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PROFILING OF ANTIBIOTIC RESISTANT BACTERIA ISOLATED FROM POULTRY LITTER OF COMMERCIAL FARMS IN KHULNA DISTRICT, BANGLADESH

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Abstract

Microbial resistance to antibiotics has become a global threat that interferes with the interaction among humans, the environment, and microbes. Our study sought to detect antibiotic-resistant bacteria in light of the growing interest in using antibiotics in the poultry sector. We collected 24 poultry liter samples from 04 different upazilas of Khulna district (Batiaghata, Dumuria, Paikgachha and Koyra). Initially, 09 bacterial isolates were selected and among them, 77.78% bacteria were found gram positive. Subsequently, to characterize these bacteria, a toal of 10 biochemical tests (methyl red test, MacConkey agar test, indole test, catalase test, triple sugar iron test, mannitol salt agar test, oxidase test, Voges-Proskauer test, citrate test and nitrate test) were carried out in this experiment. Moreover, our study also isolated and amplified bacterial DNA for Sanger sequencing and finally disclosed 09 different antibiotic resistant bacterial species, namely *Priestia aryabhattai, Bacillus cereus, Priestia megaterium, Lysinibacillus macrolides, Rossellomorea aquimaris, Mammaliicoccus sciuri, Bacillus wiedmannii, Escherichia coli* and *Citrobacter freundii.* Further, disk diffusion assays were performed following CLSI guidelines and it unveiled the susceptibility and resistant properties of isolates against commonly used 08 antibiotics (penicillin 10U, tetracycline 20 µg, nitrofuran 300 µg, clindamycin 2 µg, azithromycin 15 µg, quinolones 5 µg, tetracycline 30 µg and penicillin 30 µg) in poultry farms. All of the bacterial isolates were found resistant to at least one of the antibiotics except *C. freundii* (isolate 9). Most of the isolates (66.67%) were resistant to nitrofuran, whereas all of them were susceptible to penicillin. Lastly, this study also made an effort to understand the evolutionary relationships of the identified species through a phylogenetic tree. Thus, the findings of this study will help farmers and common people to better understand the risk of developing antibiotic-resistant bacteria as a result of excessive antibiotic usage.

Keywords: Antibiotic resistance, CLSI, PCR, sanger sequencing

Introduction

Antibiotics transformed our society by allowing previously incurable illnesses to be treated and medical treatments such as surgery and chemotherapy to be conducted safely (Barriere, 2015). The discovery of antibiotics was a defining moment in the history of humankind. Hundreds of millions of lives have been saved, and our level of living has increased significantly. However, the time we have with these medications is going out. Antibiotics have been used so widely in humans and animals that many of them are losing their effectiveness to kill germs (Ventola, 2015).

Antibiotics are used by humans in an estimated 34.8 billion doses per year, with worldwide consumption growing by 65 percent between 2000 and 2015 (Klein et al., 2018). Antibiotics are provided unnecessarily 1 in every 5 times in the United Kingdom and this figure climbs to one in three in the United States (Pouwels et al., 2019).

Animal farming in low-resource settings is critical, since many nations migrate to more intensive animal farming techniques, resulting in increased antibiotic usage and, as a result, a larger risk of antibiotic resistance exposure in animals and humans throughout the world (Landers et al., 2012). As low to middle-income nations progress toward high-income status, there will be an ever-increasing demand for high-quality animal protein sources (Henchion et al., 2017).

Poultry is one of the most widely consumed foods on the planet. With nearly 90 billion tons of chicken meat produced each year, chicken is the most widely farmed animal (Machuve et al., 2022). As the need for protein rises,

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this business will continue to expand. Antibiotics must be used frequently during this process. Farmers and stakeholders rely heavily on intensive poultry farming to satisfy current demand, which necessitates the use of vaccinations, vitamins and minerals, and, most importantly, antibiotics (Pouwels et al., 2019). Antibiotics have aided in the efficient production of chicken, allowing consumers to acquire high-quality meat and eggs at an affordable price (Mehdi et al., 2018).

