Title: Insights into Bacterial Communities: Multi Drug-Resistant and Biofilm-Forming Bacteria in Poultry Droppings

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Abstract

An ever-increasing demand for high-quality protein sources like poultry products along with excessive use of antibiotics in the animal husbandry are contributing factors to the growing global problem of antimicrobial resistance (AMR). The overuse of antibiotics in poultry and dissemination of poultry fecal waste in environment result in propagation and spread of multidrug-resistant (MDR) bacteria. We investigated bacterial diversity within poultry droppings by targeted amplicon sequencing and determined resistance pattern of 165 bacterial isolates to a range of antibiotics. We found species of the genera *Enterobacter*, *Enterococcus*, *Escherichia*, *Proteus*, *Bacillus*, and *Shigella* showed resistance against Beta-lactams, Cephalosporins, Fluoroquinolones, Sulphonamide, Nitrofurantoin, Polymyxin E, and Aminoglycosides. In addition, we detected strong biofilm-producing isolates of genus *Enterobacter*, *Bacillus*, *Proteus*, *Escherichia*, and *Enterococcus*. The detection of biofilm forming MDR bacteria in poultry droppings highlights the need for proactive measures to mitigate their growth and transmission. High throughput sequencing revealed the differential prevalence of amplicon sequence variants (ASVs) belonging to *Lactobacillus*, *Corynebacterium*, and *Bacteroides*. Functional imputations support the observed potential of biosynthesis of divergent antibiotics and drug resistance. Our findings highlight that poultry droppings harbor a diverse array of antibiotics resistant bacteria, underscoring the significance of continuous surveillance and appropriate disposal methods to counteract the escalating problem of multi drug resistance under the One Health approach.

Keywords

Poultry, Antimicrobial resistance, Biofilm, Colistin resistance, One Health

Introduction

Antimicrobial resistance (AMR) is one of the most important public health issues worldwide affecting both human and animal health sectors. Indiscriminate usage of antibiotics in animal husbandry and unregulated ad hoc disposal of inadequately treated waste effluents in the environment are contributing factors to the local and global spread of AMR[1]. India has been one of the world's largest consumers of antibiotics, with around 5071 million defined daily doses (DDD) of antibiotics consumed in 2015[2,3]. An ever-increasing demand for high quality protein products like milk, poultry, meat and fish has led to unregulated use of antibiotics in the animal production sector in India[2]. It contributes to continuing the spread of AMR, from animal food sources to the humans and environment at large, by the farm to fork approach. Widely used antibiotics in poultry feed include aminoglycosides, beta-lactams, colistin, quinolones, sulfonamides and tetracyclines[4].

The use of antibiotics in poultry feed in the conventional poultry farming practices, to assure flock health, growth promotion and prophylaxis of diseases, has led to rise in AMR worldwide[5]. Thus, the recent methods adopted by the nations under the One Health Approach, to curb the growing AMR throughout the world, use antibiotic free production (ABF) or alternative to antibiotics like probiotics, prebiotics, essential oils, enzymes, etc. in the poultry production[6]. The overuse of antibiotics in poultry production and dissemination of poultry fecal waste by direct land dumping in the environment can result in propagation and spread of multidrug-resistant (MDR) bacteria[7]. The biofilm formation potential of the bacterial strains, poses an additional health risk to humans and animals by increasing the AMR and development of fatal infections.

The World Health Organization (WHO) emphasizes surveillance studies due to the critical importance of monitoring the significant impact of AMR, often termed a silent pandemic. Considering the pivotal role of surveillance in combating AMR, our current investigation focuses on assessing the prevalence of antibiotic-resistant bacteria and their ability to form biofilms in conventionally raised poultry using culture-dependent approach. Additionally, to explore the microbial communities linked to poultry droppings in greater detail, we integrated targeted amplicon sequencing. Previous studies have highlighted the need to employ both culture dependent and culture independent approaches to attain a comprehensive understanding of the microbial diversity.

Materials and methods

Sample collection

Poultry dropping samples were collected in triplicates from five conventional poultry farms growing broiler chickens (JPA, JPB, JPC, JPD and PP) around Pune, India. Samples were collected aseptically in sterile gamma irradiated tubes and immediately processed for the isolation and community DNA extraction.

