IMMUNOLOGY, HEALTH, AND DISEASE

Study on acquisition of bacterial antibiotic resistance determinants in poultry litter

T. Sridevi Dhanarani,* C. Shankar,*† J. Park,*† M. Dexilin,* R. Rajesh Kumar,* and K. Thamaraiselvi*†[1]

*Molecular Bioremediation Division, Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli, 620 024, India; and †Department of Civil and Environmental Engineering, Yonsei University, Seoul, 120 749, South Korea

ABSTRACT Antibiotic resistance and the mode of transmission were investigated in bacteria isolated from poultry litter. Total aerobic heterotrophic bacteria were screened and identified for their resistance to different antibiotics such as ampicillin, streptomycin, erythromycin, tetracycline, chloramphenicol, kanamycin, tobramycin, and rifampicin. The distribution of bacteria found in the litter was *Staphylococcus* (29.1%), which was the predominant group, followed by *Streptococcus* (25%), *Micrococcus* (20.8%), *Escherichia coli* (12.5%), *Salmonella* (8.3%), and *Aeromonas* (4.1%). Fifty percent of these isolates were susceptible to ampicillin, 57% to erythromycin, 25% to tetracycline, 4% to chloramphenicol, 40% to kanamycin, 75% to streptomycin, 54% to tobramycin, and 4% to rifampicin. Three randomly selected isolates representing *Staphylococcus, Streptococcus*, and *Micrococcus* were examined for plasmids, and plasmid-curing and plasmid-induced transformation studies were conducted. *Streptococcus* and *Micrococcus* harbored a plasmid of 4.2 and 5.1 kb, respectively, whereas *Staphylococcus* did not harbor any plasmids. Plasmids were cured in *Streptococcus* and *Micrococcus* at a concentration of 75 and 100 μg/mL of acridine orange, respectively, and transformation of 4.2- and 5.1-kb plasmids isolated from the *Streptococcus* and *Micrococcus* to plasmid-free *E. coli* DH5α strain was possible. In conjugation experiments, the antibiotic resistance profiles of transconjugant cells were found to be the same as the donors with the exception of *Staphylococcus*. The results of this study suggest that transformation and conjugation could be an important mechanism for horizontal gene transfer between bacteria in poultry litter. An understanding of the mechanism and magnitude of resistance gene transfer may provide a strategy to reduce the potential for dissemination of these genes.

Key words: poultry litter, antibiotic, conjugation, plasmid curing, transformation

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INTRODUCTION

A wide variety of antibiotics are routinely added to animal feed in subtherapeutic doses for growth promotion of animals produced for human consumption (Vazquez-Moreno et al., 1990; Roura et al., 1992; Martin et al., 1996; Rassow and Schaper, 1996). Approximately 8,164,662 kg of antibiotics are used annually in animal farming (70% of which is used for nontherapeutic purposes such as growth promotion and disease prevention) compared with only 1,363,636 kg per year used in human medicine (Roe and Pillai, 2003). This practice may lead to a selection of resistant microbial populations (including pathogens) in the native microbiota of the animal and the local environment due to shedding in the feces (Bastianello et al., 1995; Sundin et al., 1995). Antibiotic-resistant organisms from animals reenter the human and animal populations through several pathways including natural water, irrigation water, drinking water, vegetables, and foods (Roe and Pillai, 2003). These resistant bacteria are shed in feces, where they can share extra chromosomal antibiotic resistance plasmids (r-plasmids) with native bacteria and may be disseminated to other animals. Also, antibiotics may accumulate in the tissues of animals and be ingested by consumers whose own resident microflora may become antibiotic-resistant (Kobe et al., 1995; Corpet, 1996; Kolawole and Shittu, 1997). Therefore, microbial contamination of litter should be reduced or eliminated before reutilization to minimize environmental health risks related to transfer of antibiotic-resistant bacteria to humans or other animals.

The aim of this study was to determine the antibiotic susceptibility profile of bacteria isolated from poultry

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[1]Corresponding author: kthamaraiselvi@hotmail.com

litter to 8 antibiotics. To investigate the potential effects of the shifts in antibiotic resistance and the potential risk of transmission of antibiotic resistance, the presence of plasmids associated with the acquisition of antibiotic resistance in poultry litter was investigated.