Currently, approximately 80% of all food-producing animals and birds receive medication for part or most of their lives (Truong et al., 2019). Even though the European Union has outlawed the use of antibiotics for growth promotion, there is little regulation of growth promotion drugs globally (Casewell et al., 2003). Because of the extensive use of antibiotics for growth promotion, illness prevention, and infection treatment in industrial animal food production, resistance may develop.

Antibiotic resistance develops naturally, although it has been exacerbated by overuse and abuse of antibiotics when bacteria's sensitivity to antibiotics changes and they gain the capacity to overcome the medications (Reygaert, 2018). The uprising of antibiotic-resistant bacteria results in severe health risks and economic collapse due to longer hospital stays, more medical expenses, and more fatalities that could have been avoided (Dadgostar, 2019). According to the World Health Organization (WHO), antibiotic resistance is one of the most serious health dangers to the world is facing (WHO, 2021). There is an estimated 4·95 million deaths associated with bacterial antibiotic resistance in 2019, including 1·27 million deaths attributable to resistant bacteria (Murray et al., 2022). Without immediate action, this number is expected to skyrocket. While new medications have been developed, they have not kept up with the rate of resistance (Podolsky, 2018).

The most resounding message that comes through from every new resistance study is that the pool of resistance genes and the mechanisms of resisting antibiotics, available to bacteria are effectively limitless (Hayes, 2022). However, the battle against antibiotic resistance is far from over. We must continue the struggle, which necessitates a greater understanding of the mechanisms of action of antimicrobial drugs as well as their mechanisms of resistance. But, most importantly, we must first identify the resistant bacteria.

Due to the growing use of PCR and DNA sequencing, 16S rRNA gene sequencing has become more important in clinical microbiology laboratories for the precise identification of bacterial isolates and the discovery of novel bacteria. 16S rRNA gene sequencing not only sheds light on the causes of infectious diseases, but also aids clinicians in selecting medications, estimating the length of treatment, and planning infection control measures (Woo et al., 2008). These predictions are more accurate and reasonable for bacterial identification because the intergenic spacer region of bacterial DNA encodes for 16s rRNA and this spacer region is unique for every strain of bacteria. Hence, our study shed light on profiling antibiotic resistant bacteria isolated from litter of poultry farms following both biochemical and molecular approaches. In addition, the evolutionary distances were computed among the isolated bacteria.

Materials and Methods

The sequential workflow of our research is presented in Figure 1.

Sample collection and isolation of bacterial isolates

A total of 24 poultry litter samples were collected from 04 different upazilas of Khulna district (Batiaghata, Dumuria, Paikgachha and Koyra). Dry, clean, and sterile polythene bags were used to collect samples. Later, 1g of sample was weighed out and diluted in 100 ml of distilled water followed by precipitation with blotting paper. The processed sample suspension was diluted in 10 ml test tubes until 1:100000 dilution was achieved. Samples from the 4^{th} and 5^{th} dilution tubes were selected for further processing. Subsequently, 1 ml from each dilution was aseptically spread on appropriately labeled nutrient agar plates that were previously sterilized by autoclaving at 121^{o}C for 15 mins. Finally, these nutrient agar plates were incubated at 37˚C for 24 hours. At the end of the incubation period, colonies with a specific shape, surface texture, and edge were picked and streaked on a new plate. In this process, a small amount of inoculum from each morphologically different single colony was streaked onto the nutrient agar plate and incubated at 37^{o}C for 24 h. Additionally, 40% glycerol was used to store each bacterial culture for long-term storage.

