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Clinically important microbial diversity and its antibiotic resistance pattern towards various drugs

Gowri M. Boovaragamoorthy[a], Murugadas Anbazhagan[a,b], Prakash Piruthiviraj[c], Arivalagan Pugazhendhi[d], Smita S. Kumar[e], Naif A. Al-Dhabi[f], Abdul-K. Mohammed Ghilan[f], Mariadhas V. Arasu[f], Thamaraiselvi Kaliannan[a,*]

[a] Laboratory of Molecular Bioremediation and Nanotechnology, Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India
[b] National Centre for Alternatives to Animal Experiments (NCAAE), Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India
[c] Department of Biotechnology, Sree Narayana Guru College, Coimbatore 641 1005, Tamil Nadu, India
[d] Innovative Green Product Synthesis and Renewable Environment Development Research Group, Faculty of Environment and Labour Safety, Ton Duc Thang University, Ho Chi Minh City, Viet Nam
[e] Centre for Rural Development and Technology, Indian Institute of Technology Delhi, HauzKhas 110016, New Delhi, India
[f] Department of Botany and Microbiology, College of Sciences, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

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ABSTRACT

Background: Increased use of antibiotics in poultry leads to the development of antimicrobial resistance among the commensal bacterium of broiler chickens.
Objective: In this study, we aimed at studying the effect of periodic administration of therapeutic antibiotics against the bacterial diversity in poultry litters collected from broiler chickens.
Methods: Poultry litters were collected randomly at regular intervals after administration of antibiotics (1st, 12th and 22nd day) to the chicken. Bedding material without litters served as control. Phenotypic observations showed that there is a difference in the bacterial richness isolated at regular intervals. A total of 32 bacteria were isolated from poultry letters and are grouped into ten different genus. Isolated bacterial species were further confirmed by16S rRNA sequencing.
Results: Antibiotic susceptibility profile of isolated bacterial species exhibited strong resistance towards 13 selected antibiotics. These results substantiate that administration of antibiotics leads to the alterations in bacterial diversity and development of antimicrobial resistance among the commensal bacteria of poultry litter.
Conclusion: This high selection pressure of therapeutic antibiotics may lead to species selection and development of antibiotic resistance among bacterial population. Development of such species selection may access the human and other organisms via food chain and can cause severe health defects.

Introduction

Usage of antibiotics for various human and veterinary bacterial diseases has revolutionized the medical industry for the past 70 years. However, extensive use and abuse of antibiotics triggers high selection pressure among microbes which resulted in the development of antibiotic resistance [1,2]. Development of antibiotic resistance has emerged as a serious problem in clinical trials. Emergence of antibiotic resistance has driven into the health risk because of its drastic use in non-therapeutic practices analogous as therapeutics especially in animal farming [1].

When compared to other livestock business, the growth of poultry industry has considerably increased since 1970. Consumption of chicken meat has increased by four percentage for every year [3]. As a result, poultry farms are increased and equipped to rear large number of chickens for the steady production of meat [3]. During large scale meat production, care must be taken to maintain the health of the chickens. Subsequently this situation will result in the use and abuse of antibiotics for the treatment of infectious diseases. In some cases, antibiotics are also used as growth promoting agents in poultry [4]. Although administration of antibiotics seem

* Corresponding author.
E-mail addresses: arivalagan.pugazhendhi@tdtu.edu.vn (A. Pugazhendhi), kthamaraiselvi@hotmail.com (T. Kaliannan).

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to be inevitable in both conventional and modern poultry farming, their overuse in recent days results in the development of high level of antibiotic resistance microbes.

Development of antibiotic resistance in veterinary environment has become the significant cause of concern due to its possible effects on humans. The direct contact with poultry environment may lead to antibiotic resistant bacterial accumulation in humans. Usually the poultry slaughter house farmers are at high risk of colonization of antibiotic resistant bacteria. Also the accumulation of antibiotic residues via the intake poultry products in humans leads to the highest selection pressure on human microbiota [5]. Such exchange of antibiotic resistance to the humans will arise to complications during surgeries, transplantation of organs, chemotherapy for cancer treatment, etc., [6]. Poultry environment harbors complex microbiota of chicken intestinal origin [7]. Researchers found that food additives including antibiotics influence the gut microbiota of chickens [7–9]. Increased use of antibiotics in poultry feed results in bacterial selection in chicken intestine. Reports show that the administration of antibiotics influences the growth of gram positive microflora in chicken intestine. Accordingly, gram positive bacteria produce toxins against other commensal bacteria and compete with the host nutrients [10]. Antibiotic resistant bacteria can enter the humans via direct contact, intake of animal originated foods and/or through the livestock environment [11]. Transmission of antibiotic resistance genes to human bacterial community by means of intestinal passage may contribute to the treatment failure risk [12].

