstuffs essential for human nutrition. Antibiotics like streptomycin, chloramphenicol, azithromycin, moxifloxacin, and moxclav (amoxicillin and clavulinic acid) were used to investigate their activities against bacteria in broiler litter and pond water samples where broiler farm litter was applied. Mixed-type zones of inhibition of all the antibiotics except moxclav were noticed in the case of the pond water sample. Whereas, sublethal bacterial growth inhibition was observed against all tested antibiotics in broiler farm litter samples. In this case, most of the bacteria were insensitive to various antibiotics. Streptomycin and moxclav-resistant bacteria were gram-positive as they appeared blue and were rod-shaped, and plausibly detected as *Enterococcus* as per artificial intelligence (AI) (https://openai.com/), whereas, an *Actinomyces*-like structure was observed upon Gram staining in the case of chloramphenicol-resistant bacteria. All these resistant bacteria were found to be catalase-positive and produced gas in the culture medium, but didn't produce acid as revealed by the methyl red test. Besides that, the mentioned resistant bacteria were seen to be non-motile as revealed by the 'wet mount' technique. These preliminary findings will help to study more about the multi-drug resistant (MDR) bacteria with human concern and the efficacy of antibiotics in the broiler industry.

Introduction

Global population expansion and rising affluence are driving up demand for animal protein (Belkhanchi *et al.*, 2023). One of the most popular meats consumed worldwide is poultry. The agricultural subsector growing at the fastest rate is poultry, particularly in emerging nations. The feathers of chickens are also very useful for plant growth (Paul *et al.*, 2013). The increasing demand for meat and eggs due to urbanization and population increase is expected to sustain the ongoing expansion of the global poultry business. Consequently, the industry is

dealing with several issues never encountered before (Mottet and Tempio, 2017). Globally, the fabricating of poultry meat is expected to grow 2.3% per annum until 2023 to 134.5 million tonnes, making it the main bird meat sector. According to the Department of Animal Husbandry and Dairying, India is 6th in chicken meat production in the world.

The commonly found microorganisms in the manure of industrial broiler farms can potentially be hazardous for birds, workers, and people staying near the farmhouse. *Bacillus anthracis*, *Chlamydia ornithosis*, *Salmonella choleraesuis, Campylobacter,* and *Acinetobacter spare* the most commonly found bacteria in such farms (Skora *et al.*, 2016). Antibiotics are commonly administered to the entire broiler flock to promote growth, prevent sickness, and treat illness (Roth *et al.*, 2019). In the EU in 2006, the US in 2017, and Brazil and China at the moment, antibiotic growth promoters are permitted (European Commission, 2005, Access Science Editors, 2017) (Roth *et al.*, 2019). Broiler farm manure is used to fertilize ponds to raise nutrient levels and encourage the growth of phytoplankton, which is the food of fish (Nyberg *et al.* 2024). Moreover, it has been found that pond fish treated with chicken dung had higher abundances of tetA, tetW, and other resistance genes (Nyber *et al.* 2024; Lin *et al.*, 2023). The uses of antibiotics in animal production are two major causes of antimicrobial resistance (AMR), a threat to global health. Particularly, a significant portion of chicken production has involved the use of antibiotics in compound feeds, both to avoid infectious bacterial diseases and to improve animal development rates. It is widely accepted that contamination of poultry by *Campylobacter* is a significant risk factor for human campylobacteriosis (El-Saadony *et al.*, 2023). Remarkably, an integrated poultry-fish farming system is becoming popular, where the litter of broilers is usually given in the pond, which inadvertently facilitates the spread of MDR bacteria (Adeyemi *et al.*, 2022). Here, our objectives were to find antibiotic-resistant bacteria in the broiler farm litter sample in pond water where broiler farm litter was applied to promote fish growth. To characterize the isolated bacteria gram's staining and AI (https://openai.com) were performed along with other biochemical assays.

1. Materials and Methods

1.1. Sample collection

Broiler litter and pond water samples were collected from Anandapur, Paschim Medinipur, where broiler farm litter is generally introduced to the pond to promote aquaculture. Five grams of farm litter was dissolved in 20 ml of autoclaved distilled water followed by subsequent mixing and vigorous shaking. One ml of the mixture was

taken in an Eppendorf tube and stored at 4 °C in the refrigerator. The pond water sample was also taken in an Eppendorf tube and was kept at 4 °C.