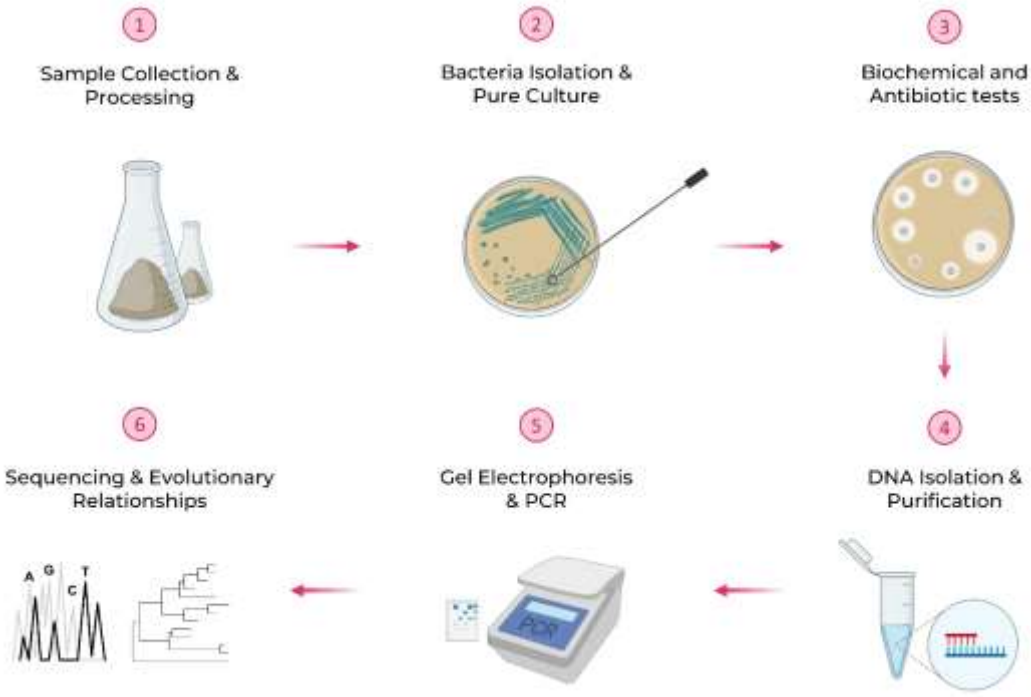

Figure 1. Schematic illustration of the overall general workflow of this study

Biochemical characterization of presumptive bacterial isolates

A standard gram staining procedure was followed to identify the gram-positive and gram-negative bacteria (Claus, 1992). However, the following biochemical tests were used to further identify and classify the isolates: methyl red test, MacConcy agar test, indole test, catalase test, triple sugar iron test, mannitol salt agar test, oxidase test, Voges-Proskauer test, citrate test and nitrate test. The outcomes from all biochemical tests were analyzed according to Bergey's manual of systematic bacteriology (Bergey, 1994).

Table 1. Class, generic and commercial names of the antibiotics with their concentrations.

Serial number	Class of antibiotics	Generic name of antibiotics	Commercial name of antibiotics	Concentration
1	Penicillin	Amoxicillin	Penvik	10U
2	Tetracycline	Oxytetracycline	Oxyvet	20 µg
3	Nitrofuran	Nitrofurantoin	Nintoin	300 µg
4	Clindamycin	Clindamycin	Clindacin	2 µg
5	Azithromycin	Azithromycin	Azith	15 µg
6	Quinolones	Ciprofloxacin	Ciprocin	5 µg
7	Tetracycline	Doxycycline	Vibramycin	30 µg
8	Penicillin	Amoxicillin	Amoxicillin	30 µg

Antibiotic sensitivity test

Eight different types of commercial antibiotic discs (6mm diameter) were used to screen out the resistant pattern among the isolated bacteria (Table 1). On the other hand, sterile blank discs impregnated with distilled water (200µl) was used as positive control. Kirby Bauer's disc diffusion, an antibiotic sensitivity test, was performed on Muller – Hinton agar (MHA) media following The Clinical & Laboratory Standards Institute (2006) guidelines. Hinton agar plates were prepared and sterilized by autoclaving at 121°C for 15 min. Standard suspensions of the isolates were adjusted to 0.5 McFarland Standard (McFarland, 1907). Immediately after standardization, 1 ml suspension was

spread on a petri dish by using a spreader, and a lawn culture was performed on the surface of the MHA plate. Then, discs were arranged on the surface of inoculated plates. The plates were incubated at 37°C for 24 hours.