In commercial poultry farms, poultry litters are accumulated in large scale [4]. Poultry litter are the mixed composition of bedding material, faeces and feathers. It also contains significant amount of nutrients and are utilized as cheap organic fertilizer for the improved crop management. Such applications of litter to the agricultural land can attribute to the manure borne resistance spread. This is because nearly 70–90% of administered poultry antibiotics at sub-therapeutic level are excreted through litters in the manure [4]. Most of the human therapeutic antibiotics used in poultry farms may be responsible for the development and outbreak of antibiotic resistance pathogens. Subsequently it becomes a threat of treatment failure when an individual is infected with such pathogens. Besides the antibiotic resistance bacteria (ARB), antibiotic resistance genes (ARGs) which accumulate in the poultry environment can also be transferred to humans. Based on these backgrounds the present study was aimed to identify the bacterial richness in poultry litter following various antibiotic administrations. Further we also aimed to observe the antibiotic resistance profile of identified microbes against selected antibiotics used in poultry farms.

Materials and methods

Sample collection

Poultry litter samples were taken from the poultry farm in Namakkal district, Tamil Nadu, India. Namakkal district alone largely meets the need of chicken meat of India. The farm reserves approximately 1500–2000 broiler chickens. Samples were collected at different days (1^{st}, 12^{th} and 22^{nd} day) after administration of antibiotics. Bedding material serves as a control. The collected poultry litter samples were labelled and preserved at −4 °C until further use.

Selection of antibiotics

Totally thirteen antibiotics from seven main antibiotic classes namely aminoglycosides (kanamycin, neomycin, tobramycin, streptomycin), amphenicol (chloramphenicol) beta (β) - lactam (amoxycillin, cxloxacillin), cephalosporin (cefalexin), fluoroquinolones (ciprofloxacin, ampicillin), macrolides (erythromycin) and tetracyclines (tetracycline, oxytetracycline) were selected based on the data of widespread usage of antibiotics in poultry farms.

Isolation and phenotypic identification of culture dependent bacteria

To isolate individual bacterial colonies, poultry litter from each day were diluted serially (10^{-1} to 10^{-9}) in distilled water and the dilutions were spread on nutrient agar plates with the following components: Peptic digest of animal tissue -5 g L^{-1}, beef extract - 3 g L^{-1}, NaCl- 5 g L^{-1} and agar - 1.5 g L^{-1}. After inoculation, plates were incubated for 24 to 48 h at 37 °C. Based on the morphological observation individual bacteria were identified and confirmed adopting Gram staining and other biochemical tests [13].

DNA extraction and 16S rRNA sequencing

For total DNA extraction, the bacterial cells were lysed in cell lysis solution (10 mMTris–HCl, 1 mM EDTA, 10% SDS, 5 mg/ml of RNase) and kept at 37 °C (45 min). DNA is resuspended in TE buffer and preserved at −4 °C until further use [14]. The obtained DNA was checked in 0.8% agarose gel electrophoresis. For 16S rRNA sequencing, universal 16S rRNA primer set 8 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′TACGGCTACCTTGTTACGACTT-3′) (OcimumBiosolutions Ltd) were used [15]. The reaction volume of 50 μl was performed with 0.1 ng of isolated DNA, 2X PCR master mix, 10 pmol of both primer and sterilized Mili-Q water. PCR condition consists of an initial denaturation (94 °C, 5 min) and 25 cycles of denaturation (94 °C, 1 min), annealing (52.3 °C, 1 min) extension (72 °C, 1 min) and final extension (72 °C 10 min). Amplified products were further purified using HiyieldTM Gel/PCR DNA extraction kit (Gene technologies, Australia). Purified PCR products were sent to Xcelris Labs Ltd, Ahmedabad, India for gene sequencing. Sequencing was carried out in ABI-PRISM automated sequencer- ABI-3730 DNA analyzer (Applied Biosystems, USA).