1.2. Media preparation

Mueller Hinton agar (MHA), nutrient broth, agar (NB and NBA), and Luria-Bertani (LB) media were prepared and autoclaved at 15 PSI for 15 min. After autoclaving MH agar medium and nutrient agar medium were poured onto plates and kept at room temperature for solidification. On the other, hand 10 test tubes were taken; 5 ml of nutrient broth was poured into the all test tubes, cotton plugged, and autoclaved.

1.3. Antibiotics used in this study

The disk of antibiotics such as moxifloxacin (5µg), azithromycin (15µg), streptomycin (40 µg), chloramphenicol (40 µg), and moxclav (40 µg) were used in this study.

1.4. Serial dilution of broiler litter and pond water sample

The broiler litter and pond water samples were diluted serially from 10^{-1} to 10^{-5}. For dilution, 0.1 ml of the above-mentioned samples were diluted with 0.9 ml of distilled water and stored at 4°C until needed.

1.5. Plating of broiler litter and pond water samples

Two nutrient agar plates were inoculated with a crude litter sample and a pond water sample. MHA plates were inoculated with diluted samples. Two plates were inoculated with 50 µL of 10^{-2} and 10^{-3} diluted litter samples. Similarly, 10^{-2} and 10^{-3} diluted pond water were plated onto NBA and MHA plates.

1.6. Antibiotics sensitivity assay

After plating, all the plates were kept for 10 min in a laminar airflow system, and the above-mentioned antibiotic disks were placed uniformly on each plate (Bauer et. al., 1966) All the plates were kept at 37 °C for 24 hours. The zone of inhibition (ZOI) of each antibiotic was measured.

1.7. Gram staining

Gram staining was performed as stated here. A loopful of culture from antibiotic-containing (streptomycin, moxclav, and chloramphenicol) NB was taken and a smear was made in the slide. The slide was heated briefly to fix the smear properly and allowed to cool. Crystal violet was applied on each slide for 1 min followed by rinsing with distilled water. Then iodine solution was applied for 1 min and rinsed with distilled water. Then decolorization of the CV-I complex was done for 3-5 seconds with 100% ethanol and immediately rinsed with water. The counter-stain safranin was added for 30 sec and again rinsed with distilled water. The slides were dried and examined under a microscope (Olympus). Here, we used artificial intelligence (https://openai.com/) and visual inspection to identify bacterial isolates.

1.8. Catalase test

Catalase is an enzyme that converts hydrogen peroxide to water and oxygen. This test was performed to decipher the aerobic or anaerobic nature of the bacteria (Baureder *et al.*, 2012). A 2% of hydrogen peroxide was prepared. Then in a grease-free slide 10 µL of hydrogen peroxide solution was taken followed by the addition of a loopful of antibiotic-resistant bacterial cultures (as mentioned earlier) were added and kept for 5 min to check the result.

1.9. Motility test

The motility test of bacteria is usually performed to determine the movement of bacteria whether it is motile or not. Here, we have used the 'wet mount' technique to identify the motility of bacteria (Demeke *et al.*, 2021). According to this technique, a drop of antibiotic-resistant bacteria suspension was placed on a grease-free slide and covered with a cover slip. After that, the slides were examined under the compound microscope (Olympus) to observe the bacterial movement.

2. Results

2.1. Analysis of antibiotic sensitivity on bacteria; population from broiler farm litter and farm litter applied pond water samples

After 24 hours of sample plating and charging of antibiotic disks (as stated in materials and methods), the zone of inhibition of microbial growth was observed (Fig. 1). Most of the bacteria isolated from broiler farm litter were found to resist the action of antibiotics. Disc diameter and the zone of inhibition of antibiotics were measured (Tables 1 and 2).

