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ORIGINAL ARTICLE

Prevalence of Antimicrobial Resistance Among the Hydrogen Sulfide Producing Bacteria Isolated on XLD Agar from the Poultry Fecal Samples

Maya Mathew[1] · Muhammed Afthab[1] · Sreejith S.[1] · Sandhya C.[2] · Jyothis Mathew[1] · Radhakrishnan E. K.[1]

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Abstract

Poultry products remain as one of the most popular and extensively consumed foods in the world and the introduction of hydrogen sulfide (H_2S) producing antibiotic resistant bacterial species into it is an emerging challenge. The current study has been designed to analyze the distribution of antibiotic resistance among the H_2S producing bacteria isolated from the fecal samples of chickens from different poultry farms. Here, twenty bacterial isolates were selected based on their ability to produce H_2S on XLD agar, and the16S rDNA sequencing was carried out for their molecular identification. The results showed the isolates as belong to *Salmonella* spp. and *Citrobacter* spp. and in the antibiotic susceptibility test (AST), three of the *Salmonella* strains were found to be resistant to antibiotics such as tetracycline, doxycycline, nalidixic acid, and amikacin. Also, fourteen *Citrobacter* strains showed resistance towards azithromycin, and furthermore, eleven of them were also resistant to streptomycin. Resistance towards tetracycline was observed among five of the *Citrobacter* strains, and seven were resistant to doxycycline. Further molecular screening by the PCR has showed three of the *Salmonella* strains along with eight *Citrobacter* isolates to have *tet*A gene along with four of the *Citrobacter* strains to have co-harbored bla_{TEM} gene. The results on biofilm formation have also demonstrated three *Salmonella* strains along with nine *Citrobacter* strains to have the ability to form moderate biofilm. The study thus describes the occurrence of H_2S producing multidrug-resistant bacteria in poultry feces, which might contribute towards the dissemination of antibiotic resistance genes to other microorganisms including human pathogens with likely risk to treat disease conditions.

Keywords Antimicrobial resistance · Hydrogen sulfide-producing bacteria · Biofilm formation · Tetracycline · Poultry

Maya Mathew and Muhammed Afthab contributed equally to this work.

✉ Radhakrishnan E. K.
radhakrishnanek@mgu.ac.in

[1] School of Biosciencess, Mahatma Gandhi University, Kottayam, Kerala 686560, India

[2] Department of Biochemistry, Kuriakose Elias College, Mannanam, Kottayam, Kerala 686561, India

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Introduction

Antimicrobial resistance (AMR) is becoming a global concern challenging the available treatment options for the infectious diseases. Usage of antibiotics for the prophylaxis and also to enhance the growth has been practiced in the poultry sector over a long period of time. The repeated and uncontrolled use of antibiotics in animal feed has already resulted in serious environmental and health risks due to the widespread dissemination of antibiotic resistance [1]. Even though the antibiotic resistance is enduring for a while, the inappropriate usage of antibiotics hastens the environmental out spread of antibiotic resistant bacteria [2]. The food-producing animal sectors have already become an AMR reservoir challenging the humans, animals, and the environment. These reservoirs could play a crucial role in the spread of AMR genes through the animal waste released [3].