Isolation of bacteria and identification

Bacterial isolation was performed using serial dilution method on Mueller Hinton Agar (Himedia, India) plates. Distinct bacterial colonies were purified after incubating the plates at 37°C for 48 hours and further processed for the molecular identification targeting the 16S rRNA gene. For the molecular identification, genomic DNA extraction was performed by the standard phenol-chloroform method followed by PCR amplification of the 16S rRNA gene as described previously[8]. The amplified products were purified and sequenced on the 3730xl Genetic Analyzer platform (Applied Biosystems, USA) available in-house[9]. The taxonomic assignment was achieved using the EzBiocloud server[10]. The 16S rRNA gene sequences have been deposited at the GenBank (accession

numbers OP027886-OP028050) and the pure bacterial isolates were preserved in glycerol stocks at the National Centre for Microbial Resource- National Centre for Cell Science for further use[11].

Tests for antimicrobial susceptibility and biofilm formation

Antimicrobial susceptibility testing was performed using the disc diffusion method to determine resistance of the bacterial isolates against 12 antibiotics mentioned in supplementary Table S1 (Dodeca Universal II disc, HiMedia, India). The resistance and sensitivity of the bacterial isolates was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[12,13]. The isolates belonging to *Enterobacterales* showing resistance to colistin were further tested using the standard Colistin Agar Test as described[12]. Further, the biofilm formation of the bacterial isolates was assessed using the modified microtitre plate test in accordance with Stepanović[14]. Biofilm formation by the isolates was tested using tryptone soy broth containing 1% glucose. The resultant biofilms were stained using 0.1% crystal violet solution, followed by washing and air-drying. The dye bound to the cells was resolubilized with 30% glacial acetic acid, and the optical density (O.D.) was measured at 570 nm using SpectraMax Plus 384 Microplate reader (Molecular Devices, USA)[14].

Targeted amplicon sequencing

The community DNA from poultry dropping samples, was extracted using Qiagen's QIAamp PowerFecal Pro DNA kit (Qiagen, The Netherlands). The concentration and quality of the DNA was checked using Nanodrop One Spectrophotometer (Thermo Scientific, United States). Targeted amplification of the V4 region of the 16S rRNA gene using the specific primer set 515F and 806R was carried out. Subsequently, the library preparation and amplicon sequencing were carried out on the in-house Illumina MiSeq platform (California, USA) as described previously[15]. The raw sequences generated in this study were submitted to NCBI SRA under BioProject ID PRJNA804333.

Bioinformatics and statistical analysis

The resultant raw reads, generated from the Illumina high-throughput sequencing, were processed by correcting amplicon error till the clustering amplicon sequence variant (ASV) table using the DADA2 package v1.16 in R environment[16]. Taxonomic assignments were performed using SILVA 138.1 database (https://www.arb-silva.de/documentation/release-1381/). Further downstream analysis was performed in R using R-packages ggplot2, phyloseq, microbiome and tidyverse. The bacterial communities associated with the poultry droppings were also examined for their potential of harbouring drug resistance genes by using PICRUSt2 pipeline[17].

Results and Discussion

According to the WHO, the continuous surveillance of antimicrobial resistance (AMR) is a vital step in preventing the drastic rise of deaths caused by AMR, currently estimated at 4.95 million annually[18]. The development of bacterial biofilms, triggered by quorum sensing, can be detrimental to various industries, including healthcare and food production[19]. Low to middle-income countries (LMIC) are particularly affected by AMR due to the overuse of antibiotics in animal production which can then be passed on to humans. It is essential to monitor the spread of AMR-causing pathogens in order to reduce mortality rate and prevent the projected 10 million deaths predicted by 2050[18]. It is important for developing nations to shift to eco-friendly methods, of utilizing alternatives to antibiotics or antibiotic free poultry (ABF) production under the One Health approach, rather than the conventional production practices of depending on antibiotics, to reduce the development of AMR which eventually leads to increase in mortality rates in infected humans and animals causing socio- economic losses[6].