Phylogenic tree construction for isolated bacteria

The nucleotide sequences were initially examined in Blast-n site of NCBI server (http://www.ncbi.nlm.nih.gov./BLAST), matching sequences were downloaded and multiple sequences were aligned using Clustal X software program [16]. The percentage of replicate trees where the related taxa clustered in the bootstrap test (100 replicates) were expressed next to the branches [17]. The phylogenetic tree was constructed using neighbor joining method in MEGA software [18].

Antibiotic susceptibility test

Antibiotic susceptibility test was determined using disc diffusion method [19] by culturing each strain in Mueller Hinton agar (MHA) medium with varying concentrations of selected antibiotics. Culture adjusted to 0.5 NTU McFarland standards were inoculated on MHA medium and antibiotic discs were placed using aseptic technique. Then the plates were incubated at 37 °C for 16–18 h for the observation of zone formation. Susceptibility and resistance cut- off criteria of zone formation of the tested isolates were determined on the basis of Clinical and Laboratory Standard Institute's interpretative criteria [20].

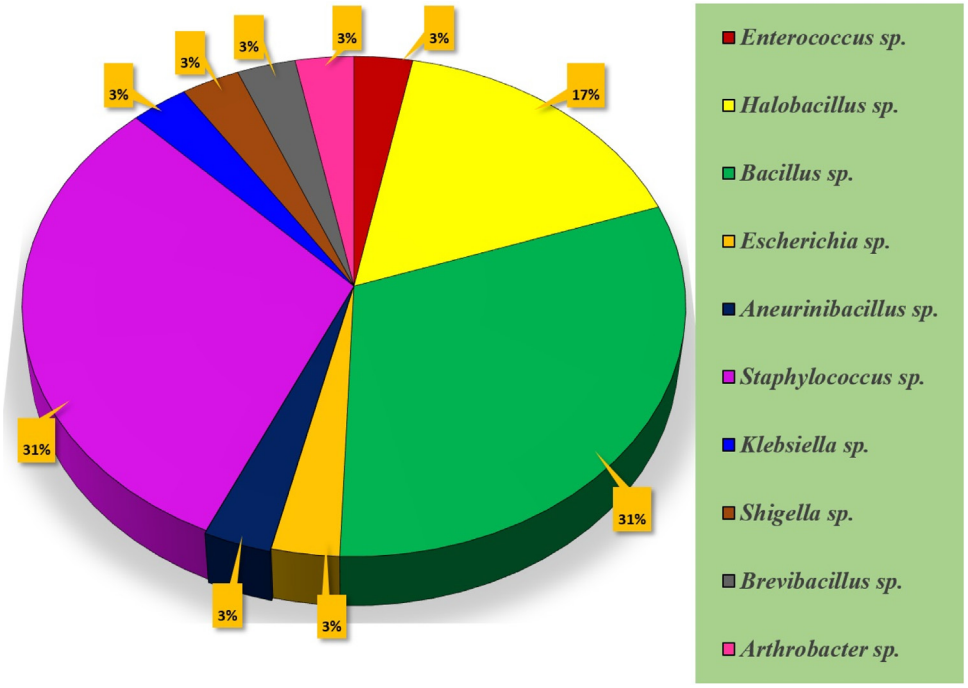

Fig. 1. Distribution of bacterial diversity in poultry litter samples.

Results and discussion

Phenotypic identification of bacterial isolates

Addition of antibiotics in animal feed leads to species selection and/or emergence of antibiotic resistance microbes in the surrounding environment [21,22]. Emergence of antibiotic resistance microbes is a serious cause of concern for both animal and human health. Hence, the present study is conducted in a view to understand the bacterial richness and antibiotic resistance behaviour of bacteria isolated from poultry litters after administration of antibiotics.

A total of 32 bacterial isolates were identified in this study. Among them six are from the bedding material and the remaining are from poultry litters. Based on the morphological assessment and various biochemical assays the bacterial isolates were identified and broadly classified into ten different genera viz., *Bacillus, Staphylococcus, Escherichia, Klebsiella, Enterococcus, Shigella, Aneurinibacillus, Brevibacillus, Arthobacter* and *Halobacillus*. The most predominant genus in the isolates was *Bacillus* and *Staphylococcus* with 31% distribution followed by *Escherichia* species with 17%. Other species contribute to 3% of distribution (Fig.1). Morphological and biochemical examination reveals that most of the bacteria isolated from the poultry litter are gram positive as mentioned in previous reports [23,24]. However, the significant finding from this study is the alterations in the microbial diversity following various phase of antibiotic administration.

