ISSN Online: 2349-7785

Antibiotic Resistance Genes as Emerging Environmental Contaminants in Poultry Farm Environment from Various Districts in Tamil Nadu, South India-A Pilot Study

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Received 06 August 2016; accepted in revised form 29 August 2016

Abstract: Extensive use of antibiotics as growth promoters in poultry farms will select for antimicrobial resistance genes among bacterial isolates of broiler origin and may spread to environment and individuals in close contact thereby posing a threat to human health. Soil samples were collected from different poultry farms in various districts of Tamil Nadu to detect the percentage of antibiotic resistance and the resistance determinants in soil. Mean resistance levels were highly variable ranging between 59-93 % for tetracycline and 23-78 % for erythromycin respectively. The most frequent gene was *erm(A)* (56.2 %) followed by *tet(K)* (43.7 %) and *erm(C)* (32.2 %). Higher tetracycline and macrolide resistance determinants were observed inside the farms compared to outside.

Key words: Poultry farm,Tetracycline resistance, Erythromycin resistance.

Introduction

Poultry industry in India is a fast growing and dynamic subsector of agriculture and has been recognized as a vital sector for sustainable employment and income generation that ensures food security through egg and meat. India ranks third in poultry egg and meat production in the world. However, the poultry sector currently faces newer challenges related to evolution of antimicrobial resistance [1]. In modern poultry industry, antibiotics are used extensively not only for therapy and prophylaxis, but also as antimicrobial growth promoters (AMGPs) in animal feeds which will eventually select for resistant forms of bacteria, resulting in development of antimicrobial resistance (AMR) particularly in intestinal microbiota of broilers. A significant amount of the antibiotics administered are excreted by broilers, making their manure a potential source of both antibiotics and antibiotic-resistant bacteria which can enter soil and groundwater.Therefore, broiler manure is a source of AMR contamination and poses a potential risk to human health. This raises an important question of whether the use of antibiotics in animal food production poses a threat to human health [2]. In particular, the worry is that resistant forms of bacteria may spread from animals and/or the environment to humans. On a global scale, the use of antibiotics as animal growth promoters differs dramatically. In India, broilers are often exposed to β-lactam antibiotics, macrolides and tetracyclines. Tetracyclines and the MLS_B group of antibiotics (macrolides, lincosamides, and streptogramin B) are inexpensive and broadly effective and have been used at both therapeutic and non therapeutic levels in chickens for decades. Resistance to these antibiotics has frequently been observed in bacterial isolates from animal litter and farm soil [3]. Hence, the present study was undertaken to detect the percentage of antibiotic resistance and the resistance determinants in soil of various poultry farms

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from Tamil Nadu, South India.

Materials and methods

Eight broiler farms (housing over 5,000 birds) located in Nammakal, Salem and Thiruvallur districts of Tamil Nadu were included for the study. Soil samples were collected from different locations in each farm within 2 m of each other. The control soil was collected from soil sample which had no anthropogenic antibiotic input. All samples were collected in sterile plastic containers packed in dry ice and returned to the laboratory and then stored at 4°C [4]. Enumeration of resistant soil bacteria was done by spread plate technique in agar plates amended with either tetracycline (8 µg/ml) or erythromycin (8 µg/ml) [5]. Further, soil DNA was extracted by the FavorPrep Soil DNA Isolation Kit. This Soil DNA Isolation Mini Kit is designed for the isolation of total DNA from soil sample. Using this simple and rapid process, the soil samples were homogenized and lysed by the buffer containing glass beads, proteinase K and detergents. Finally, the purified DNA was eluted by low-salt elution buffer. DNA concentration and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). The eluent containing soil DNA was used in the PCR assay to screen for tetracycline [(*tet(K) & tet(M)*] and macrolide [*erm(A) & erm(C)*] resistant determinants. PCR was carried out in a 25 µl reaction mix containing 200 µM of dNTPs, 1X PCR buffer (Tris HCl [10 mM]; KCl [50 mM]; $MgCl_2$ [1.5 mM] and 0.5U *Taq* DNA polymerase (New England Biolabs, Inc, U.K), primers (10 pmol each) (table 1) and 5 ng of template DNA. Amplification was done using Mastercycler Gradient (Eppendorf) with the following cycling conditions (one cycle of initial denaturation at 95°C for 5 min, 29 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 55°C and extension at 72°C for 1 min followed by final extension at 72°C for 5 min [6]. The amplified products were run on 1.5 % agarose gel in Tris-Acetate EDTA (TAE) buffer for 45 min. The ethidium bromide stained bands were examined under an ultraviolet transilluminator (Carestream Gel Logic 212 PRO).