Culture dependent study

The 16S rRNA gene-based identification of cultured isolates revealed the presence of genera *Shigella, Escherichia, Bacillus, Enterobacter, Enterococcus, Staphylococcus, Proteus, Klebsiella, Limosilactobacillus* and *Microbacterium* as described in the supplementary table S2. The members of the Gram-positive family *Enterococcaceae*, such as *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Enterococcus durans* and *Enterococcus casseliflavus* are known for their presence in poultries[7]. The Gram-negative bacterial isolates from poutry, *Shigella* sp. and *E. coli*, which commonly cause diarrhoea and urinary tract infection, were found to harbour the extended-spectrum beta-lactamases (ESBLs) of SHV type while *Klebsiella pneumoniae* has been found to contain Amp-C type beta-lactamases and ESBLs[20,21]. These bacteria are thus considered as potential pathogens due to their ability to resist antibiotics. New Delhi Metallo-β-lactamase- 1 (NDM- 1) was first identified in *Klebsiella pneumoniae* and *Escherichia coli* followed by *Enterobacter cloacae* and *Proteus mirabilis* of the *Enterobacteriaceae* family[22]. The detection of these Gram-negative bacteria, belonging to the priority pathogens list by the WHO, correlates to the emergence of AMR pathogens which is possibly a reason of uncontrolled use of antibiotics in poultry and other animal production sectors. Members of the *Staphylococcaceae* family, such as *Staphylococcus arlettae* isolates identified in our study have also been reported in poultry from Belgium in 1984 and found to be resistant to Novobiocin and Beta lactam antibiotics[23,24]. Many staphylococci, including *S. epidermidis* known as potential AMR pathogens were prevalently found in chickens[25]. Further, *Bacillus* sp. identified in the current study are well known for their presence in poultry wastes[26]. The presence of these isolates depicts the enrichment of potential multidrug resistant bacteria in poultry and also highlights the associated human health risk due to its plausible transmission to different tropical levels via consumption of contaminated meat.

Antimicrobial susceptibility testing and biofilm formation

The bacterial isolates assessed for their antimicrobial resistance pattern showed resistance towards various classes of antibiotics including the class Polymyxin E containing the last resort antibiotic, colistin. Bacteria belonging to the phylum *Pseudomonadota* and *Bacillota* were found to follow the decreasing order of resistance towards the antibiotics. Isolates belonging to genera *Bacillus*, *Enterobacter, Shigella, Escherichia, Proteus, Klebsiella* and *Staphylococcus* showed resistance towards majority of the antibiotics. We reported the presence of *Escherichia* sp. and *Klebsiella* sp. resistant to colistin, fluoroquinolones and third-generation cephalosporins as noticed in the earlier study[2]. Isolates belonging to *Bacillaceae* family such as *Bacillus cereus* and other *Bacillus* sp. were found to be resistant to beta lactams, sulphonamide, and aminoglycosides[27]. Enterococci were reported to be intrinsically resistant to Amikacin, Gentamicin, Netilin, Co-trimoxazole, Ceftriaxone, and Cefotaxime antibiotics as per CLSI guidelines[12] however we observed antibiotic susceptibility in the enterococcal strains.

Colistin agar test, performed for the isolates belonging to the *Enterobacteriaceae* family showed Colistin resistance among *Enterobacter cloacae*, *Escherichia fergusonii, Escherichia marmotae, Klebsiella pneumoniae, Proteus mirablis* and *Shigella flexneri* (supplementary Table S3). The increased oral administration of colistin in poultry farms, for the prevention of infections and growth promotion are reported as the driving factors for colistin resistance in members of *Enterobacteriaceae* like *Escherichia* sp. and *Klebsiella* sp.[28]. The presence of plasmid mediated genes, like the mobilized colistin resistance (mcr) genes in the pathogenic Gram-negative bacilli and their transfer via horizontal gene transfer, has been reported for development and spread of colistin resistance in the animals, humans, tertiary care hospitals and the environment[29].

The Gram-positive bacteria lack an outer membrane and are thus intrinsically resistant to colistin. It is in agreement with our data for the strains of *Bacillus, Enterococcus* and *Staphylococcus* that showed resistance towards this last resort antibiotic at ≥4 μg/mL concentration[30]. Though colistin does not possess antibacterial activity against the Gram-positive bacteria as described in previous studies[31], but it has been proven to cause intensive oxidative damage by enhancing the NADH metabolism in *Bacillus* sp.[32]. Thus, expanding our knowledge about the antibacterial mode of action of colistin by inhibition of the respiratory chain in the Gram-positive bacteria.