Molecular characterization of bacterial isolates

The most reliable bacterial identification is the integration of morphological methods with molecular applications (Franco-Duarte et al., 2019). Thus in this experiment, total DNA was isolated by an automated DNA extractor (Maxwell 16, Promega, USA). Broad range universal primers (27F: AGAGTTTGATCMTGGCTC AG and 1492R: GGTTACCTTGTTACGACTT) were used to recognize conserved sequences within the 16S rRNA gene and amplified the intervening variable regions (Weisburg et al., 1991). Polymerase chain reactions (PCR) were carried out in a total volume of 20 uL containing 25 ng genomic DNA, 15 pMol of forward and reverse primers, and 10uL master mix. Reactions were run on a Gene Amp PCR system 2700 thermocycler (PE Applied Biosystems) programmed with the cycling conditions of: one cycle for 3 min at 95 °C, 35 cycles of 30 sec at 95 °C, 1 min at the appropriate annealing temperature at 48°C and 90sec at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were then separated on a 1.0% agarose gel containing 1 mM ethidium bromide for visualization on a UV light box. The amplified gene fragments were purified with QIAquick Gel Extraction Kit and sequenced from Invent Technologies Ltd., Dhaka, Bangladesh.

Analysis of sequence and construction of a phylogenetic relationship

The raw sequence data from Sanger sequencing in ABI chromatogram file format was observed in finch TV software version 1.4.0. We converted them into FASTA format by ABI to FASTA Converter version 1.1.2. The FASTA format files were used to find out the strains from NCBI. Then we opened the FASTA files in MEGA 7.0 and aligned them using the muscle tool (Kumar et al., 2016). The extra parts were trimmed out. The tree was constructed following the neighbor-joining statistical method where the number of bootstrap replications was 1000. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (Kimura, 1980; Saitou & Nei, 1987). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated. There were a total of 1133 positions in the final dataset.

Results and discussion

Isolation and cultivation of unknown bacterial isolates

A total of 9 bacterial isolates (Isolate 1 to Isolate 9) were selected according to their distinct physical appearance (Figure 2). Later, they were subjected to biochemical and antibiotic susceptibility tests. The results of gram staining test revealed that the majority (77.78%) of the isolated bacteria were gram-positive whereas the rest of the isolates were found gram-negative (22.22%). Later, they were subjected to biochemical and molecular characterization.

Biochemical and molecular characterization of bacterial isolates

Results of all biochemical tests are summarized (Table 2) and illustrated (Figure 2) below. Additionally, comparative analysis of the sequences obtained from isolated bacterial DNAs with an already available database (NCBI BLAST) finally revealed all the bacterial species (Table 3). Among them, *Bacillus cereus*, *Lysinibacillus macrolides*, *Mammaliicoccus sciuri*, *Escherichia coli* and *Citrobacter freundii* were highly pathogenic for animal. *B. cereus* causes non-gastrointestinal infections, respiratory tract infections, and nosocomial infections ((Bottone, 2010). *M. sciuri* has been discovered as an opportunistic pathogen linked with mastitis, dermatitis, and exudative epidermitis on rare occasions (D et al., 2022). At least six separate pathotypes of *E. coli* are responsible for causing enteric illnesses like diarrhea or dysentery, while other pathotypes are responsible for extra-intestinal diseases like meningitis and urinary tract infections (Kaper et al., 2004).