Results and discussion

Tamil Nadu is leading the state in broiler production and ranks second in the country's egg production with a production of 10.8 billion eggs. Tamil Nadu accounts for 17.71 per cent of the poultry population of the country. More than 90 per cent of poultry or poultry products exported from India originates from Tamil Nadu. At present poultry production is restricted to certain poultry belts such as Namakkal, Erode, Coimbatore and Thiruvallur districts. Antibiotics usage for longer duration in food-producing animals such as broilers in feed increases the rate of weight of poultry broiler and improves the efficiency of converting feed to meat. Development of antibiotic resistance is a major threat due to the extensive usage of β-lactam antibiotics,macrolides and tetracyclines antibiotics in poulty farms. Resistance to these antibiotics has frequently been observed in bacterial isolates from animal litter and farm soil [3].

In our study, we quantified antibiotic-resistant bacteria in soils at farms using antibiotics for non-therapeutic purposes viz., as growth promoters.

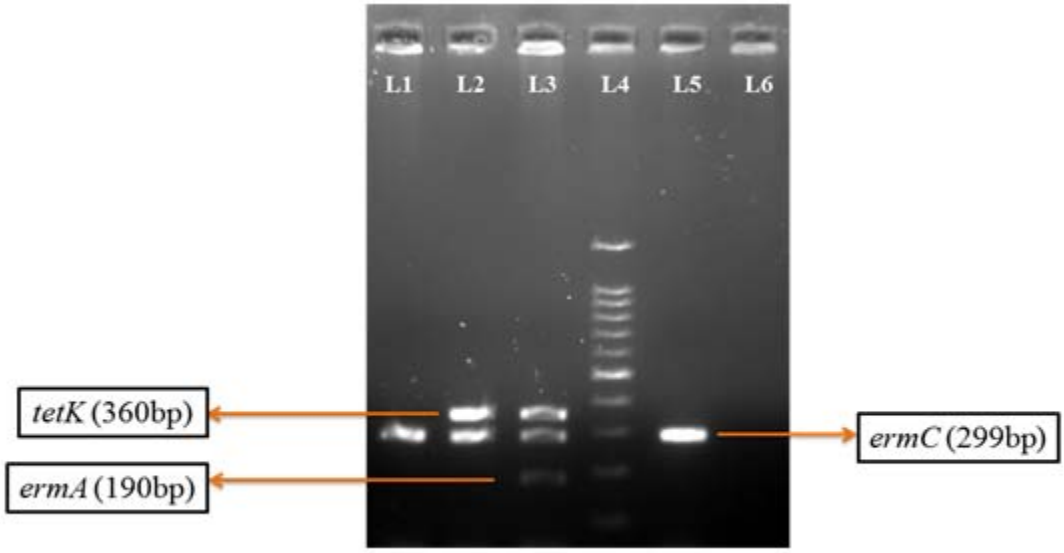
Table 1 List of Primer sequences used

Target gene	Primer sequences	Size
tet (K)	5' - GTA GCG ACA ATA GGT AAT AGT-3'	360 bp
	5' - GTA GTG ACA ATA AAC CTC CTA-3'	
tet (M)	5' - AGT GGA GCG ATT ACA GAA-3'	158 bp
	5' - CAT ATG TCC TGG CGT GTC TA-3'	
erm(A)	5' - AAG CGG TAA ACC CCT CTG A-3'	190 bp
	5' - TTC GCA AAT CCC TTC TCA AC-3'	
erm (C)	5' - AAT CGT CAA TTC CTG CAT GT-3'	299 bp
	5' - TAA TCG TG AAT ACG GGT TTG-3'	

The aim of this study was to discern the impact of antibiotic use on the occurrence and abundance of cultivable antibiotic-resistant bacteria in nearby soils exposed to animal waste [5]. This study investigated the prevalence and persistence of antimicrobial resistance genes such as tetracycline resistant determinants and erythromycin resistant determinants from poultry farm soil sample.