Additionally, poultry farms have also been described as sources of antibiotic resistance genes (ARGs) of aminoglycoside and sulfonamide classes of antibiotics[33]. Due to the presence of ESBLs, resistance against cell wall synthesis inhibitors like cefotaxime and other cephalosporin drugs has been observed in members of the family *Enterobacteriaceae*[34]. The presence of NDM-1 aids hydrolysis of penicillins, cephalosporins and carbapenems enabling escape of bacterial strains against the action of these antibiotics[22]. *Staphylococcus arlettae* has been found to produce novel beta-lactamase bla_{ARL} making it extensively resistant to Penicillin whereas *Staphylococcus epidermidis* has been found to contain ARGs against 9 antibiotic classes that might pose a serious challenge to safeguard the public health[24,25].

Biofilm formation, known to provide protection to bacteria against the harsh environmental conditions and various antibiotics, is an emerging health issue. Considering this issue, we assessed biofilm formation by the bacteria isolated from poultry droppings and observed that 47 were strong biofilm producers while 21 were moderate biofilm producers. Remaining isolates showed weak biofilm formation efficiency except the 4 isolates which were unable to form biofilm. The majority of the isolates showing biofilm formation belonged to phyla *Pseudomonadota* (~70%) and *Bacillota* (~30%) as reported earlier by Rampadarath[35]. Our data revealed *Enterobacter cloacae*, *Proteus mirabilis*, *Enterococcus faecalis, Klebsiella pneumoniae*, *Escherichia* sp., *Bacillus* sp. and *Staphylococcus* sp. as strong biofilm producers which are in agreement with the earlier studies[36,37].

Biofilm formation by antibiotic-resistant bacteria in humans contributes to chronic infections posing a serious health risk. *Klebsiella pneumoniae* strains are known to be responsible for causing urinary infections, abdominal abscesses in renal transplant patients, skin abscess and cholecystitis in hepatic transplant patients, whereas *Enterobacter cloacae* cause bacteraemia in renal transplant recipients due to biofilm formation[38]. Biofilm forming resistant strains of *Enterobacter cloacae* have also been isolated as contaminants from meat and processed food[39]. Infections caused due to biofilm-forming *Shigella sp.* are alarming health issues across the globe. *Staphylococcus epidermis* known to form biofilms on medical devices and in nosocomial infections previously, is also detected in the poultry droppings in the current study[40]. The multidrug-resistant pathogens belonging to *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae* families from poultry dropping samples found in our study and their biofilm formation efficiency are shown in Fig. 1.

Culture-independent study

The poultry dropping samples were assessed using the 16S rRNA gene-based targeted amplicon sequencing on the Illumina MiSeq platform. The high-throughput sequencing generated a total of 107,385 paired-end reads and after sequence denoising and chimera removal, 60,437 reads were retained from five samples. The alpha diversity estimates based on the Shannon index showed divergence between the samples wherein samples followed the descending order of diversity richness and evenness viz. JPD>JPA>PP>JPB>JPC. In total, twelve distinct

bacterial phyla were observed of which four phyla, *Bacillota* (54.6%), *Actinomycetota* (21.1%), *Pseudomonadota* (17.6%) and *Bacteroidota* (5%) were found to be dominant in all samples. Taxonomic profiling of the bacterial families revealed the abundance of *Enterobacteriaceae, Corynebacteriaceae, Lactobacillaceae and Enterococcaceae* families, which include opportunistic and obligate pathogens exhibiting multiple ARGs[5]. The significant positive association of pathogens belonging to family *Enterobacteriaceae* and their linked ARGs have been described and linked with aminoglycoside, tetracycline, vancomycin, phenicol and MLSB resistance[5]. At the genus level, we noted enrichment of 118 unique ASVs having divergent phylogenetic relationship and abundances wherein *Escherichia-Shigella* (16.7%) showed the maximum abundance followed by *Corynebacterium* (14.5%) and *Ligilactobacillus* (12.2%) (Fig. 2a). Our attempt to find the common signature of these ASVs in different samples resulted in filtering of 23 ASVs which were differentially prevalent in the poultry droppings (Fig. 2b). The top five prevalent ASVs (≥80%) belonged to the genus *Ligilactobacillus*, *Lactobacillus*, *Escherichia-Shigella, Corynebacterium* and *Enterococcus.* These genera harbour several members considered as priority pathogens which cause life-threatening infections in humans. Further investigating the shared bacterial communities by retrieving the lowest taxonomic rank (i.e., species) revealed ASV50 being affiliated to *Lactobacillus gasseri,* a potent human pathogen. There are many diverse reports on the *L. gasseri* indicating its role in bacteremia[41]. Another prevalent ASV includes ASV4 affiliated to *Corynebacterium stationis*, a human pathogen isolated from infant fecal sample. Thus, disseminating these multidrug-resistant bacteria and their ARGs through horizontal gene transfer via poultry fecal waste poses a risk to public health. Collective observations from the cultivable and uncultivable approaches highlight that poultry droppings might act as reservoirs of antimicrobial-resistant priority pathogens harbouring the various ARGs.