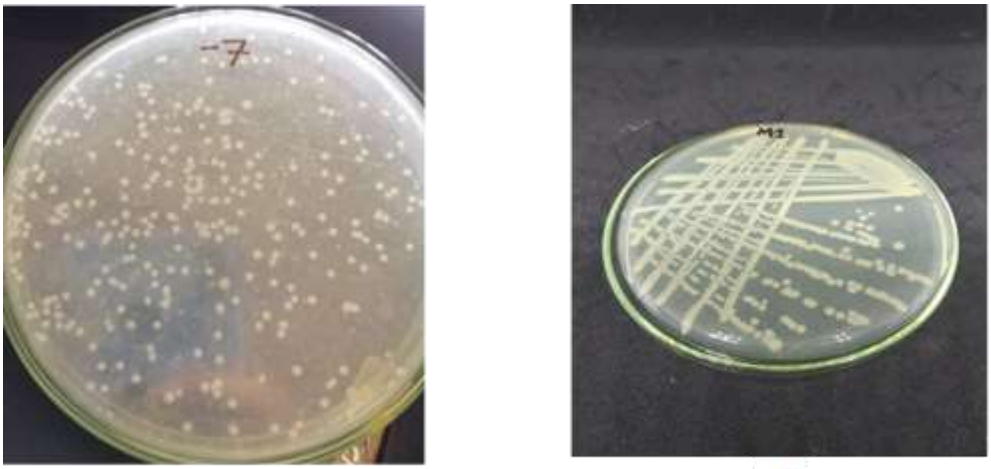

Figure 2. Bacteria was initially isolated on crowed plates (A) and then pure culture were prepared from those isolates (B).

Table 2. Results of biochemical tests for bacterial isolate identification including gram staining.

Strain	MR	MAC	VP	IND	TSI	CAT	NIT	OXI	CIT	MAN	GS
Isolate 1	-	-	-	-	K/K	+	-	-	-	-	+
Isolate 2	-	-	-	-	K/K	+	-	-	-	-	+
Isolate 3	+	-	-	-	K/A	+	+	+	+	-	+
Isolate 4	-	-	-	-	K/K	+	-	+	+	-	+
Isolate 5	-	-	-	-	K/K	+	+	-	-	-	+
Isolate 6	+	-	-	-	K/K	+	+	+	+	+	+
Isolate 7	+	-	-	-	K/A	+	+	-	-	-	+
Isolate 8	+	+	-	+	A/A	+	+	-	-	-	-
Isolate 9	+	+	-	-	K/A	+	+	-	+	-	-

Here, MR: Methyl Red; VP: Voges-Proskauer; TSI: Triple Sugar Iron; CAT: Catalase; IND: Indole; OXI: Oxidase; NIT: Nitrate; MAC: MacConkey; CIT: Citrate; MAN: Mannitol; GS: Gram Staining.

Table 3. Bacterial species identified by 16S rRNA gene sequencing of isolates obtained from poultry liter (mostly 98% identity).

Isolate No.	Scientific Name	Strain	Accession	% of similarity
Isolate 1	*Priestia aryabhattai*	WTB77	MK241860.1	98.73
Isolate 2	*Bacillus cereus*	NA-28	MN882654.1	99.51
Isolate 3	*Priestia megaterium*	Ni2_2	MH762123.1	98.88
Isolate 4	*Lysinibacillus macrolides*	KPB6	MH542661.1	99.35
Isolate 5	*Rossellomorea aquimaris*	AP BFT2	MK934550.1	96.31
Isolate 6	*Mammaliicoccus sciuri*	AA1	MT275460.1	98.88
Isolate 7	*Bacillus wiedmannii*	B16-2	MT256061.1	99.26
Isolate 8	*Escherichia coli*	JCD1	MH517447.1	98.66
Isolate 9	*Citrobacter freundii*	DSR1	MH181794.1	98.29

