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Phenotypic and Genotypic Characterization of Antimicrobial Resistance of Enterobacteriaceae Isolated from Free Range Chicken in Andhra Pradesh

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A B S T R A C T

Antimicrobial resistance in bacteria from the family *Enterobacteriaceae* is an important indicator of the emergence of resistant bacterial strains in the gut microbiota. A total of 150 cloacal swabs were collected from free range chickens, 217 *Enterobacteriaceae* isolates were identified. This study investigated the antimicrobial susceptibility and resistance of commensal *Enterobacteriaceae* from free-range chickens to 14 antimicrobial agents using the disc diffusion method. Hundred per cent phenotypic resistance of the isolates was noticed against bacitracin, colistin, nitrofurazone, furazolidone, virginomycin, vancomycin and tylosine. Moderate resistance (70-25%) of isolates was noticed against doxycycline HCl, ampicillin, cefotaxime, ciprofloxacin and chloramphenicol. Least resistance (<25%) of isolates was recorded against enrofloxacin and gentamicin. The genotypic resistance of the *Enterobacteriaceae* to tetracycline was detected by targeting *tet-A* gene. About 90.9% of phenotypic resistant samples were found to harbour *tet-A* gene. Resistance to ampicillin was detected by targeting *bla*$_{TEM}$ gene and 90.55% of phenotypic resistant samples against ampicillin were found to have *bla*$_{TEM}$ gene.

Introduction

Backyard poultry keeping indeed constitutes an integral part of many households in rural areas. It plays an important role in providing additional income and high quality protein with negligible production input from the farmer. Free-range chickens reared under an extensive management system scavenge for food and receive little or no antimicrobials as feed additives. They are also highly resistant to different infectious diseases. Because of this reason and also of the rich taste and flavour of desi chicken there is preferential consumption of desi chicken meat and eggs.

In commercial poultry antimicrobials are used as growth promoters and also used for the

treatment of infections caused by common poultry pathogens such as *Salmonella*, *E.coli*, *Campylobacter* and *Clostridium*. There is emergence of multi drug resistant bacteria which are excreted in the faeces and gain entry in to the poultry litter. The poultry litter is usually used as fertilizer in agricultural fields, there by multi drug resistant bacteria can enter in to the environment. From the environment humans and other birds may acquire these resistant bacteria. Scavenging free-range chickens may also be asymptomatic carriers and shedders of resistant bacteria in their faces, thereby contaminating the environment. These pathogens can thus be transmitted to other animal hosts and humans where they produce disease.

Materials and Methods

Sample collection

A total of 150 cloacal swabs were collected from different villages in and around Tirupati, A.P. which include Chittoor, Venkatagiri, Tirupati, Nagalapuram, Pallam, Vampalli, B.Kandriga and Kalahasti.

Isolation and identification of bacteria

Cloacal swab samples from broth cultures were incubated aerobically at 37˚C for 24 hours and loop full of enriched broth culture was streaked onto nutrient agar plates. Isolated single colonies were identified and further streaked onto MacConkey agar. Characteristic lactose-fermenting and non-lactose fermenting colonies on MacConkey agar were picked up and further streaked onto EMB and XLD agar plates, respectively.

Members of *Enterobacteriaceae* were presumptively identified based on catalase (positive) and oxidase (negative) reactions. Lactose fermenting pink colonies on MacConkey agar and green metallic sheen colonies on EMB agar were presumptively identified as *E.coli* and were confirmed by IMVIC (++--) and TSI agar (Y/Y/H_2S –ve) tests. Lactose fermenting mucoid colonies on MacConkey agar and dark centered pale mucoid colonies on EMB agar were presumptively identified as *Klebsiella* spp. and were further confirmed by capsular staining, motility (negative) and IMVIC (--++) tests.

Lactose non-fermenting pale colonies on MacConkey agar, red or opaque colonies on BGA and black centered colonies on XLD agar were presumptively identified as *Salmonella* spp. and were confirmed by IMVIC (-+-+) and TSI agar (R/YH_2S +ve) tests.