The sulfate-reducing bacteria which are one of the oldest forms of bacterial species on earth utilize the inorganic sulfur substrates for anaerobic respiration and produce H_2S as the end product [4]. The H_2S produced can create offensive odor resulting in the malodorous emissions from the poultry and other animal livestock areas [5], and it has also been reported to protect the bacterial species from the mechanism of action of antibiotics [6]. At the same time, H_2S can also be produced through the biodegradation of antibiotics and hence is as an emerging beta-lactam metabolic marker for screening the antibiotic resistance among the bacterial species [7]. Among the H_2S producing bacterial species, *Salmonella enterica* serotypes are one of the most common causes of food borne diseases in humans [8]. Along with the *Salmonella* spp., *Citrobacter* spp. are also H_2S producers having significance to get considered as emerging resistance carriers. However, *Citrobacter* spp. are very less studied in the context of antimicrobial resistance with respect to the poultry settings [9, 10]. The presence of these resistant organisms in the poultry products can generate significant concern to the food supply chain up to the end consumers [1, 9]. The gut microbiome of various food-animals can act as the reservoirs of antibiotic resistant pathogens such as *Salmonella* spp., and these account for a major source for antibiotic resistance genes (ARGs) in livestock farms even though these are least investigated [11, 12]. Even if the biofilms have been evolved as organized forms to protect the bacteria from extreme environmental conditions [13], it has close association with drug resistance transmission as in *Salmonella* spp. [14]. Poultry originated *Salmonella enterica* is one of the most common food contaminants, owing to their broad range distribution and is a primary cause of gastroenteritis in humans [15]. Among the *Salmonella enterica*, non-typhoidal *Salmonella* is predominantly introduced into the food products from the intestine of animals [16]. Non-typhoidal *Salmonella* can cause gastroenteritis, vomiting, diarrhea, nausea, fever, and abdominal cramps. These bacteria can even cause invasive infections in people with immunocompromised systems [17]. At the same time, in an extensive observational study, *Citrobacter* spp. were reported to cause 0.8% of all Gram-negative infections and has also been identified as predominant pathogens in patients with underlying illnesses or immunocompromised conditions [18]. They can get introduced into the food animals and are frequently associated with the gastrointestinal tract of both humans and animals [19]. As the multidrug resistance including the extended spectrum beta-lactamase (ESBL) production is becoming common among bacteria, the distribution of these properties among the bacteria associated with the intestinal tract of animals are crucial to be considered. This is because the food-producing animals have

already been reported to be the potential source of ESBL genes [20]. In a previous study, transmission of ESBL from the intestine of food-producing animals to humans and thereby resulting infections with ESBL producing bacteria has also been reported [21].

The beta-lactam drugs are broadly used to treat the infections caused by Gram negative bacteria (GNB) in humans and animals, and hence the production of beta-lactamase among GNB generates significant challenges therapeutically [22]. The beta-lactam resistance is achieved mainly through the enzymatic inhibition, and the most common enzymes among *Salmonella* species produced for the same are extended spectrum beta-lactamase (ESBL) and restrict spectrum beta-lactamase (Amp C) [23]. At the same time, the broad-spectrum tetracyclines with activity against a range of Gram-positive and Gram-negative bacteria have been used commonly for the human infection therapy and also as veterinary medicine for the infection prevention and control [24]. Due to its wide use, the tetracycline resistance conferring *tet*A gene has been reported in our previous study to be commonly associated with the poultry sector [25]. At the same time, tetracycline resistance has also been reported to be provided by *tet*A, *tet*B, *tet*D, *tet*E, *tet*M, *tet*W, *tet*O and *tet*G genes. Majority of these genes can rapidly be transmitted from resistant bacteria to susceptible one through the horizontal gene transfer which thereby can make more bacteria to be resistant to the tetracyclines. As the bacterial infections can only be controlled therapeutically by using the antibiotics, the wide range transmission of resistance will have life threatening impact as the same process happens with all the antibiotics used for the prophylaxis and therapy. Broader dissemination of beta-lactam and tetracycline resistance has previously been reported to be mediated by the food producing animals [26, 27]. Even though *Salmonella* spp. and *Citrobacter* spp. are already known to cause malodorous in poultry food products due to H_2S production, only limited studies are reported on the resistance development in them. Hence, the current study focused on the occurrence of antibiotic resistant *Salmonella* spp. and *Citrobacter* spp. distributed in chicken feces collected from poultry farms. The findings from the study show the occurrence of H_2S producing bacteria to have multidrug resistance with associated occurrence of *tet*A and bla_{TEM} genes in many of them. This provides insights into the need for a detailed study to understand the association between H_2S production and antibiotic resistance.