Functional analysis using the PICRUSt2 revealed that bacterial communities contain seven major drug resistance gene families. The bacterial communities show predominance of genes for the biosynthesis of diverse antibiotics such as streptomycin, tetracycline, ansamycins, vancomycin, penicillin and cephalosporin. Additionally, bacterial communities harbour genes for beta- lactam resistance and *Staphylococcus aureus* infection (Fig. S1a & b). Poultry samples viz. JPC (Shannon Index= 4.22) and JPB (Shannon Index = 4.75) depicting the relatively lower estimates of the alpha diversity indices contained higher abundance of the drug resistance gene families. Sample JPC contained an average 0.08-fold higher abundance for four out of seven gene families under study. Similarly, JPB harbours an average 0.48-fold higher abundance for three out of seven gene families that include beta- lactam resistance and penicillin and cephalosporin biosynthesis as the prominent families. The observation was supported by the linear correlation indicating inverse relationship between the bacterial diversity and major drug resistance families (Fig. 3). The incidence of alteration in bacterial diversity (especially in case of dysbiosis) and subsequent increase in the potential pathogenic microbiota has been shown by multiple studies focusing on the human and environmental microbiome[18]. We readily acknowledge the limitation of our observation of increase in abundance of drug resistance genes with decrease in bacterial diversity (Shannon Index) which is based on limited samples. At the same time, if it holds a weight, it raises serious concern mitigating the hazards to animal and public health. This study underscores the importance of proper monitoring and disposal of antibiotics used in poultry operations to prevent the spread of multidrug-resistant bacteria. Alarmingly, some strains have even developed resistance to colistin, the last-resort antibiotic. To improve infection control and mitigate further dissemination of antibiotic-resistant microorganisms, further investigation is needed into production facilities and experimental poultry houses. In alignment with the 'One Health' approach for food safety and nutrition, the data suggests that regulating

antibiotic usage in poultry production and conducting routine surveillance of these resistant pathogens is crucial. Also adopting alternative methods of poultry production which replace antibiotics with alternatives or do not use them at all (ABF) is of utmost importance to win the battle against the rising AMR worldwide.

Author Contributions

The study was ideated and designed by AS, AZ, and KJ. Sample collection was carried out by SK and AK. The experiments and data analysis were performed by NJ, SK, AK, CM, MC and MJ. Interpretation of the data was done by SK, KJ, NJ, AZ and AS. Manuscript was drafted by NJ, SK, KJ, CM, AVK, AZ and AS. AS-Administration, Funding acquisition and Supervision. All the authors approved the final manuscript.

Ethics declarations

All the authors declare no conflict of interest.

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Figure Legends

Fig. 1: Heat map representing the response of bacterial isolates to various antibiotics and their biofilm formation efficiency (For response against the antibiotics, S: Susceptible, I: Intermediate, R: Resistant; for biofilm formation, St: Strong biofilm producer, M: Moderate biofilm producer, N: No biofilm formation). Colistin data in the heat map represents all the organisms including bacteria known for intrinsic resistance.

Fig. 2: Bacterial community composition at the taxonomic rank of genera (**2a**) indicates the phylogenetic distribution and the abundant bacterial genera. (**2b)** Depicts the common bacterial genera found across studied poultry droppings.

Fig. 3. Relationship between the bacterial diversity and the observed major drug resistant families. The linear correlation indicates the bacterial diversity is inversely proportional to the abundance of drug resistance gene families.