Antimicrobial sensitivity test

All the identified isolates were subjected to antimicrobial sensitivity testing by disc diffusion method on Muller Hinton (MH) Agar (Bauer *et al*., 1966). Inhibition zone diameters were interpreted according to CLSI (2014) guidelines (M100-S24). MacFarland standard (0.5) equivalent to an approximate cell density of 1.5×10^8CFU/mL was used as reference to adjust turbidity of microbial suspensions. Commonly used antibiotics in commercial poultry farming were selected and ABST was performed.

A total of 14 antibiotic discs were selected in the present study. The discs used include ampicillin, bacitracin, cefotaxime, chloramphenicol, ciprofloxacin, colistin, doxycycline HCL, gentamicin, enrofloxacin, vancomycin, furazolidone, nitrofurazone, virginomycin and tylosine.

DNA extraction

DNA extraction was carried out by boiling and snap chilling method as described by Rao (2009) with minor modifications.

PCR

Resistance to tetracycline was detected by targeting *tet-A* gene as per the procedure described by Randall *et al.,* (2004). The *tet-A* gene was amplified by uniplex PCR using specific primers (F: GGTTCACTC GAACGACGTCA and R: CTGTCCG ACAAGTTGCATGA). Resistance to ampicillin was detected by targeting bla_{TEM} gene as per the procedure described by Dallenee *et al.,* (2010). bla_{TEM} gene was amplified by using specific primers (F:CATTTCCGTGTCGCCCTTATTC and R:CGTTCATCCATAGTTGCCTGAC) in uniplex PCR.

Results and Discussion

Isolation and Identification of *Enterobacteriaceae*

In this study, a total of 217 isolates with reference to the family *Enterobacteriaceae* were obtained from the faeces of free-range chickens**.** A total of 130 (59.9%) were characterized as *E. coli*, 42 (19.35%) were characterized as *Salmonella* spp. and 45 (20.73%) isolates were characterized as *Klebsiella* spp. In a study conducted by Ojo *et al.,* (2012) in Nigeria, out of 184 isolates tested, the most prevalent bacteria of free range chicken were reported to be *E.coli*, *Klebsiella* spp. and *Salmonella* spp.

Phenotypic antimicrobial resistance of *Enterobacteriaceae*

Overall, Hundred percent phenotypic resistance was noticed against bacitracin, colistin, nitrofurazone, furazolidone, virginomycin, vancomycin and Tylosine. Moderate resistance (70-25%) was noticed against Doxycycline HCl, ampicillin, cefotaxime, ciprofloxacin and chloramphenicol. Least resistance (<25%) was recorded against enrofloxacin and gentamicin (Table 1 and Fig. 1). In a similar study conducted by Odomene and Enwere (2018) from chicken slaughter houses of Nigeria, higher phenotypic resistance levels were reported to chloramphenicol (75.9%), amoxicillin (73.4%), gentamicin (44.3%) and ciprofloxacin (20.3%).

Antimicrobial resistance of *E.coli*

All the *E.coli* isolates were found to be resistant to bacitracin, colistin, furazolidone, nitrofurazone, virginomycin, vancomycin and tylosine. A total of 101 (77.69%) isolates showed resistance to Doxycycline HCl. 93 out of 130 (71.53%), 20 (15.38%), 25 (19.23%), 38 (29.23%) and 20 (15.30%) isolates were resistant to ampicillin, cefotaxime, chloramphenicol, ciprofloxacin and enrofloxacin respectively. Lowest resistance of 10.76% (14 isolates) was observed against gentamicin (Table 2). In a study conducted by Obeng *et al.,* (2012) from Nigeria, two hundred fifty one strains of *E.coli* were isolated from backyard chicken. 40.6 and 26.7% isolates were resistant to tetracycline and ampicillin. *E.coli* isolates from free range chicken of Tanzania showed 75% resistance to tetracycline followed by 63.63% resistance to ampicillin. 54, 53 and 29% resistance was observed against ofloxacin, cotrimoxazole and cefotaxime. The least resistance was observed against chloramphenicol (5%) and cefoxitin (6%) (Hamisi *et al*., 2014).