Materials and Methods

Rappaport Vassiliadis broth (M1491) and XLD Agar (M031) used were purchased from HiMedia labs Mumbai. Nucleospin Microbial DNA extraction kit used was obtained from Machery-Nagel: (Cat. no. 740235.50). Antibiotic discs used were from HiMedia labs Mumbai which includes amikacin 30mcg (SD035), trimethoprim/sulfamethoxazole 25mcg (SD010), nalidixic acid 30mcg (SD021), doxycycline 10mcg (SD120), levofloxacin 5mcg (SD216), tetracycline 30mcg (SD037), chloramphenicol 30mcg (SD006), azithromycin 15mcg (SD204), amoxyclav 30mcg (SD063), tobramycin 10mcg (SD044), streptomycin 10mcg (SD031), cefuroxime 30mcg (SD061), cefotaxime 5mcg (SD040), ampicillin 10mcg (SD002), meropenem 10mcg (SD727), ertapenem 10mcg (SD280), imipenem 10mcg (SD073), and piperacillin/tazobactam 30/6 mcg (SD292E).

Sample Collection

The poultry fecal samples ($n = 30$) were collected from six different places of Kottayam, Kerala, India. Here, sterile scoops were used to collect fresh droppings of 6 to 40 day old chickens and were immediately transferred into 50 mL sterile tubes. The samples collected were immediately transported to the laboratory and kept under refrigerated conditions. From the samples, 1 mg was taken from each tube and inoculated into 10 mL of sterilized Rappaport Vassiliadis broth in triplicates and incubated for 18–24 h at 37 °C as per the previous report [28].

Isolation and Identification of Bacteria

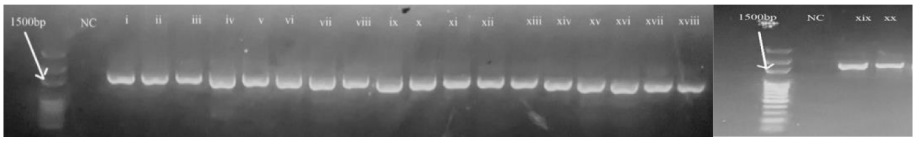
The above enriched culture was inoculated onto xylose lysine deoxycholate (XLD) agar followed by incubation at 37 °C for 18-72 h [29]. The H_2S producing colonies which appeared as black and pink with black center were selected for further studies. The selected isolates were further cultured in LB broth and incubated overnight at 37 °C and subjected to DNA extraction using NucleoSpin Microbial DNA Isolation Kit as per the manufacturer's instructions. Isolated DNA samples were further confirmed by agarose gel electrophoresis (AGE) and were then subjected to 16S rDNA amplification [30] using the universal primers 16SF (5′--AGAGTTTGATCMTGGCTC--3′) and 16SR (5′--AAGGAGGTGWTC CARCC--3′). The PCR was programmed for 35 cycles using Wee-32™ Thermal Cycler (HiMedia, Mumbai). The conditions used were initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 58 °C), and extension (2 min at 72 °C) with a final extension at 72 °C for 7 min. After the PCR, the product formation was confirmed by agarose gel electrophoresis. The products were further sequenced at AgriGenome Labs Pv.t Ltd. Kochi using Sanger sequencing, and the sequence data were further analyzed by BLASTn to identify its percentage similarity for identification [31].

Antimicrobial Susceptibility Testing

Here, a total of 20 bacterial isolates were tested against 18 antibiotics (amikacin, trimethoprim/sulfamethoxazole, nalidixic acid, doxycycline, levofloxacin, tetracycline, chloramphenicol, azithromycin, amoxyclav, tobramycin, streptomycin, cefuroxime, cefotaxime, ampicillin, meropenem, ertapenem, imipenem, and piperacillin/tazobactam) on sterilized Mueller Hinton agar plates by inoculating the culture and introducing the antibiotic discs followed by the observation of zone of inhibition after 24-h incubation at 37 °C as per the Clinical Laboratory Standards Institute (CLSI) guidelines. A heatmap was generated in Morpheus online software to generate dendrogram showing heatmap signatures of the isolates to determine the resistant, intermediate, and susceptible categories of the bacterial isolates studied.