16S rRNA sequence analysis

The genus of bacterial isolates was reconfirmed to species level using 16S rRNA gene sequence analysis. For all the isolates the identification is based on the similarity percentage ranges from 90 to 99%. After BLAST analysis the sequences were deposited in NCBI and the accession number is provided in Tables 1–4. These results indicated that short sequence 16S rRNA sequencing could offer an appropriate identification amongst the isolated strains.

The evolutionary relationships of bacterial isolates at different day of sampling were inferred using Neighbor-Joining method using MAGA X software. The optimal phylogeny with sum of branch length was shown in Fig. 2. This phylogenetic tree with branch length in the same units infers the evolutionary distances of bacterial taxa. Maximum composite Likelihood computation of evolutionary distances results in the units of the number of base substitutions per site. The phylogeny derived from the bacterial isolates from each day is used to compare the bacterial diversity alteration during various days of antibiotic administration.

Bacillus*group*

A total of 10 different *Bacillus* strains (*B. korlenis, B. firmus, B. niacini, B. endophyticus, B. tequilensis, B. subtillis, B. megatarium, Bacillus* species, *B. aryabhattai* and *B. koreensis*) were identified in this group. Among the isolated strains first five strains were predominantly found in bedding material. *B. subtillis* were identified in 1st day, *B. megatarium, Bacillus* species *and B. aryabhattai* were identified in 12th day and *B. koreensis* was identified in 22nd day.

Staphylococcus *group*

A total of 10 *Staphylococcus* strains belonging to 5 different *Staphylococcus* species (*S. sciuri*, *Staphylococcus* species, *S. saprophyticus*, *S. qallinarum* and *S. arlettae*) were identified in this study. *S. sciuri* and *Staphylococcus* species are identified from 1st day. While *S. saprophyticus* and *S. qallinarum* strains were present in 12th day. *Staphylococcus* species was again seen in 22nd day along with *S. arlettae*.

Other species

Other species identified in this study are *Escherichia coli, Klebsiella pneumonia*, *Enterococcus faecium*, *Shigella flexneri*, *Aneurinibacillus aneurinilyticus*, *Brevibacillus* species, *Arthobacter nicotianae* and *Halobacillus blutaparonensis*. *Bacillus* species alone were found in control samples. Likewise, *Escherichia coli* strains were dominantly found in the 1st day and only one was present in 12th and 22nd day. Among the remaining, *Enterococcus fae-*

Table 1
Phylogeny based identification of bacteria in the control sample.

Bacterial strains identified	BLASTn similarity in %	Strain name	NCBI accession number
Bacillus niacini	99%	KTSMBNL-44	KM099167
Bacillus korlenis	98%	KTSMBNL-45	KM099168
Halobacillusblutaparonensis	99%	KTSMBNL-46	KM099169
Bacillus endophyticus	99%	KTSMBNL-47	KM099170
Bacillus firmus	99%	KTSMBNL-48	KM099171
Bacillus tequilensis	99%	KTSMBNL-49	KM099172