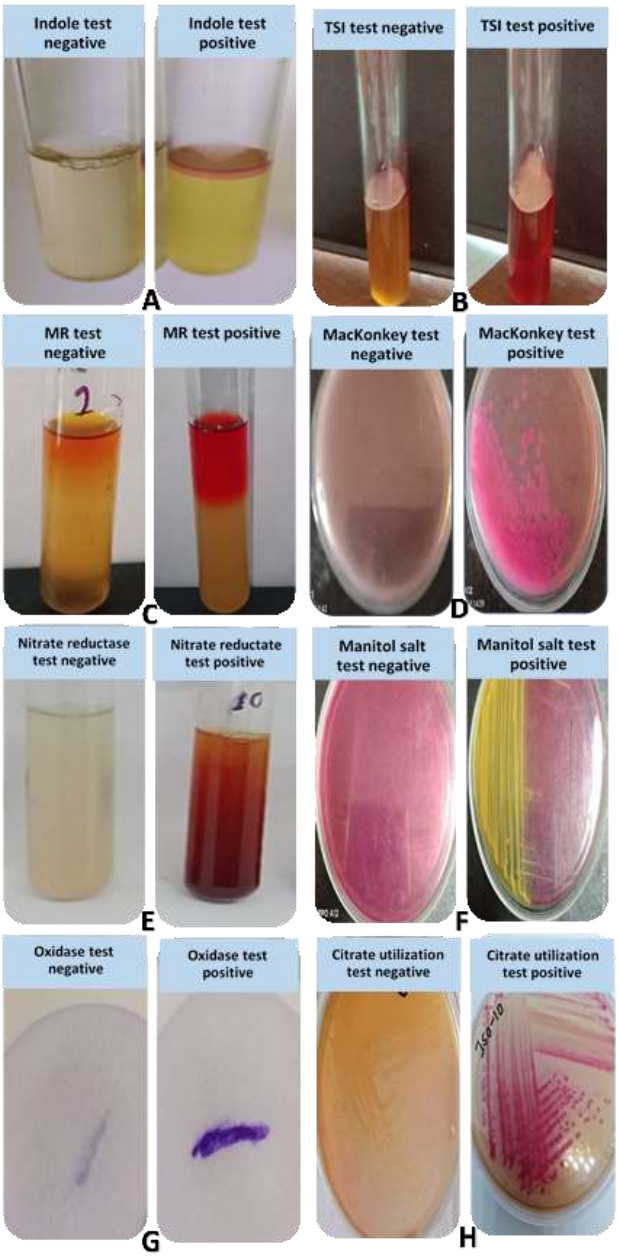

Figure 3: Figure showing the positive and negative outcomes of biochemical tests: Indole test (A), Triple sugar iron test (B), Methyl red test (C), MacConkey agar test (D), Nitrate reductase test (E), Mannitol salt test (F), Oxidase test (G) and Citrate utilization test (H).

Antibiotics susceptibility profiling of isolated bacteria

All the isolates were subjected to antibiotic susceptibility test against 9 commercially available antibiotics following CLSI guidelines (Figure 4). The obtained result revealed high level of multi-drug resistance among the isolates. It was observed that majority of the bacterial isolates were resistant to nitrofurantoin (66.66%), followed by doxycycline (55.55%), azithromycin (33.33%), oxytetracycline (22.22%) and amoxicillin (22.22%) (Table 4; Figure 5).

The resistance of gram-negative bacteria to nitrofurantoin is 100% followed by amoxicillin 50%. *E.coli*, a gram-negative bacteria showed multiple antibiotic resistance, namely nitrofurantoin, doxycycline, and amoxicillin. In the present study, none of the gram-negative isolates showed resistance against ciprofloxacin. This was different from other studies that have been performed in Bangladesh (Hasan et al., 2020). Among Gram-positive bacterial isolates, the most striking multiple drug-resistant isolates, which were resistant to 4 antibiotics, was *B. cereus* (isolate-2*)* followed by *L. macroides (*isolate-4*)*. On contrary, the only multi-resistant gram-negative bacterial isolate was *E.coli* (isolate-8).