MATERIALS AND METHODS

Bacterial Identification

Poultry litter samples were collected from a poultry farm at Salem, Tamil Nadu, India. Samples were collected with sterile sponge swabs premoistened with sterile buffered peptone water and were stored at 4°C. The samples were serially diluted in 0.5% NaCl and the dilutions were pour-plated on nutrient agar. To isolate individual bacterial colonies, a nutrient medium was prepared using peptic digest of animal tissue (5 $g \cdot L^{-1}$), beef extract (3 $g \cdot L^{-1}$), NaCl (5 $g \cdot L^{-1}$), and 1.5 g of agar for 1,000 mL of medium. The isolates were screened by colonial morphology, diffusible pigment production, and gram stain. For further identification, biochemical and physiological tests were carried out. The isolated cultures were confirmed by selective medium such as mannitol salt agar for *Staphylococcus* sp., blood agar for *Streptococcus* sp., *Salmonella-Shigella* agar for *Salmonella* sp., and *Aeromonas* agar for *Aeromonas* sp. The inoculated plates were incubated at room temperature (30 to 35°C) for 48 h. Cultures were stored on nutrient agar slants at 4°C and frozen at −70°C with 15% (vol/vol) glycerol.

Antimicrobial Susceptibility Testing

Disk Diffusion Method. Antimicrobial resistance tests were performed by the agar disk diffusion method (Bauer et al., 1966). Microbial isolates were diluted to a turbidity of 0.5 nephelometric turbidity units (**NTU**) on the McFarland scale, the broth was swabbed evenly onto the surface of Muller-Hinton agar using sterile cotton swabs, and the covered plates were allowed to dry. Antibiotic-impregnated filter paper disks were placed on the surface of the agar and incubated at 37°C for 24 h. Four disks were placed on the agar surface for each isolate, for a total of 8 disks (1 for each antibiotic tested) using a disk dispenser. Antibiotic doses used were as follows: kanamycin (30 μg), tetracycline (30 μg), erythromycin (15 μg), ampicillin (25 μg), tobramycin (10 μg), streptomycin (10 μg), rifampicin (5 μg), and chloramphenicol (30 μg). According to Barnhart and Pancorbo (1992), the antibiotic doses were chosen. Antibiotic resistances were determined by comparing bacterial isolate inhibition zone diameters with NCCLS criteria (NCCLS, 2004).

Determination of Minimum Inhibitory Concentration. Minimum inhibitory concentration (**MIC**) was determined for randomly selected strains such as *Staphylococcus, Streptococcus*, and *Micrococcus.* Isolates were reactivated in bacterial medium [peptic digest of animal tissue (5 $g \cdot L^{-1}$), beef extract (3 $g \cdot L^{-1}$), and NaCl (5 $g \cdot L^{-1}$) at 37°C for 18 h and diluted in Muller-Hinton broth to a turbidity of 0.5 NTU on the McFarland scale. A stock solution of 10 mg/mL for each antibiotic (Sigma, St. Louis, MO) was prepared and diluted in a concentration range of 10 μg to 300 μg/mL. An aliquot containing 50 μL of diluted antibiotics and 100 μL of double-strength Muller-Hinton broth and 50 μL of isolated cultures at 0.5 NTU on the McFarland concentration (total volume of 200 μL per well) were taken and incubated at 37°C for 24 h. Growth was assessed spectrophotometrically in an ELISA plate reader at λ 540 nm (Moreira et al., 2004).

Isolation and Detection of Plasmid DNA

Plasmid DNA was isolated in randomly selected strains of *Staphylococcus, Streptococcus*, and *Micrococcus* by the alkaline lysis method (Birnboim and Doly, 1979). The lysates were separated by horizontal electrophoresis at 80 V, 50 mA for 3 h in 0.8% agarose gels prepared with Tris acetate buffer (Sambrook et al., 1989). The gels were stained with ethidium bromide solution (0.5 $\mu g \cdot mL^{-1}$) for 30 min and plasmid bands were viewed with a UV transilluminator. The reference molecular mass marker used was λ DNA double-digested with *Eco*RI and *Hin*dIII.

Curing of Plasmid DNA

Curing of plasmid was carried out in plasmid-positive *Streptococcus* sp. and *Micrococcus* sp. (Silhavy et al., 1984). Plasmid-positive isolates were cured by exposure to different concentrations of acridine orange (25, 50, 75, 100, and 125 μg/mL) in nutrient broth and incubated at 37°C for 24 h. The plasmid DNA from the randomly selected clones was isolated and analyzed by agarose gel electrophoresis to verify the plasmid loss. The cells were then tested for antibiotic resistance after the determination of plasmid content. The susceptibility of the plasmid-cured isolates to antibiotics was investigated by the disk diffusion method.