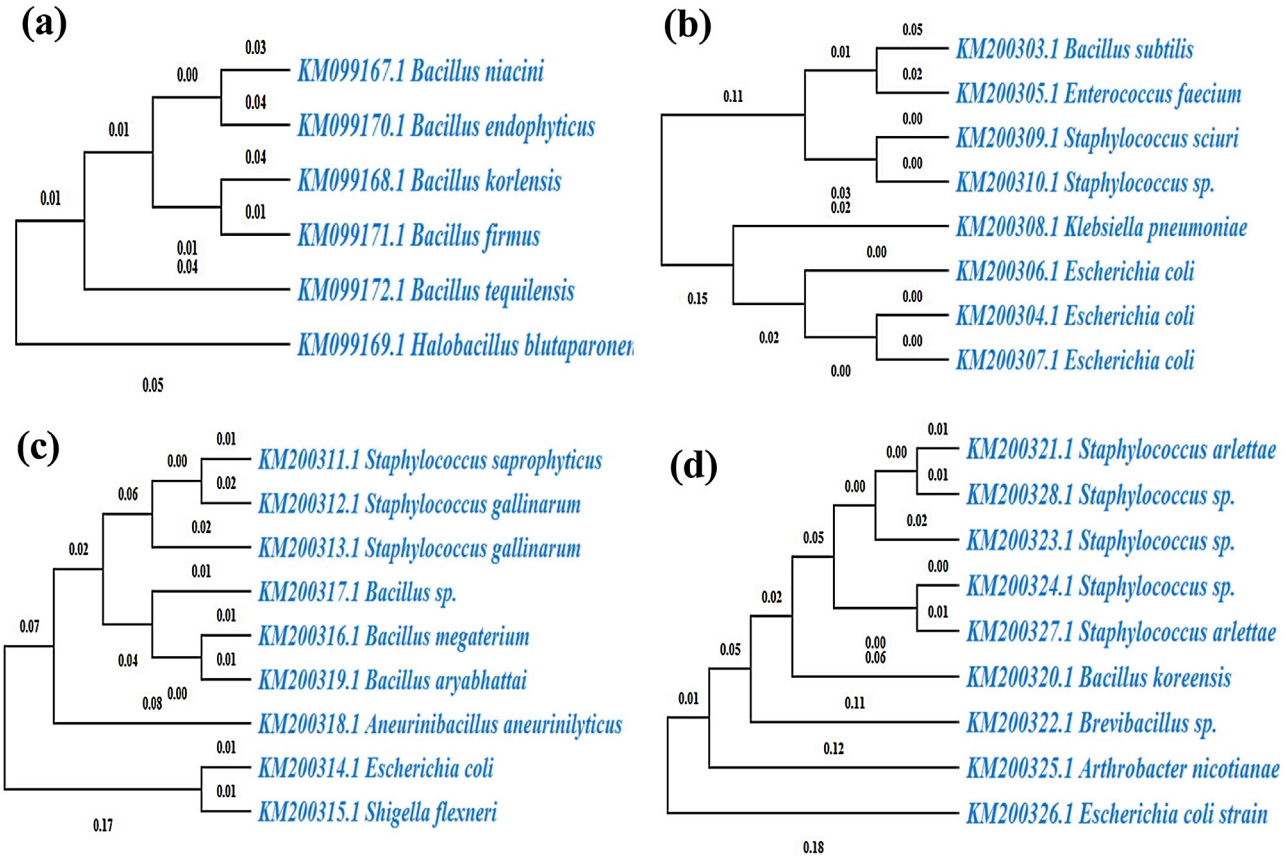

Fig. 2. (a) Phylogenetic tree of bacteria isolated from control sample, (b) Phylogenetic tree of bacteria isolated from 1st day poultry litter sample, (c) Phylogenetic tree of bacteria isolated from 12th day poultry litter sample, (d) Phylogenetic tree of bacteria isolated from 22nd day poultry litter sample.

Table 2
Phylogeny based identification of bacteria in the 1st day poultry litter sample.

Bacterial strains identified	BLASTn similarity in %	Strain name	NCBI Accession number
Bacillus subtillis	100	KTSMBNL-50	KM200303
Escherichia coli	99	KTSMBNL-51	KM200304
Enterococcus faecium	99	KTSMBNL-53	KM200305
Escherichia coli	99	KTSMBNL-54	KM200306
Escherichia coli	99	KTSMBNL-55	KM200307
Klebsiellapneumoniae	99	KTSMBNL-56	KM200308
Staphylococcus sciuri	100	KTSMBNL-57	KM200309
Staphylococcus sp.	100	KTSMBNL-59	KM200310

Table 3
Phylogeny based identification of bacteria in the 12th day poultry litter sample.

Bacterial strains identified	BLASTn similarity in %	Strain name	NCBI accession number
Staphylococcus saprophyticus	99	KTSMBNL-60	KM200311
Staphylococcus qallinarum	98	KTSMBNL-61	KM200312
Staphylococcus qallinarum	97	KTSMBNL-62	KM200313
Escherichia coli	98	KTSMBNL-64	KM200314
Shigellaflexneri	99	KTSMBNL-65	KM200315
Bacillus megatarium	99	KTSMBNL-66	KM200316
Bacillus sp.	98	KTSMBNL-67	KM200317
Aneurinibacillusaneurinilyticus	98	KTSMBNL-68	KM200318
Bacillus aryabhattai	98	KTSMBNL-69	KM200319

Table 4
Phylogeny based identification of bacteria in the 22nd day poultry litter sample.