Molecular Screening of Selected Bacteria for *bla*$_{TEM}$ and *tet*A Genes

The genomic DNA extracted from the selected bacterial isolates were screened to detect the presence of *bla*$_{TEM}$ and *tet*A genes. For the *bla*$_{TEM}$ gene amplification, the previously described primers were used which included *bla*$_{TEM}$-F (5′--ATAAAATTCTTGAAGACG



A B

Fig. 1 Agarose gel analysis of PCR amplified 16S rDNA of selected bacteria from poultry feces. **A** (Ladder; NC, negative control; i, S1A3; ii, S4B2; iii, S1E3; iv, S3B1; v, S3B2; vi, S3C1; vii, S3D3; viii, S3F1; ix, S5E2; x, S4B2a; xi, S3F2; xii, S4C3; xiii, S3B3; xiv, S4C2; xv, S3C2; xvi, S3F3; xvii, S4B2b; xviii, S3E3); **B** (Ladder; NC, negative control; xix, S4C1; xx, S3D1)

AA--3′) and bla_{TEM}-R (5′--GACAGTTACCAATGCTTAATC--3′) [32]. Here, the PCR was programmed for 30 cycles with initial denaturation at 94 °C for 5 min followed by cyclic denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, and cyclic extension at 72 °C for 60 s followed by the final extension at 72 °C for 10 min. Similarly, for the *tet*A gene amplification, *tet*A-F (5′--GGCGGTCTTCTTCTTCATCATGC--3′) and *tet*A-R(5′--CGG CAGGCAGAGCAAGTAGA--3′) primers were used as described before [33]. The PCR was programmed for 35 cycles with initial denaturation at 94 °C for 5 min, cyclic denaturation at 94 °C for 30 s, annealing at 62 °C for 60 s, and cyclic extension at 72 °C for 30 s and with the final extension at 72 °C for 5 min [33, 34]. The amplicons formed in both the PCR reactions were separated on 1.2% agarose gel and sequenced at AgriGenome Labs Pvt Ltd., Kochi using Sanger sequencing, and the results were analyzed by Blastx.

Screening of Selected Bacteria for the Biofilm Formation

Here, overnight grown bacterial broth cultures were transferred in to fresh 10 mL of Trypticase Soy Broth (TSB). The optical density (O.D.) was adjusted to be equivalent to 0.5 McFrland standard, and the cultures were diluted as 1:100 with fresh sterile TSB. From this, 0.2 mL aliquots of each bacterial suspension was added into the wells of 96-well sterile tissue culture plate in triplicates along with sterile media as control. After the incubation at 37 °C for 24 h, the contents of each of the wells were removed carefully, and the wells were washed four times with 0.2 mL of phosphate-buffered saline (PBS, pH 7.2) to remove the free bacteria. The plates were then stained with 0.2 mL of (w/v) 0.1% crystal violet stain and were further incubated at room temperature for 15 min. Each well was further washed repeatedly with PBS to remove the excess stain. The plates were then dried, and 200μL of 90% ethanol was added into each of the wells, and after shaking for a few minutes, the O.D. readings were taken at 570 nm using ELISA plate reader (Bio Rad imark) [35, 36].

Table 1 Summary of 16S rDNA sequence based identification of 20 bacteria isolated in the study

Sl. no	Isolate ID	Identified as	Closest NCBI Accession no	Percentage of similarity	Accession no. of sequences submitted
1	S3F2	*Citrobacter* sp.	BBMW01000025.1	98.01	OP848186
2	S3F3	*Citrobacter* sp.	BBMW01000025.1	100	OP848187
3	S3E3	*Citrobacter* sp.	AJ233408.1	99.71	OP848188
4	S3B3	*Salmonella enterica* sp.	EU014685.1	99.39	OP848189
5	S3F1	*Salmonella enterica* sp.	EU014688.1	100	OP848190
6	S3B1	*Citrobacter* sp.	BBMW01000025.1	97.66	OP848191
7	S3B2	*Citrobacter* sp.	AJ233408.1	99.79	OP848192
8	S3D1	*Salmonella enterica* sp.	EU014688.1	99.56	OP848193
9	S1A3	*Citrobacter* sp.	MN548424.1	100	OP848194
10	S1E3	*Citrobacter* sp.	MN548424.1	99.05	OP848195
11	S4B2a	*Citrobacter* sp.	MN548424.1	99.37	OP848196
12	S4B2	*Citrobacter* sp.	MN548424.1	99.87	OP849664
13	S3C1	*Citrobacter* sp.	BBMW01000025.1	98.46	OP848197
14	S5E1	*Citrobacter* sp.	MN548424.1	99.08	OP848198
15	S5E2	*Citrobacter* sp.	MN548424.1	98.99	OP848199
16	S3C2	*Citrobacter* sp.	BBMW01000025.1	99.22	OP848200
17	S4C2	*Citrobacter* sp.	MN548424.1	99.25	OP848201
18	S4C1	*Citrobacter* sp.	FLYB00000000.3	98.75	OP848202
19	S4B2b	*Citrobacter* sp.	MN548424.1	100	OP848203
20	S4C3	*Citrobacter* sp.	NAEW01000064.1	99	OP848204