Transfer of Drug Resistance

Transformation was carried out according to Sambrook et al. (1989) using *Streptococcus* sp. and *Micrococcus* sp. as the donor and a plasmid-free competent cell, *Escherichia coli* DH5α, as the recipient, which is sensitive to all of the previously tested drugs. Plasmid transformation was checked through electrophoresis and the transformants were also tested for antibiotic susceptibility.

Conjugation

Conjugation was carried out in selected *Staphylococcus, Streptococcus*, and *Micrococcus* using the double-selection method (Govender, 2002). Antibiotic sensi-

tivity-resistance patterns of the transconjugant were determined and compared with those of the donor (isolated strain) before conjugation.

RESULTS AND DISCUSSIONS

Screening and Identification of Microorganisms in Poultry Litter

One hundred twenty bacteria were isolated from the poultry litter sample. The distribution of genera among the 120 strains is shown in Table 1. The predominant organisms were *Staphylococcus* (29.1%), *Streptococcus* (25%), and *Micrococcus* (20.8%), which are all gram-positive organisms. A similar report for chicken intestinal microflora also demonstrated that the predominant organisms were gram-positive (Gong et al., 2002). Many other genera have also been found in poultry processing waste such as *Staphylococcus, Streptococcus, Clostridium, Aeromonas, Pseudomonas*, and *Yersinia* (Goyal and Hoadley, 1979; Dodd et al., 1988; Barnhart and Pancorbo, 1992; Bongers et al., 1995; Corpet, 1996; Kolawole and Shittu, 1997). *Yersinia enterocolitica* has been isolated from poultry litter during litter storage and reutilization (Kelley et al., 1994, 1995). *Campylobacter jejuni* has also been isolated from poultry litter and is an emerging agent of foodborne enteritis in humans (Shane, 1991; Kelley et al., 1994, 1995; Koenraad et al., 1995).

Antimicrobial Susceptibility Testing

Disk Diffusion Method. Isolated bacterial strains (120 isolates) were tested in vitro to determine their antibiotic susceptibility pattern by an antibiotic disk diffusion method. The majority of the strains showed antibiotic resistance to one or more antibiotics. The overall resistance pattern showed that all 120 isolates had different patterns of resistance to antibiotics. The resistance pattern was as follows; kanamycin (40%), tetracycline (25%), erythromycin (56.6%), ampicillin, (50%), tobramycin (54.1%), streptomycin (75%), rifampicin (45.8%), and chloramphenicol (3.33%) (Figure 1). Similarly, *Aeromonas hydrophila* has been isolated from poultry processing plants, and the antibiotic resistance of these isolates was examined (Barnhart and Pancorbo, 1992). Antibiotic resistance of *Pseudomonas aeruginosa* isolated from internal organs of poultry has been reported (Koncicki and Szubstarska, 1988). Antibiotic resistance of isolates from broiler processing locations was resistant to ampicillin and cephalothin. This difference may be due to different environmental conditions of broiler production and processing (Kelley et al., 1998). Previous study suggests that use of low-

Table 1. Distribution of genus groups among the 120 strains isolated from poultry litter

Genus	No. of isolates (%)
Staphylococcus	35 (29.1)
Streptococcus	30 (25)
Micrococcus	25 (20.8)
Escherichia coli	15 (12.5)
Salmonella	10 (8.3)
Aeromonas	5 (4.1)

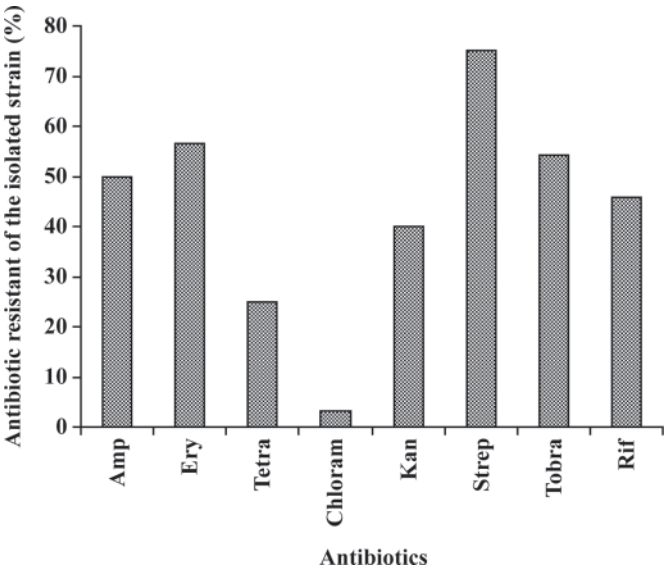

Figure 1. Antibiotic susceptibility profile for the isolated bacterial species. Amp = ampicillin; Ery = erythromycin; Tetra = tetracycline; Chloram = chloramphenicol; Kan = kanamycin; Strep = streptomycin; Tobra = tobramycin; Rif = rifampicin.