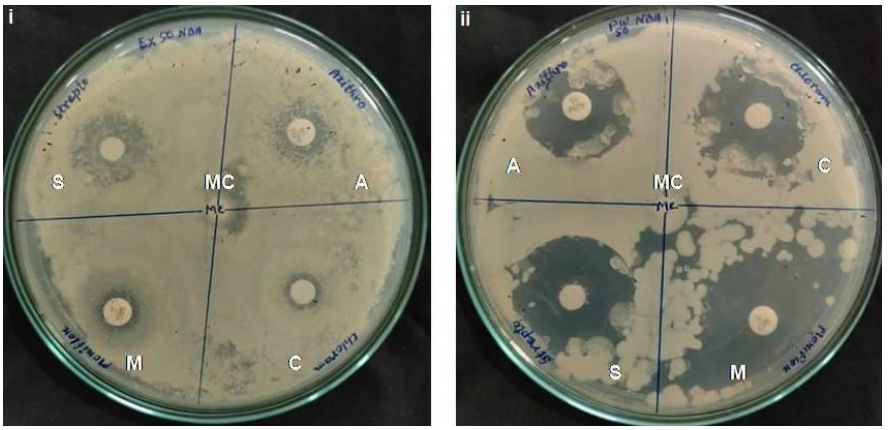

Fig. 1. **i.** Antibiotic sensitivity assay of broiler farm litter sample. **ii.** Antibiotic sensitivity assay of pond water where broiler farm litter sample was introduced. A, C, M, S, and MC indicate azithromycin, chloramphenicol, moxifloxacin, streptomycin, and moxclav respectively.

Table 1. Effect of various antibiotics on bacterial population from broiler farm litter.

Antibiotic Disc Used	Diameter of Disc (mm)	Total zone of inhibition (mm)	Average zone of inhibition (ZOI) excluding disk diameter (mm)
Streptomycin (40µg)	6	16	10
Azithromycin (15µg)	7	25	18
Moxclav (40µg)	6	14	8
Chloramphenicol (40µg)	6	23	17
Moxifloxacin (5µg)	7	19	12

Table 2. Effect of various antibiotics on bacterial population from pond water where broiler farm litter was applied.

Antibiotic Disc Used	Diameter of Disc (mm)	Total zone of inhibition (mm)	Average zone of inhibition (ZOI in mm) excluding disk diameter (mm)
Streptomycin (40µg)	6	26	20
Azithromycin (15µg)	7	25	18
Moxclav (40µg)	6	00	00
Chloramphenicol (40µg)	6	26	20
Moxifloxacin (5µg)	7	32	25

2.2. Characterizations of bacteria: Gram staining of antibiotic-resistant bacteria

Gram staining of chloramphenicol, streptomycin, and moxclav-resistant bacteria was performed which revealed that both streptomycin and moxclav-resistant bacteria were Gram-positive and remained in a cocci-shaped arrangement. With the help of artificial intelligence (https://openai.com/), these bacterial isolates were determined as *Enterococcus* plausibly. Whereas, an *Actinomyces*-like bacterial structure was seen in the case of chloramphenicol-resistant bacteria plausibly (Fig. 2).

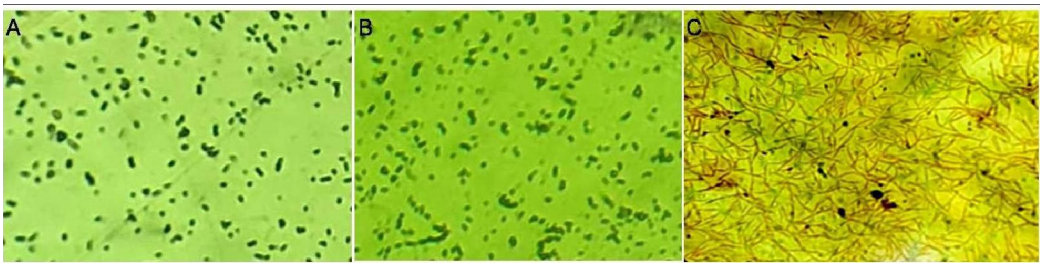

Fig. 2. A. Streptomycin resistant bacteria **B.** Chloramphenicol resistant bacteria.

C. Moxclav-resistant bacteria. All these bacteria were isolated from broiler litter and examined under 40X magnification in a microscope (Olympus).