Result

Isolation and Identification of Bacteria

From the broiler chicken fecal materials, a total of 20 bacterial isolates which appeared as black and pink colored with black center on XLD agar were selected for DNA extraction followed by 16S rDNA PCR for the identification. The gel analysis of PCR products showed the presence of products with a 1500 bp size range (Fig. 1). The sequenced data of PCR products when analyzed using NCBI BLAST gave their percentage of similarity to the 16S rDNA sequences of bacteria available in the database (Table 1). Among the 20 isolates, 3 were identified as *Salmonella* spp. and 17 were identified as *Citrobacter* spp.

Antimicrobial Susceptibility Testing

Here, all the 20 bacterial isolates were tested against 18 antibiotics. Among these, 8 isolates (S3F1, S3F2, S3F3, S3C1, S3C2, S3B1, S3B2, and S3B3) were found to be multidrug resistant against doxycycline, nalidixic acid, and ampicillin. The resistance, sensitivity, and intermediate responses of all the isolates against the 18 antibiotics were plotted as a heatmap (Fig. 2). Here, seven of the isolates (S3F1, S3F2, S3F3, S3C1, S3C2, S3B2, and S3B3) were found to be resistant to tetracycline and azithromycin. Four isolates (S3F2, S3C1, S3B2, and S3F1) were identified to be resistant to levofloxacin, while another four

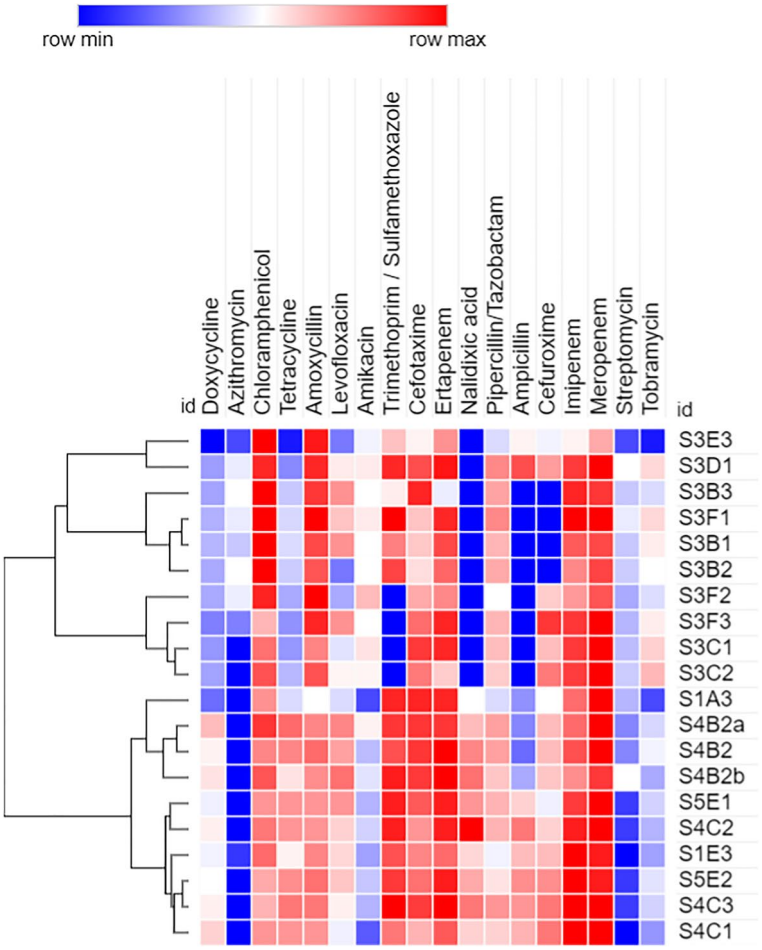


Fig. 2 Heatmap showing the response of 18 antibiotics (*X* axis) against 20 selected bacterial isolates (*Y* axis). Responses were marked as blue and light blue for the resistant, white and light peach for the intermediate resistant and dark peach to red for the susceptible