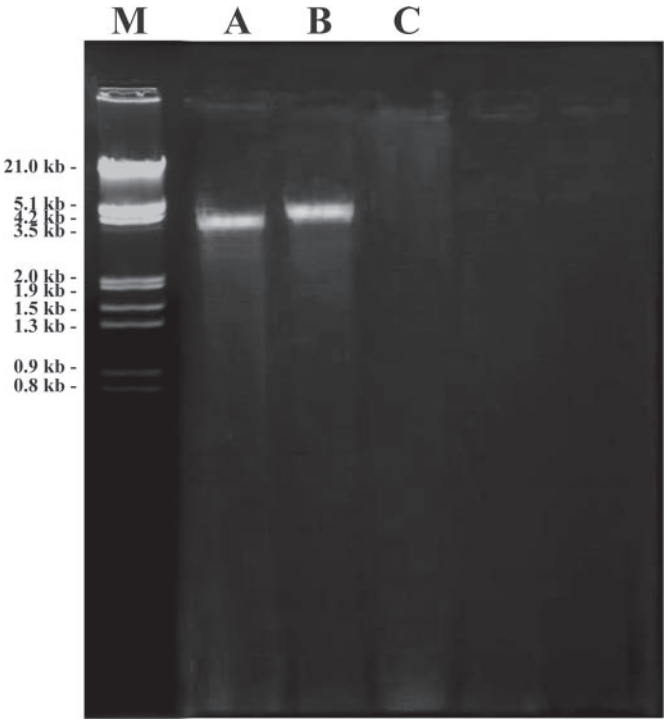

Figure 2. Plasmid DNA electrophoresis of *Strepotococcus, Micrococcus*, and *Staphylococcus* species. Percentage = 1%; M = λ DNA double-digested with *Hin*dIII and *Eco*RI; A = plasmid isolated from *Streptococcus*; B = plasmid isolated from *Micrococcus;* C = plasmid isolated from *Staphylococcus*.

Table 2. Minimum inhibitory concentration (MIC) for 3 predominant genus groups

	MIC (μg/mL)		
Antibiotics (μg/mL)	*Streptococcus*	*Micrococcus*	*Staphylococcus*
Kanamycin	60	80	110
Tetracycline	3	50	270
Erythromycin	1	100	240
Ampicillin	110	120	230
Tobramycin	1	20	10
Streptomycin	70	90	260
Rifampicin	3	70	110
Chloramphenicol	60	10	80

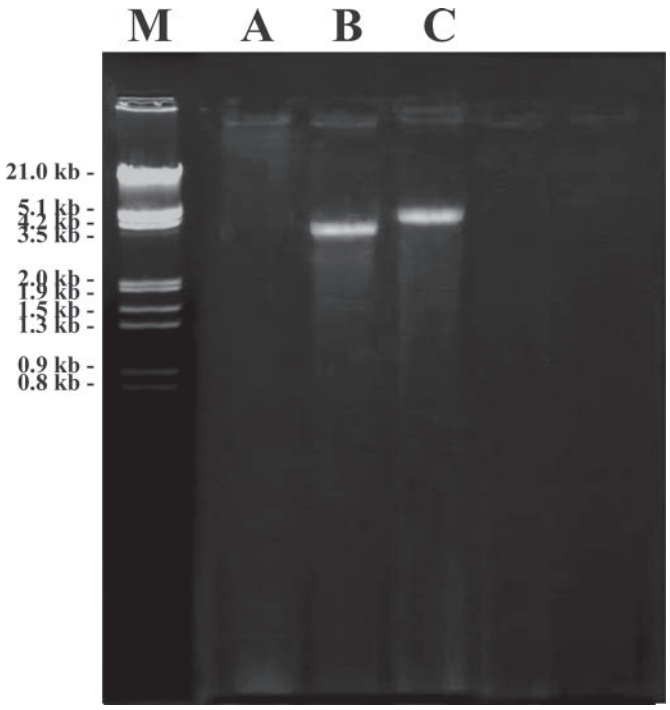

Figure 3. Plasmid DNA electrophoresis of transformants containing the plasmid of *Streptococcus* and *Micrococcus* species. Percentage = 1%, M = λ DNA double-digested with *Hin*dIII and *Eco*RI; A = plasmid isolated from *Escherichia coli* DH5α; B = transformants containing the plasmid of *Streptococcus*; C = transformants containing the plasmid of *Micrococcus* species.