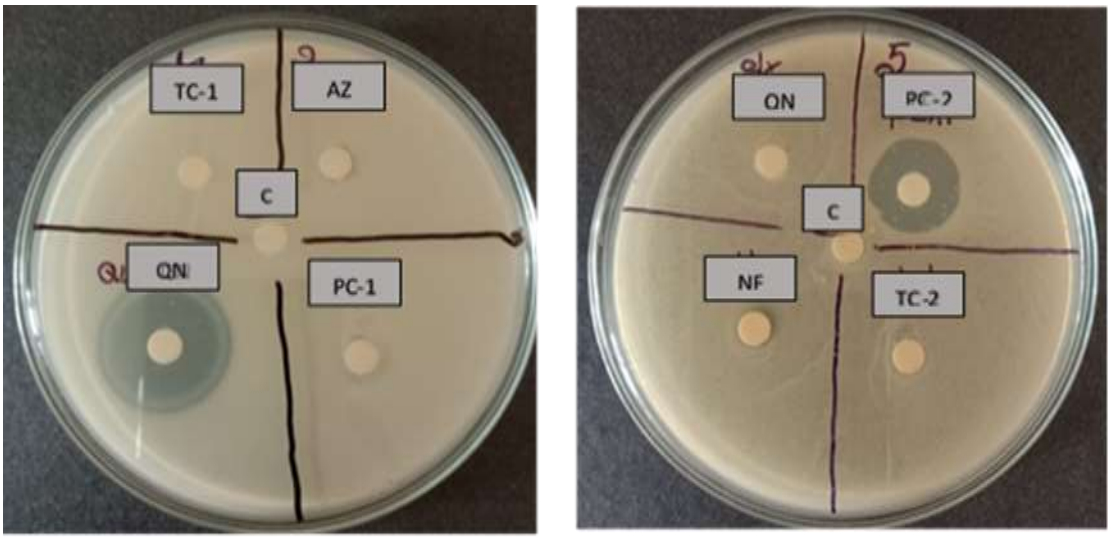

Figure 4. Antibiotic treatments against *B. cereus* (A) and *L. macrolides* (B). Here, TC: Tetracyclin; AZ: Azithro-mycin; QN: Quinolones; PC: Penicillin; NF: Nitrofuran and C: Control.

Table 4. Sensitivity of different bacterial isolates to different antibiotics.

Isolates	Antibiotics							
	Penicillin	Tetracycline	Nitrofuran	Clindamycin	Azithromycin	Quinolones	Tetracycline	Penicillin
P. aryabhattai	S	S	R	S	R	S	R	S
B. cereus	R	R	S	S	R	S	R	R
P. megaterium	S	S	R	S	R	S	S	S
L. macrolides	S	R	R	S	S	R	R	S
R. aquimaris	S	S	R	S	S	S	S	S
M. sciuri	R	S	S	S	S	S	S	S
B. wiedmannii	R	S	R	S	S	S	R	S
E. coli	S	S	R	S	S	S	R	R
C. freundii	S	S	S	S	S	S	S	S

Here, S= Susceptible and R= Resistant

Construction of evolutionary relationship among the isolates

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figure 6). There are 3 main clades in the tree. They are two *Bacillus* bacteria (clade1), two *Priestia* bacteria (clade 2), and *Citrobacter* and *Escherichia* (clade 3). *Rossellomorea*, *lycinibacillus,* and *mammalicoccus* are three outgroups. From the tree, we can see that *R. aquimaris* and *C. freundii* have gone through the most genetic change.

One of the main two branches of the tree is gram-negative (*C. freundii* and *E. coli*) and the other one is gram-positive which also supports our gram staining results.