(S3F2, S3F3, S3C1, and S3C2) were resistant to trimethoprim/sulfamethoxazole, and four other isolates (S3B3, S3F1, S3B1, and S3B2) were resistant to cefuroxime. The percentage of isolates resistant to each antibiotic was plotted in a graph with resistance, intermediate resistance, and sensitive which were highlighted in different colors (Supplementary Fig. 1).

Molecular Screening for *bla*TEM and *tet*A gene

Here, four *Citrobacter* strains (S3F2, S3F3, S3C1, and S3C2) were found to harbor *bla*TEM resistance gene due to the formation of PCR product with 850 bp size corresponding to the amplicon of the *bla*TEM gene. The Blastx analysis of its sequences further confirmed it to have 100% identity with the beta-lactamase TEM gene reported from

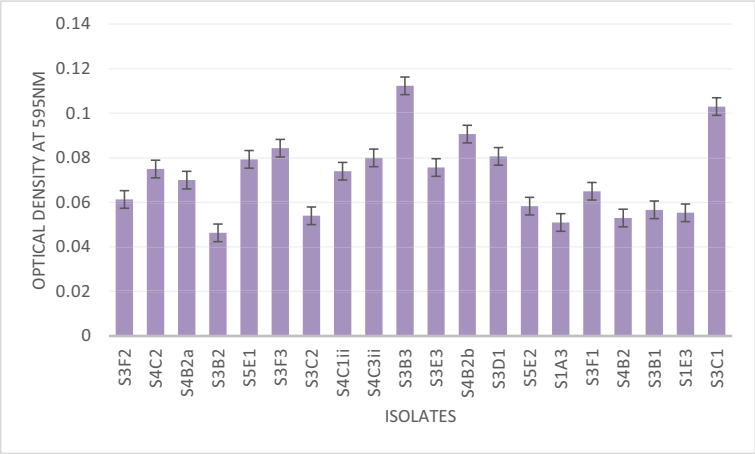


Fig. 3 Biofilm formation by bacteria isolated in the study as analyzed by tissue culture plate assay

other organisms including those with accession numbers KSZ04438.1 (Class A beta lactamase [*Klebsiella pneumoniae*]), AMM70781.1 (TEM family class A beta-lactamase [*Escherichia coli*]), KSZ04438.1 (Class A beta lactamase [*Klebsiella pneumoniae*]), and AXH80245.1 (beta-lactamase TEM-1 variant [*Escherichia coli*]) respectively. Among the 20 isolates, the t*et*A gene was found to be present in 11 isolates due to the formation of PCR product of 501 bp size. Here also the Blastx analysis of sequence data showed it to have similarity percentage of 100 with t*et*A family of tetracycline resistance MFS efflux pump. The *tet*A gene sequences of *Citrobacter* strains S3F2, S3F3, S3B1, S3C1, and S3C2 were found to have maximum identity with accession numbers EJS83793.1 (TetA, partial [*Pasteurella multocida*])QUW44562.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]), QUW44583.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]), ALZ59061.1 (tetracycline efflux protein TetA [*Shigella sonnei*]), and QUW44564.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]), respectively. In the case of *Salmonella enterica* strains S3F1 and S3D1 the *tet*A gene sequences were found to have maximum identity with accession numbers QUW44564.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]) and ALZ59061.1 (tetracycline efflux protein TetA [*Shigella sonnei*]), likewise in *Citrobacter* strains S3E3 and S3B2, *tet* A was found to have maximum identity with accession numbers ACP28869.1 (tetracycline resistance protein class A, partial [*Escherichia coli*]) and QUW44562.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]). In the same way, *Salmonella enterica* strain S3B3 and *Citrobacter* strain S4B2a were found to have *tet*A gene with maximum identity towards accession numbers ACP28869.1 (tetracycline resistance protein class A, partial [*Escherichia coli*]) and QUW44562.1 (tetracycline efflux MFS transporter TetA, partial [*Escherichia coli*]) (Supplementary Table 1).