level, nontherapeutic, antibiotic feed supplements may contribute to selection of antibiotic-resistant bacterial populations in the environment and animals (Kawano et al., 1996). Bacitracin, chlortetracycline, tylosin, avoparcin, neomycin, oxytetracycline, and others are used as growth-promoting antibiotics, and their doses are lower than those required for therapeutic use. Inappropriate use of these growth-promoting antibiotics is the major contributor to the emergence of antibiotic-resistant bacteria (Khachatourians, 1998). In the European Union and many other countries, drugs that have been registered for therapeutic use in humans or animals, or both, are not allowed to be used as growth promoters. However, many of the compounds used as growth promoters are analogs and show cross resistance with therapeutic antibiotics (Bogaard and Stobberingh, 1999). Development of antibiotic resistance in bacteria is mainly based on 2 factors, namely the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 1997). Extensive application of antibiotics in veterinary medicine is not only for treatment but also used for growth promotion (WHO, 1997), which leads to the evolution and enrichment of antibiotic-resistant bacteria.

MIC. The predominant strains, namely *Staphylococcus, Streptococcus*, and *Micrococcus*, were selected for the determination of MIC. The MIC determined for *Streptococcus*, *Micrococcus*, and *Staphylococcus* sp. were 60, 80, and 110 μg/mL for kanamycin; 3, 50, and 270 μg/mL for tetracycline; 1, 100, and 240 μg/mL for erythromycin; 110, 120, and 230 μg/mL for ampicillin; 1, 20, and 10 μg/mL for tobramycin; 70, 90, and 260 μg/mL for streptomycin; 3, 70, and 110 μg/mL for rifampicin; and 60, 10, and 80 μg/mL for chloramphenicol, respectively (Table 2). All of the isolates were resistant to all of the antibiotics. These isolates had their resistances to these drugs confirmed based on the breakpoints of NCCLS (2004). Resistance to antibiotics is extremely high in poultry litter due to utilization of antibiotics used in the prevention of infectious disease and as a growth promoter in poultry. A similar trend was reported in *Aeromonas* isolated from food and clinical samples (Pesavento et al., 2007).

Isolation and Curing of Plasmid

Staphylococcus, Streptococcus, and *Micrococcus* were examined for the presence of plasmids. The results indicated that *Streptococcus* and *Micrococcus* harbored a low molecular weight plasmid of 4.2 and 5.1 kb, where-

Table 3. Plasmid curing and its antibiotic susceptibility for the selected strains

	Streptococcus		*Micrococcus*	
Antibiotics	Before curing (mm)	After curing (mm)	Before curing (mm)	After curing (mm)
Kanamycin	16	30	0	22
Tetracycline	16	23	10	30
Erythromycin	0	13	0	30
Ampicillin	12	17	10	30
Tobramycin	12	28	12	26
Streptomycin	10	25	0	20
Rifampicin	6	20	0	18
Chloramphenicol	26	40	26	34

Table 4. Transformation and antibiotic susceptibility for 2 predominant strains

	Streptococcus		*Micrococcus*	
Antibiotics	Before transformation (mm) DH5α	After transformation (mm)	Before transformation (mm) DH5α	After transformation (mm)
Kanamycin	30	12	30	0
Tetracycline	36	16	36	10
Erythromycin	15	0	15	0
Ampicillin	50	10	50	8
Tobramycin	25	12	25	12
Streptomycin	28	10	28	0
Rifampicin	25	8	25	8
Chloramphenicol	33	13	33	22