Bacteria resistant to tetracycline and erythromycin were detected in soil samples of eight different farms. Antibiotic resistance levels were calculated as the ratio of bacteria growing on plates supplemented with antibiotics compared to the number of bacteria growing on plates without antibiotics. The average resistance levels for each of the three soil samples collected at a site were used to calculate mean and standard deviation (s.d.) of resistance levels at a site. Mean resistance levels were highly variable for both antibiotics, ranging between 59-93 % and 23-78 %, respectively (Table 2). Statistical significance was observed among the 8 sites based on resistance levels. Enumeration of resistant bacteria showed that elevated levels of tetracycline and erythromycin resistance were quantified in the soils from inside the farms, but not outside the farms. When elevated levels of antibiotic-resistant bacteria were enumerated, significant shifts were also observed in types of genes that encode resistance within these soil sample DNA. However, a farm from Nammakal district showed elevated level of both phenotypic and genotypic erythromycin resistance even in soil outside the farm. This shows the significance of over usage of antibiotics which leads to dissemination of resistant bacteria outside the farm environment. Even though there are several genes encoding resistance to tetracycline and erythromycin resistance, in this limited study, we focused only on efflux mediated tetracycline resistance *tet (K)* & *tet (M)* and target site modification mediated erythromycin resistance *erm (A)* & *erm (C)* (Figure 1).

Among the genes tested, *erm(A)* was found in the soil of all 8 farms, whereas *tet(K)* (87.5 %) was found in 7 farms and *erm(C)* (75 %) was found in 6 farms. *tet(M)* was not detected in any of the samples. Soil samples collected outside two farms showed the presence of resistance determinants. Although the isolation rate of tetracycline and erythromycin resistant bacteria was high from soil samples, corresponding resistance genes were not detected among all the resistant samples indicating the presence of novel resistance determinants. Presence of the resistance determinants could make that poultry farm a potential reservoir of tetracycline and erythromycin resistance thereby causing environmental contamination.



L1: *ermC* positive; L2: *tetK* + *ermC* positive; L3: *tetK* + *ermC* + *ermA* positive;
L4: 100bp ladder; L5: *ermC* positive; L6: Negative Control

Figure 1. Multiplex PCR for detection of *tet K*, *erm A* and *erm C* genes

Table 2. Resistance levels in poultry farm environment

Sample Site		Phenotypic detection		Genotypic detection			
		Tetracycline resistant Level (%)	Erythromycin resistant Level (%)	Tetracycline resistant determinants		Macrolide resistant determinants	
				tet K gene	*tet M* gene	*erm A* gene	*erm C* gene
Farm-1	A1 (Inside)	88 ± 1.0	42 ± 1.5	Positive	Negative	Positive	Positive
(Namakkal district)	A2 (Outside)	n.d.	n.d.	Negative	Negative	Negative	Negative
Farm-2	B1(Inside)	67 ± 9.2	73 ± 5.3	Positive	Negative	Positive	Negative
(Namakkal district)	B2(Outside)	64 ± 3.5	n.d.	Negative	Negative	Negative	Negative
Farm-3	C1(Inside)	80 ± 1.5	49 ± 3.2	Positive	Negative	Positive	Negative
(Namakkal district)	C2 (Outside)	n.d.	n.d.	Negative	Negative	Negative	Negative
Farm-4	D1 (Inside)	59 ± 7.7	51 ± 1.0	Positive	Negative	Positive	Positive
(Salem district)	D2 (Outside)	n.d.	n.d.	Negative	Negative	Negative	Negative
Farm-5	E1 (Inside)	82 ± 7.5	23 ± 9.0	Positive	Negative	Positive	Positive
(Salem district)	E2 (Outside)	n.d.	n.d.	Negative	Negative	Positive	Negative
Farm-6	F1 (Inside)	89 ± 2.0	53 ± 5.8	Negative	Negative	Positive	Negative
(Thiruvallur district)	F2 (Outside)	n.d.	n.d.	Negative	Negative	Negative	Negative
Farm-7	G1 (Inside)	87 ± 2.5	66 ± 6.1	Positive	Negative	Positive	Positive
(Thiruvallur district)	G2 (Outside)	n.d.	n.d.	Negative	Negative	Negative	Negative
Farm-8	H1 (Inside)	93 ± 2.5	78 ± 8.7	Positive	Negative	Positive	Positive
(Thiruvallur district)	H2 (Outside)	86 ± 4.0	53 ± 3.2	Negative	Negative	Negative	Negative

n.d. - not detected

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