Biofilm Formation

Here, the bacterial isolates with O.D. value > 0.12 were considered as strong biofilm producers [37]. Those with values between 0.06 and 0.12 were moderate biofilm producers

and with less than 0.06 were non-biofilm producers. Among the 20 isolates, 12 were moderate biofilm producers (Fig. 3), and 8 were weak biofilm producers.

Discussion

Uncontrolled use of antibiotics in the food-producing animals has recently been identified to have life-threatening impact on public health. The repetitive usage of antibiotics in various forms can enrich the animal gut microbiota with the resistance genes, and hence resistant bacteria can get released from animals to the environment continuously. The extent of multidrug resistant pathogens disseminated through the food-producing animals indicates the need for effective implementation of guidelines for the proper administration of antibiotics. The practice of buying poultry meat from freshly slaughtered wet markets also increases the risk of cross contamination of meats with antibiotic resistant bacterial species [38]. An extensive understanding about the antibiotics administered in the poultry sector can hence reflect the magnitude of resistance genes transmitted from poultry to the environment. In the past decade, MDR pathogens were significantly studied in Europe (EFSA, 2019) and sub-Saharan Africa [39]. Bacterial metabolic features can also have varying impact on AMR evolution and dissemination. In a previous study, H_2S producing bacteria have already been reported from the poultry sector [40]. Such organisms have also been reported to have the mechanisms for protecting themselves from the antibiotic mediated cell disruption [41]. In another study, the biogenesis of H_2S has been reported to be linked with the bacterial resistance development against different classes of antibiotics including beta lactam group [42]. In a previous study, both the endogenously produced H_2S and the exogenously administrated sulfide salts were demonstrated to provide protection against a wide range of antibiotics [43]. As a confirmatory to such observation, *P. aeruginosa* PA14 H_2S mutant strain has experimentally been proven to be sensitive to antibiotics even though the wild strain was antibiotic resistant [43]. However, detailed mechanistic insight into the same is limiting. As per the available information, H_2S has also been reported to react with the reactive oxygen species produced by bacteria under antibiotic stress [41]. It has also been reported that H_2S may likely to activate genes associated with antibiotic resistance by functioning as a signaling molecule [44].

In another study conducted on bacteria from the poultry products, resistance towards multiple antibiotics was found to be high in H_2S producing *Salmonella* spp. [45]. In another study, the H_2S producing *Citrobacter* spp. from chicken have also been reported to have associated with multiple antibiotic resistance. This indicates the dissemination of antibiotic resistance from H_2S producers to the environment which ultimately makes the treatment of diseases to be highly challenging. Since the XLD agar is used as a selective medium for the H_2S producing *Enterobacteriaceae*, the current study has used the same for the isolation of bacteria from poultry feces. Further to this, antibiotic resistance pattern of selected bacterial species was performed along with the identification of selected antibiotic resistance genes.

Among the 20 bacterial isolates, three *Salmonella enterica* strains S3B3, S3F1, and S3D1 showed complete resistance towards doxycycline, tetracycline, nalidixic acid, and amikacin. The results were comparable to the report on *Salmonella enterica* serovar *Indiana* and *California* isolates studied previously from chicken samples [46]. From *Citrobacter* spp., fourteen (S3F3, S3E3, S3B1, S1E3, S4B2a, S4B2, S3C1, S5E1, S5E2, S3C2, S4C2, S4C1, S4B2b, and S4C3) displayed resistance towards azithromycin which is comparable to the resistance of *Citrobacter* spp. reported in a previous study from Ethiopia [47]. Also, seven *Citrobacter*