2.3. Analysis of catalase activity of antibiotic-resistant bacteria

The chloramphenicol, streptomycin, and moxclav-resistant bacteria were analyzed for oxygen formation by using hydrogen peroxide. All the above-mentioned bacteria produced oxygen bubbles indicating that these bacteria were catalase-positive, aerobic, and contained the gene for catalase (Fig. 3).

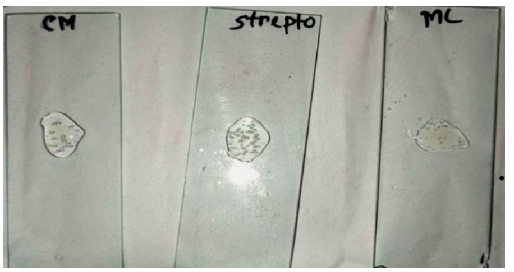

Fig. 3. The chloramphenicol (CM), streptomycin (strepto), and moxclav (MC) resistant bacteria were found to produce air bubbles (oxygen) in the presence of hydrogen peroxide.

2.4. Motility test of antibiotic-resistant bacteria

By the 'Wet mount' technique, under the microscope, it was noticed that the chloramphenicol, streptomycin, and moxclav-resistant bacteria remained in a stationary condition. Hence, it was determined that these resistant bacteria are non-motile (data not shown here).

2.5. Acid and gas production analysis of antibiotic-resistant bacteria

The chloramphenicol (CM), streptomycin (Strepto), and moxclav (MC) resistant bacteria were found to produce gas in the Durham's tube in nutrient broth medium (Fig. 4), although it was noticed that no color change of methyl red in the same culture medium. This result indicated that these resistant bacteria produce gas but not acid (Fig. 5).

Fig. 4. The moxclav (MC), chloramphenicol (CM), and streptomycin (strepto) resistant bacteria were found to gas in the LB broth.

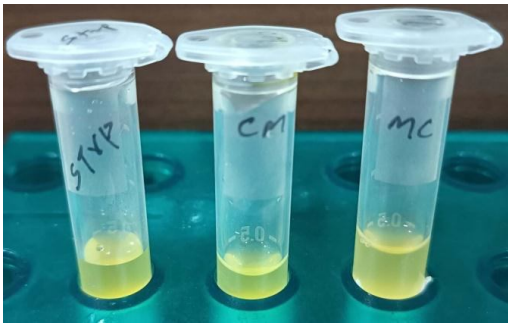

Fig. 5. The streptomycin (strp), chloramphenicol (CM), and moxclav (MC) resistant bacteria were found to produce no acid as the color of methyl red was not turned into red.

3. Discussion

Globally, there is a growing demand for products made from broiler meat, and chicken species are widely acknowledged as a major source of human food (Zampiga *et al.*, 2021). The animal production sector, particularly poultry production, symbolizes one potential source of multidrug-resistant bacteria, which hold plasmid-mediated resistance genes (Xexaki *et al.*, 2021). The majority of the broiler chickens are more likely to carry *Salmonella* primarily, proper litter execution during the pre-harvest stage must be essential to the general welfare of each broiler flock and for food safety. (Plumblee Lawrence *et al.,* 2021).

It is concerning that the majority of the antibiotics employed in raising broilers or poultry are classified as essential drugs for human use. (Mansaray *et al.,* 2022). Many investigations have found extensive resistance to antibiotics of broiler litter bacterial isolates like ampicillin, chloramphenicol, and tetracycline. Such bacterial isolates have the potential to spread antibiotic-resistance genes to bacteria which could infect humans. Antibiotics that are considered essential for human use, such as streptomycin and erythromycin, are included in the urgent medicine category (Mansaray *et al.,* 2022).