Identified bacterial strains	BLASTn similarity in %	Strain name	NCBI accession number
Bacillus koreensis	99	KTSMBNL-70	KM200320
Staphylococcus arlettae	99	KTSMBNL-71	KM200321
*Brevibacillus*sp.	97	KTSMBNL-72	KM200322
Staphylococcus sp.	99	KTSMBNL-73	KM200323
Staphylococcus sp.	99	KTSMBNL-74	KM200324
Arthobacternicotianae	98	KTSMBNL-76	KM200325
Escherichia coli	99	KTSMBNL-77	KM200326
Staphylococcus arelettae	99	KTSMBNL-78	KM200327
Staphylococcus sp.	99	KTSMBNL-79	KM200328

cium and *Klebsiella pneumonia* were isolated in 1st day. *Shigella flexneri* and *Aneurinibacillus aneurinilyticus* were found in 12th day alone. Wherea *Brevi bacillus* species and *Arthobacter nicotianae* were present in 22nd day.

It is documented in this study that the bedding material reserves large number of *Bacillus* species. Besides *Bacillus* species, majority of the bacteria identified after 1st day of antibiotic administration were *E. coli*. However, the trend has been changed to *Staphylococcus* species in the subsequent days after antibiotic administration. From this observation, it is revealed that administration of antibiotics to the poultry chickens alters the bacterial richness in the chicken intestine [25]. This finding is in concurrent with the earlier reports of [26] where the ingestion of lupulone had adverse effect on bacteria species like *Lactobacillus* and *C. perfringens*. Similarly, in another study, treatment with antibiotics such as streptomycin and tetracycline leads to changes in composition of chicken microbiota. Streptomycin and tetracycline dramatically decreases the operational taxonomic unit (OTU) microbiota up to 55 and 94%, respectively. Since the chickens were retained under the same settings, it is believed that such changes in gut microbial composition upon antibiotic administration were direct results of the antibiotic treatment and not of random variation in gut