Antimicrobial resistance of *Salmonella*

A total of 42 *Salmonella* isolates showed resistance to bacitracin, colistin, furazolidone, nitrofurazone, vancomycin, virginomycin and tylosine. For Doxycycline HCl, 31 (73.80%) isolates showed resistance. 16 (38.09%), 20 (47.61%), 15 (35.71%), 12 (28.57%) and 10 (23.80%) isolates were resistant to ampicillin, cefotaxime, ciprofloxacin, chloramphenicol

and enrofloxacin respectively. Lowest resistance of 16.6% (7 isolates) was noticed against gentamicin (Table 2). In a similar study conducted by Samanta *et al.*, (2014) antibiotic resistance patterns of *Salmonella* spp. from backyard poultry showed 100% resistance against chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, norfloxacin and oxytetracycline. In a study conducted by Ghoddusi *et al*., 2015 in Northern Iran, 44 *Salmonella* were isolated from backyard chicken. 100% resistance was observed against tetracycline and doxycycline followed by chloramphenicol (79%) and florfenicol (72%) respectively.

Antimicrobial resistance of *Klebsiella*

All the *Klebsiella* spp. isolates were resistant to bacitracin, colistin, furazolidone, nitrofurazone, vancomycin, virginomycin and tylosine. A total of 22 (48.8%) isolates were resistant to Doxycycline. 18 (40%), 18 (40%), 14 (31.1%), 12 (26.6%) and 12 (26.6%) isolates were resistant to ampicillin, cefotaxime, ciprofloxacin, chloramphenicol and enrofloxacin respectively. For gentamicin eleven (24.4%) isolates showed resistance (Table 2). In a study conducted by Hyati *et al.*, (2019) from East Java, *Klebsiella* spp. showed 100% phenotypic resistance against oxytetracycline, ampicillin and amoxicillin. 90.9, 54.5, 27.3 and 18.2% resistance was observed against colistin, doxycycline Hcl, ciprofloxacin and enrofloxacin respectively.

Genotypic resistance of *Enterobacteriaceae*

In the present study genotypic resistance of tetracycline and ampicillin was studied. As on today, 38 tetracycline resistance genes have been identified in various bacterial organisms. Out of which, 23 genes are related to efflux proteins such as *tet A*, *tet B* etc. The efflux genes are the most commonly reported genes for tetracycline resistance in *Enterobacteriaceae* isolates obtained from environment, animals and humans. *Tet A* gene is the most important and prevalent gene among all the resistance genes (Sigirci *et al*., 2019).

All the phenotypically tetracycline resistant *Enterobacteriaceae* isolates (154) were subjected to PCR, targeting 577 bp region of *tet A*. Out of 154 phenotypically tetracycline resistant *Enterobacteriaceae* isolates, 140 samples were found to harbour *tet A* gene and 14 samples did not possess *tet A* gene (Table 3 and Fig. 2). Out of 101, 22 and 31 *E.coli*, *Klebsiella* and *Salmonella* tested, 91 (90.09%), 20 (90.09%) and 29 (93.54%) were found to possess *tet A* gene respectively. 90.9% positive isolates harboured *tet-A* gene. Tetracycline resistance of avian pathogens has been reported by several workers (Kolar *et al.*, 2002; Omoya and Ajayi, 2016; Ayandiran *et al*., 2018). In a similar study conducted by Obeng *et al.*, (2012), 32.6% (63/193) of the *E.coli* isolates from free range chicken in Australia were phenotypically tetracycline resistant. But *tet A* was detected in only 76% (48/63) of the tetracycline resistant isolates. Al Bahry *et al.*, (2013) isolated 46 *E.coli* from Oman and screened for tetracycline resistance. About 85 and 80% of the samples possessed *tet A* and *tet B* genes respectively.

In the present study, PCR was standardized targeting 800 bp of bla_{TEM} gene. Out of 127 samples which were phenotypically resistant to ampicillin, bla_{TEM} could be detected in 115 (90.55%) samples (Table 3 and Fig. 3). 12 samples which were phenotypically resistant to ampicillin didn't show the presence of bla_{TEM} gene. Out of 93, 18 and 16 *E.coli*, *Klebsiella* and *Salmonella* tested, 85 (91.39%), 14 (77.77%) and 16 (100%) were found possess bla_{TEM} gene respectively. Around 90.55% of phenotypically positive isolates harboured bla_{TEM} gene.