Here we have assayed various antibiotic sensitivity on broiler farm litter samples alone and pond water samples where broiler or poultry farm litter is usually given to increase the growth of fish. We have used streptomycin, azithromycin, moxclav, chloramphenicol, and moxifloxacin. Where moxifloxacin, streptomycin, chloramphenicol, and azithromycin revealed a greater zone of inhibition in the case of both of the samples, indicating the effectiveness of these antibiotics. Although, streptomycin, moxclav, and chloramphenicol-resistant bacteria appeared. In contrast, moxclav was proved to be very less effective against the bacteria isolated from the broiler litter sample and the pond water sample (Tables 1 and 2). Earlier reports revealed similar efficacy and resistance against these drugs of human use by MDR bacteria of broiler farm litter (Furtula *et al.,* 2013; Agyare *et al.,* 2018; Abreu *et al.,* 2023).

These resistant bacterial isolates grow well in the aforementioned antibiotic-

containing LB broth. All these bacterial isolates were analyzed in a microscope upon Gram staining. All the resistant bacteria were Gram-positive and by artificial intelligence (https://openai.com/), the resistant bacteria were characterized in which streptomycin and moxclav-resistant bacteria were probably *Enterococcus* sp, showed typical Gram-positive characteristics. Similar findings were found by Furtula *et al.* in the year of 2013. Streptomycin-resistant *Enterococcus* is a most significant human pathogen and is responsible for urinary tract infections, bacteremia, intra-abdominal infections, ophthalmic infections, and endocarditis (Fiore, 2019). *Enterococcus* are now the third most common hospital-acquired pathogen, causing 14% of hospital-acquired infections in the United States between 2011 and 2014 (Weiner *et al.,* 2014). Typical *Actinomyces*-like filamentous bacteria was observed in the case of chloramphenicol, this bacteria was also Gram-positive. *Actinomyces* is very common in broiler farm litter samples (Chen and Jiang, 2014; Gorliczay *et al.*, 2021). Human actinomycosis, a chronic, granulomatous infectious disease mostly mediated by *Actinomyces israelii* has been recognized for a long time and is mostly involved in dental caries and periodontal diseases (Kononen and Wade, 2015). Their common niche in the human body are the gut, genitourinary tract, and skin, and cause numerous infections which are difficult to treat (Li *et al.*, 2018; Kononen and Wade, 2015).

Our result showed that both the above-mentioned bacterial species were catalase-positive. An earlier report revealed that in some cases *Entercoccous* showed weak catalase activity, whereas, *Actinomyces viscous*, *A. naeslundii*, and *A. naeslundii* were also catalase-positive (Agger and Kowalski, 2010). So, our result is in line with the earlier findings. It is well known that both of these bacterial isolates are non-motile and we have obtained similar results by hanging mount microscopic analysis (Li *et al.*, 2018; Krawczyk *et al.*, 2021). Surprisingly, we have found gas in the LB medium containing streptomycin and moxclav-resistant *Enterococcus* sp. Some heterofermentive *Enterococcus* produce gas which is helpful for their growth (Cai, 1999). It is remarkable that *Actinomyces* also produced gas in the said medium which plausibly helped their growth in the LB medium containing chloramphenicol. Both these bacterial isolates were found to produce no acid in the LB medium, this may be due to the non-availability of anaerobic conditions in our experimental conditions, and sustaining in respective antibiotic selection pressure (Ramos *et al.*, 2020). Although we have investigated the sensitivity of various antibiotics against broiler farm litter bacterial isolates at a preliminary level, this study requires more research to identify those

bacterial isolates more specifically. This study is also very significant in terms of overuse and misuse of antibiotics for human uses which is a great concern about the efficacy of antibiotics as therapeutics for humans.

4. Conclusion

Our study aimed primarily to identify the antibiotic-resistant bacteria isolates from commercial broiler farm litter and pond water where such litter is usually applied to promote aquaculture. In our experimental conditions, we have identified streptomycin, moxifloxacin, azithromycin, chloramphenicol, and moxclav-resistant bacteria. Here, we have also found streptomycin, chloramphenicol, and moxclav-resistant *Enterococcus* and *Actinomyces*-like bacteria. These antibiotic-resistant bacteria were non-motile and aerobic. Surprisingly, these bacteria produce gas but not acid. Due to a limited time, we were unable to characterize those bacteria completely. Further study is needed to characterize these bacteria as they are an alarming threat to broiler meat consumers and farm workers.

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Conflicts of interest: There are no conflicts of interest.

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