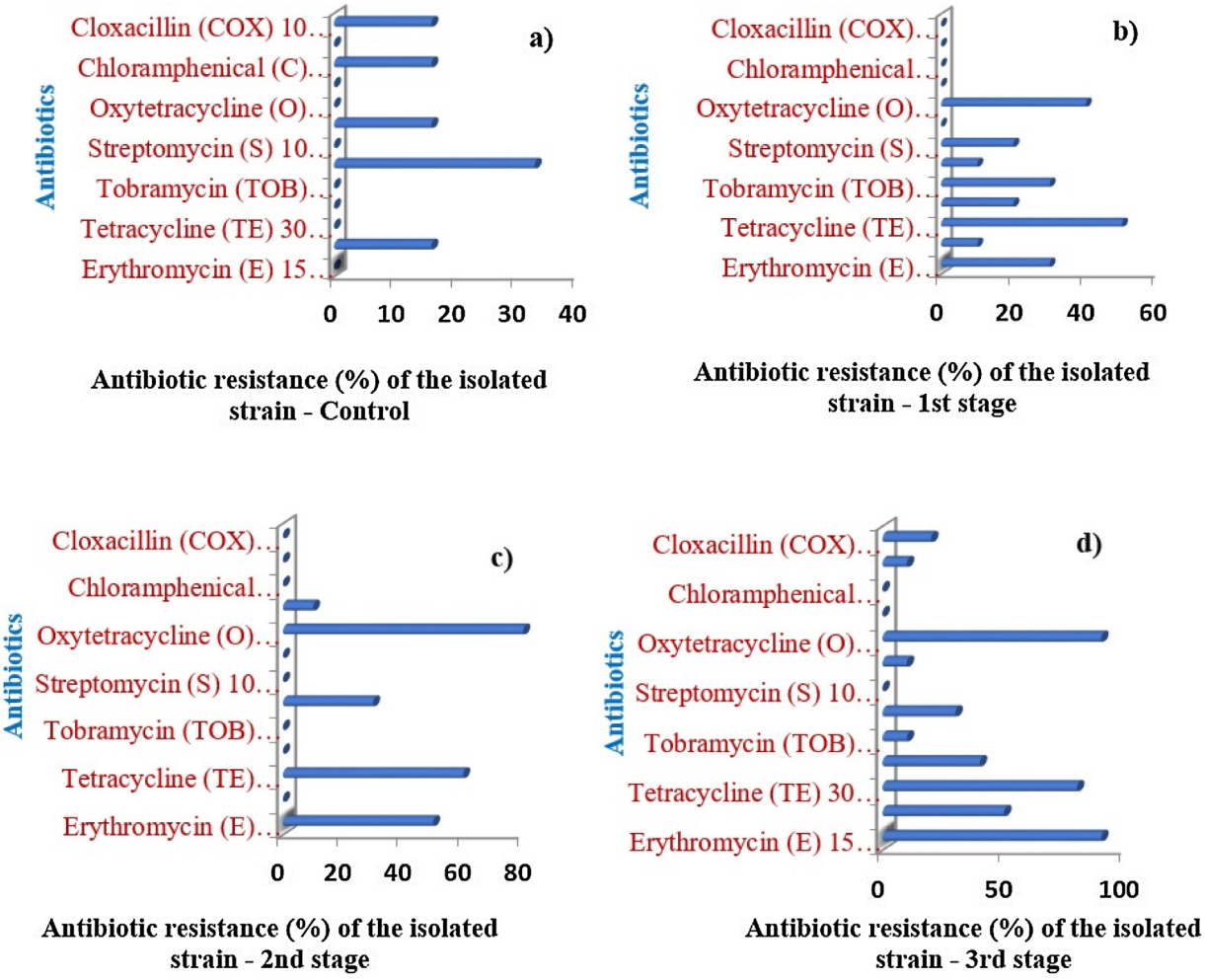

Fig. 3. (a) Antibiotic susceptibility profile of bacteria isolated from control sample, (b) Antibiotic susceptibility profile of bacteria isolated from 1st day poultry litter sample, (c) Antibiotic susceptibility profile of bacteria isolated from 12th day poultry litter sample, (d) Antibiotic susceptibility profile of bacteria isolated from 22nd day poultry litter sample.

microbial composition and it also reflects in the poultry litter [27].

However certain bacterial species like *Enterococcus* and *Escherichia* were restored in chicken intestine with response to antibiotic treatment. Hence it is believed that stomach is the potential reservoir for specific microbial community. Even though the animal gut microbiota has random fluctuation in their composition, antibiotic administration may be the cause of concern for changes in gut microbiota [28].

Antibiotic susceptibility test

To determine the antibiotic susceptibility, 13 antibiotics were tested in this study. According to clinical laboratory standard institute it has been defined that the isolates capable of inhibiting feasible concentration of antimicrobial agents are referred as susceptible and resistant organisms are not inhibited by achievable concentrations of agents. Bacterial isolates from the bedding material exhibited resistance towards ampicillin (35%) followed by cloxacillin, chloramphenicol, amoxicillin and ciprofloxacin (15%) (Fig. 3a). It is observed that the bacterial isolates from poultry litter of 1st day exhibits strong resistance to tetracycline (50%), whereas, bacterial isolates from 12th day exhibits strong resistance to oxytetracycline (80%) and bacterial isolates from 22nd day shows strong resistance to oxytetracycline and erythromycin (90%) (Fig. 3 b, c, d). It is interesting to see that antibiotic susceptibility profile of bacterial isolates from day 12 and 22 exhibited strong resistance towards oxytetracycline. This clearly indicates that administration of antibiotics leads to the emergence of antibiotic resistance pathogens.

Resistance of microbes to these antibiotics may be due to development of antibiotic resistance genes developed by the microbes following continuous administration of antibiotics. This clearly indicates that as far as the antibiotics are being administered into the host, it creates high selection pressure on them, which leads to the existence of selected species or emergence of antibiotic resistance population.

Conclusion

The detailed analysis of poultry litter bacterial community will give insights into vital performers in the spreading of antimicrobial resistance. The study helps in facilitating the necessary and appropriate prevention measures to deal with antibiotic-resistant bacteria in the litter which is reused as fertilizer. More research is needed in the future perspective to determine the potential consequence of the veterinary medicines on microbial flora in the environment. The present study will be the ground to examine the presence of source-oriented significant antibiotic resistant bacterial release within the poultry environment.

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