Table.1 Phenotypic antimicrobial resistance in gut microbiota of desi chicken

S.No	Name of antibiotic	No. of samples tested	No. Resistant (%)
1.	Bacitracin	217	217 (100)
2.	Colistin	217	217 (100)
3.	Furazolidone	217	217 (100)
4.	Nitrofurazone	217	217 (100)
5.	Vancomycin	217	217 (100)
6.	Virginomycin	217	217 (100)
7.	Tylosine	217	217 (100)
8.	Doxycycline Hcl	217	154 (70.96)
9.	Ampicillin	217	127 (58.52)
10.	Cefotaxime	217	58 (26.72)
11.	Ciprofloxacin	217	67 (30.8)
12.	Chloramphenicol	217	49 (22.5)
13.	Enrofloxacin	217	38 (17.5)
14.	Gentamicin	217	35 (16.1)

Table.2 Antimicrobial resistance in *E.coli*, *Salmonella* spp. and *Klebsiella* spp.

	E.coli		***Salmonella* spp.**		***Klebsiella* spp.**	
Name of antibiotic	**No. of samples tested**	**No. Resistant (%)**	**No. of samples tested**	**No. Resistant (%)**	**No. of samples tested**	**No. Resistant (%)**
Bacitracin	130	130 (100)	42	42 (100)	45	45 (100)
Colistin	130	130 (100)	42	42 (100)	45	45 (100)
Furazolidone	130	130 (100)	42	42 (100)	45	45 (100)
Nitrofurazone	130	130 (100)	42	42 (100)	45	45 (100)
Vancomycin	130	130 (100)	42	42 (100)	45	45 (100)
Virginomycin	130	130 (100)	42	42 (100)	45	45 (100)
Tylosine	130	130 (100)	42	42 (100)	45	45 (100)
Doxycycline Hcl	130	101 (77.69)	42	31 (73.80)	45	22 (48.8)
Ampicillin	130	93 (71.53)	42	16 (38.09)	45	18 (40)
Ciprofloxacin	130	38 (29.23)	42	15 (35.71)	45	14 (31.1)
Chloramphenicol	130	25 (19.23)	42	12 (28.57)	45	12 (26.6)
Cefotaxime	130	20 (15.38)	42	15 (35.71)	45	18 (40)
Gentamicin	130	14 (10.76)	42	7 (16.6)	45	11 (24.4)
Enrofloxacin	130	20 (15.38)	42	10 (23.80)	45	12 (26.6)

Table.3 Genotypic resistance to tetracycline (*tet A* gene) and ampicillin (bla_{TEM}gene) in enteric bacteria of free range chicken

Gut microbiota	No. of samples tested	Resistance to *tet A* (%)	No. of samples tested	Resistance to bla_{TEM} (%)
E.coli	101	91 (90.09)	93	85 (91.39)
***Salmonella* spp.**	31	29 (93.54)	16	16 (100)
***Klebsiella* spp.**	22	20 (90.09)	18	14 (77.77)