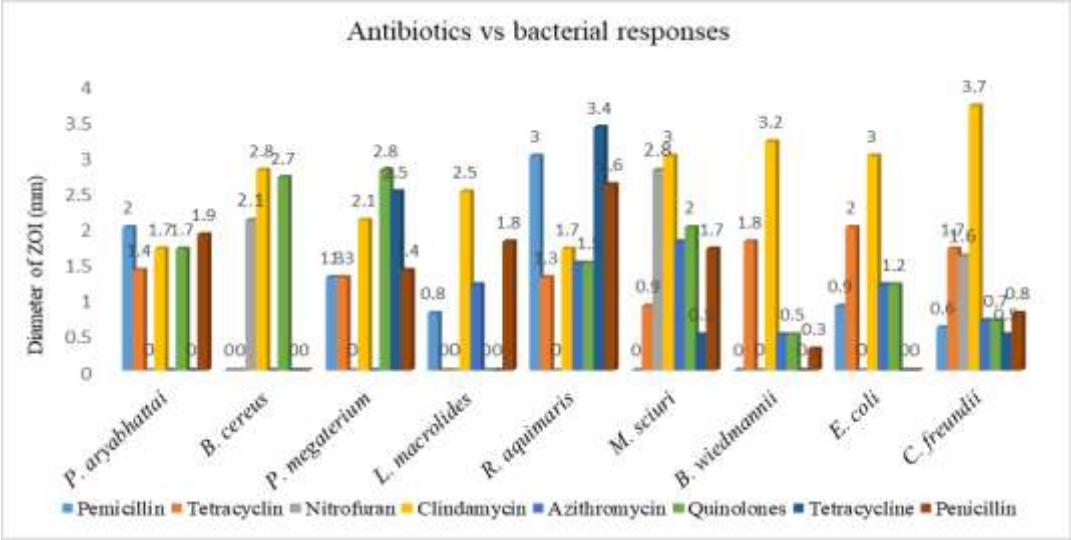

Figure 5. Bar diagram showing the responses of bacterial isolates against different antibiotics. Zone of inhibition (ZOI) was measured (mm) for antibiotic sensitive bacteria.

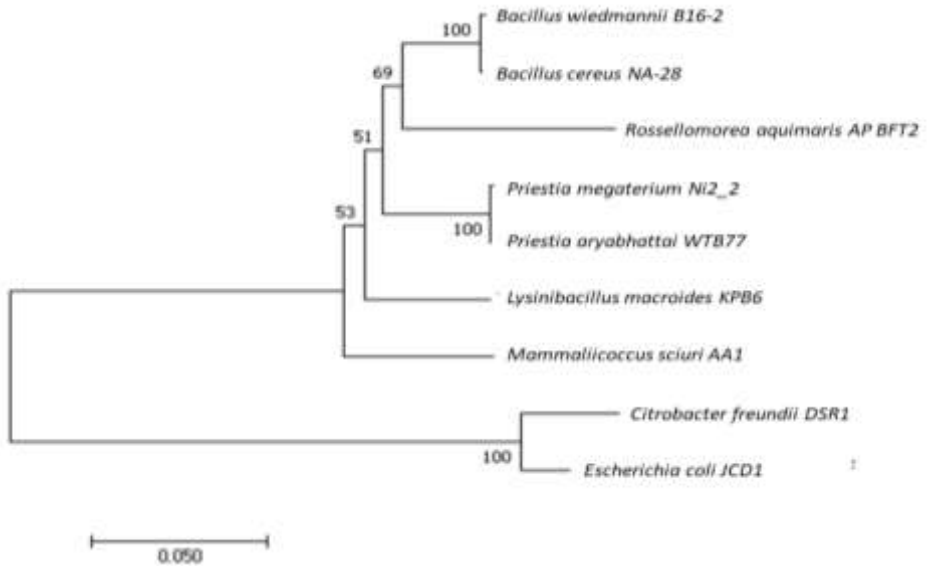

Figure 6. Evolutionary relationships of taxa. The optimal tree with the sum of branch length = 0.49176152 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Conclusion

We can infer from our experiment and the aforementioned data that the bacteria in the various poultry farms in the Khulna district have alarmingly evolved antibiotic resistance. Given that the majority of the chicken farms in our nation use excessive antibiotics, there is a great likelihood that the same situation will occur there as well. The majority of the bacteria we've discovered in our experiments are pathogenic, meaning they can result in serious

illnesses in people like meningitis, brain abscesses, endophthalmitis, pneumonia, gas gangrene, tropical ulcer formations, dermal and/or respiratory infections, mastitis, dermatitis, and exudative epidermitis, infections of the skin, soft tissues, CNS, and urinary tract, among other conditions. We found that the majority of the bacteria and their evolution are similarly based on sequencing analysis and phylogenetic relationships. This study's results are scientific evidence and could justify that excessive antibiotic usage can lead to produce several antibiotic-resistant bacteria. However, there is a need for further study on the antibiotic resistance genes and genetic relatedness of these isolates.

Authors' contributions

AH designed and prepared the proposal, analyzed data, wrote and revised the manuscript. SR conducted the field and laboratory work, analyzed data and revised the manuscript. MHP analyzed data, reviewed the manuscript and edited the language. AH and MH reviewed the manuscript, analyzed data and edited the language. All authors read and approved the final manuscript.

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Conflict of Interests

The author declares no conflict of interest.

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