as *Staphylococcus* did not harbor a plasmid (Figure 2). Similar results have been reported in *P. aeruginosa* isolated from hospitalized burn patients (Shahid et al., 2003). In *Streptococcus* sp., the plasmids were cured at a concentration of 75 μg/mL of acridine orange, and in the case of *Micrococcus* sp., the plasmids were cured at a concentration of 100 μg/mL. The antibiotic susceptibility of the plasmid-cured isolates is shown in Table 3. The loss of antibiotic resistance was associated with the loss of the plasmid. Another report suggests that loss of 6.4- and 3.8-kb plasmids in *Bacteroides fragilis* C68c was related to antibiotic resistance (Nakano et al., 2004). According to Pestana et al. (1999), the absence of plasmid suggests that the resistances are chromosomally mediated. Similarly, Radu et al. (2003), analyzing *Aeromonas* spp. isolated from fish, also found tetracycline-resistant strains harboring plasmids of about 3 and 15.7 kb, which were to be correlated with drug resistance. Antimicrobial resistance has been correlated with plasmids in clinical isolates of *Aeromonas* spp. (Adams et al., 1998; Casas et al., 2005).

Transformation

Isolated *Streptococcus* sp. and *Micrococcus* sp. bearing 4.2- and 5.1-kb plasmids were transformed to *E. coli* DH5α. Transformed *E. coli* DH5α showed that they encode resistance to antibiotics, and it is transferable to other genera. Transfer of plasmid associated with antibiotic resistance was attempted through transformation, and it was seen that the transformant (*E. coli* DH5α) acquired resistance to antibiotics after the transformation experiments. Transformed colonies from strain DH5α containing the plasmid of *Streptococcus* presented an inhibition halo of 12, 16, 0, 10, 12, 10, 8, and 13 mm and in *Micrococcus* presented an inhibiotion halo of 0, 10, 0, 8, 12, 0, 8, and 22 mm of kanamycin, tetracycline, erythromycin, ampicillin, tobramycin, streptomycin, rifampicin, and chloramphenicol, respectively, which were changed from 30, 36, 15, 50, 25, 28, 25, and 33 mm when the plasmid was transformed into the plasmid-free strain (Table 4). Agarose gel electrophoresis of the transformant showed the presence of the transforming plasmid (Figure 3). Similar results were observed in clinical isolates of *S. aureus,* harboring a plasmid of 23 kb when transferred to *E. coli* LE392 strain, which developed drug resistance (Rahman et al., 2005).

Conjugation Analysis

In the conjugation experiments, *Streptococcus* sp. and *Micrococcus* sp. successfully transferred antibiotic resistance to the recipient (*E. coli* DH5α). Antibiotic-resistant patterns of the transconjugates were the same as those of the donor (isolates) with the exception of *Staphylococcus* sp. Transconjugates of *Streptococcus* presented an inhibition halo of 10, 6, 18, 26, 17, 12, 13, and 8 mm; for *Micrococcus* 6, 6, 10, 24, 6, 6, 13, and 8 mm; and for *Staphylococcus* 42, 14, 28, 30, 30, 24, 25, and 23 mm of ampicillin, erythromycin, tetracycline, chloramphenicol, kanamycin, streptomycin, tobramycin, and rifampicin, respectively, from 50, 15, 36, 33, 30, 28, 25, and 25 mm of conjugates (Table 5). In *Staphylococcus* sp., there was no clear correlation of antibiotic resistance pattern when conjugated with the recipient,

Table 5. Antibiotic resistance pattern of *Escherichia coli* (recipient) and its transconjugates

Antibiotics	*E. coli* (mm)	*Streptococcus* – transconjugates (mm)	*Micrococcus* – transconjugates (mm)	*Staphylococcus* – transconjugates (mm)
Ampicillin	50	10	6	42
Erythromycin	15	6	6	14
Tetracycline	36	18	10	28
Chloramphenicol	33	26	24	30
Kanamycin	30	17	6	30
Streptomycin	28	12	6	24
Tobramycin	25	13	13	25
Rifampicin	25	8	8	23

which may be due to absence of plasmids in these isolates. Successful transconjugates of *Streptococcus* sp. and *Micrococcus* sp. showed clear antibiotic resistance patterns. A similar observation was seen in *Aeromonas salmonicida,* in which conjugation or mobilization of plasmids encoding tetracycline resistance alone or together with other drugs has been described and may contribute to the dissemination of plasmids (Schmidt et al., 2001; L'Abee-Lund and Sorum, 2002).

Based on the results of this study, it was concluded that this study might be helpful in understanding the microbiota in poultry litter environments. Further results indicated that plasmids are responsible for antibiotic resistance, suggesting the potential role of plasmids in horizontal transfer of antibiotic resistance in poultry litter environment.

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