Fig.1 Antibiotic resistance in desi chicken of enteric bacteria

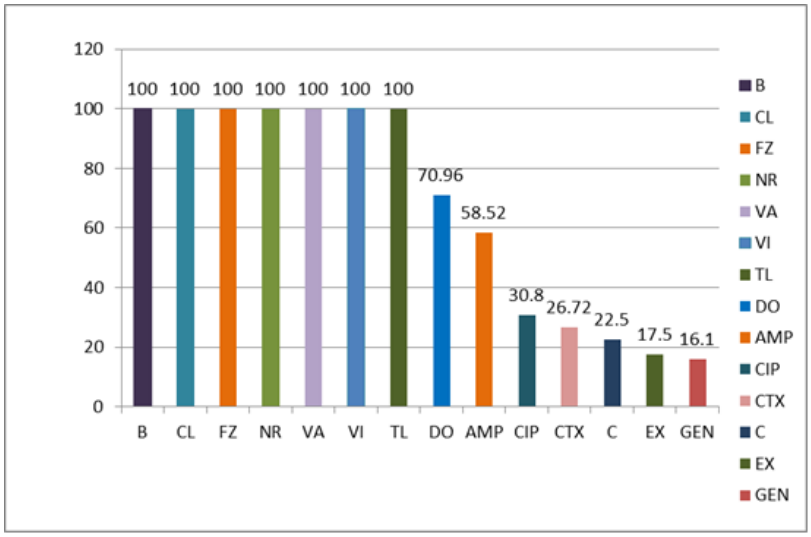
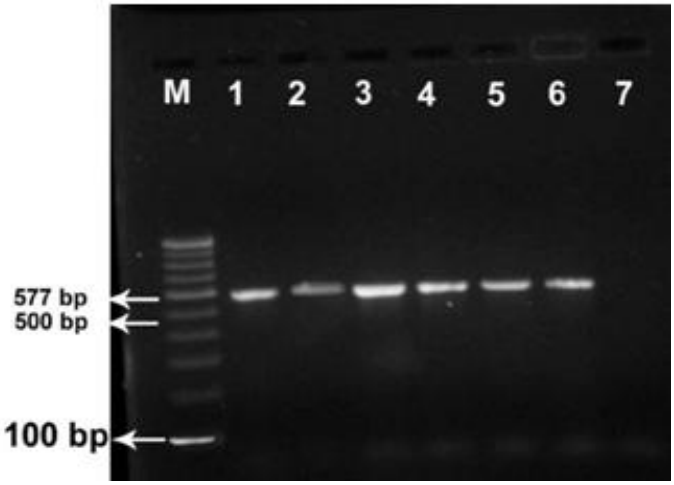
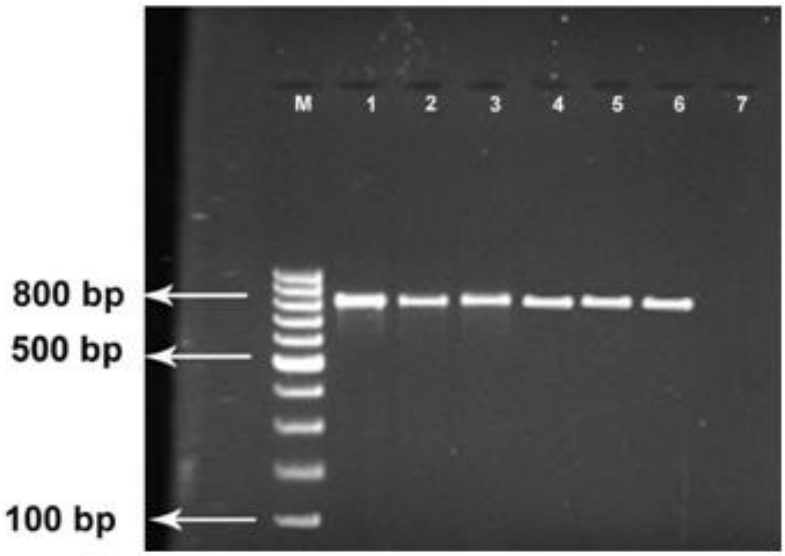


Fig.2 Detection of *tet A* gene in *Enterobacteriaceae* of desi chicken



Lane M Molecular weight marker (100bp)
Lane 1 Positive control for *tet A* gene (577bp)
Lane 2 to 6 Desi chicken microbiota carrying *tet A* gene and
Lane 7 Negative control

Fig.3 Detection of *bla- TEM* gene in *Enterobacteriaceae* of Desi chicken



Lane M : Molecular weight marker (100bp)
Lane 1 : Positive control for *bla-TEM* gene (800bp)
Lane 2 to 6: Desi chicken microbiota carrying *bla-TEM* gene
Lane 7 : Negative control

Similar findings of ampicillin resistance were reported in chicken worldwide (Kar *et al*., 2015; Chishimba *et al*., 2016 and Sailu *et al*., 2017). Younis *et al*., (2017) from Egypt reported 78% resistance to ampicillin among the *E.coli* isolates of poultry origin by PCR targeting bla_{TEM} gene. In a study conducted by Langata *et al*., (2019) in Nairobi, 43% of phenotypically ampicillin resistant *E.coli* isolates were positive for bla_{TEM} gene in PCR.

In conclusion, the results of the present study revealed the phenotypic and genotypic antibiotic resistance in the enteric microbiota of desi chicken. Though antibiotics are not being used in the rearing of desi chicken, they might have acquired the resistant bacteria from the environment and may spread these bacteria to other animals and humans. Enteric bacteria especially, the organisms belonging to family *Enterobacteriaceae* serve as the indicator organisms for assessing the antimicrobial resistance.

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