strains (S3F2, S3F3, S3E3, S3B1, S3B2, S3C1, and S3C2) displayed resistance towards nalidixic acid and doxycycline. No other studies so far showed *Citrobacter* spp. to be resistant to doxycyclines even though first-generation tetracycline resistance was reported in many along with resistance to nalidixic acid [48]. The resistance towards levofloxacin was observed in two *Salmonella enterica* strains S3F1 and S3D1 and five *Citrobacter* strains S3F2, S3E3, S3B2, S3C1, and S3C2 and similar observations were reported previously in a study conducted on chicken meat isolates from Iraq. The study pointed out the resistance towards levofloxacin in *Salmonella enterica* sp. only, whereas no significant data was found on the same towards *Citrobacter* spp. [49]. In a study conducted to explore the diversity and antibiogram of *Salmonella enterica* isolated from different species of poultry in India, high resistance to antibiotics such as ampicillin, nalidixic acid, and tetracycline have been reported to be prevalent in the poultry sector [50]. In another study on the antimicrobial resistance of *Citrobacter* spp., high prevalence of resistance towards aminoglycosides, tetracyclines, sulfonamides, penicillin, and quinolones has been reported [51]. The resistance observed among *Salmonella* and *Citrobacter* strains isolated in this study are comparable with the results of previous studies [47, 48 & 50]. Another study has also reported the frequent occurrence of MDR pathogens in the poultry sector due to the administration of amikacin and azithromycin [52] which is similar to the results observed in the current study. The resistance towards tetracycline is a key concern in the poultry sector since the feed is usually supplemented with it [25]. From the results of the current study, resistance towards the first and second-generation tetracyclines was observed to be present at an alarming magnitude. Screening for the *tet*A gene as conducted in the study has revealed its presence in eleven of the isolates (S3F2, S3F3, S3E3, S3B3, S3F1, S3B1, S3B2, S3D1, S4B2, S3C1, and S3C2) and at the same time the antibiotic susceptibility test of these isolates against tetracycline indicated eight (S3F2, S3F3, S3E3, S3B3, S3F1, S3D1, S3C1, and S3C2) to have resistance, two (S3B1 and S3B2) to have intermediate resistance and one (S4B2) to have no resistance.

In this study, the resistance observed for *Citrobacter* spp. towards ampicillin, cefuroxime, and cefotaxime was 50, 40, and 30%, respectively, and was in accordance with the results reported previously [51]. Based on the results of antibiotic susceptibility test, a heatmap with dendrogram was generated to comprehend the magnitude of data in two dimensions as described before [53]. The overall trend of resistance observed in the current study is highlighting the prevalence of tetracyclines, ampicillin, amikacin, nalidixic acid, and doxycycline resistance and is in par with the previous reports on *Salmonella* and *Citrobacter* spp. [51, 52]. The study also focused on the biofilm formation properties of the selected 20 isolates as the antibiotic resistance has also been reported to be related to the biofilm formation [54]. From the results of the study, two *Salmonella enterica* isolates (S3F1 and S3D1) along with two *Citrobacter* spp. isolates (S3F3 and S3E3) were found to be moderate biofilm forming, and these were resistant to doxycycline, azithromycin, tetracycline, and nalidixic acid along with 80% resistance towards levofloxacin. The *Salmonella* spp. S3F1 also exhibited resistance towards ampicillin. The biofilm formation in these isolates may favor rapid dissemination of resistance and acquisition of resistance towards other antibiotics leading to difficult-to-treat infections.

The study highlighted the occurrence of antibiotic resistance among bacteria like *Citrobacter* spp. which are the least investigated agents disseminating environmental AMR. At the same time, increasing resistance observed among the pathogens of *Salmonella* spp. as observed in the study generates concern with the management of foodborne diseases caused by them. The results of the study also indicate the importance of monitoring and controlling the prophylactic usage of antibiotics in the poultry sector.

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Author Contribution Maya Mathew: conceptualization, methodology, data curation, original draft preparation. Muhammed Afthab: methodology, data curation. Sreejith S.: methodology. Sandhya C.: supervision. Jyothis Mathew: supervision. Radhakrishnan E.K.: supervision, conceptualization, methodology, validation, reviewing and editing. All authors approved the final manuscript.

Data Availability All data generated or analyzed during this study are included in this article.

Declarations

Ethical Approval This study was performed in line with the principles of the Institutional ethical committee. Approval was granted by IBSC/RCGM with no. IBSC/10/2022 dated 04/07/2022.

Consent to Participate This is not applicable because this study does not involve human participants.

Consent for Publication This is not applicable because this study does not involve human participants.

Competing Interests The authors declare no competing interests.

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