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# Tetracycline efflux pump genes in *Escherichia coli* from retail chicken in central Kerala

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**Abstract**

*The rearing of chicken in India has undergone a drastic change from backyard production to commercial intensive farming. This has led to the use of antibiotics in therapy, metaphylaxis and as growth promoters. Escherichia coli are commensals that inhabit the gut of man and animals. The detection of virulent pathotypes of E. coli in chicken is a huge threat to human health. The present study assessed 200 retail chicken sold in central Kerala for the presence of virulent E. coli and studied the tetracycline susceptibility followed by detection of tetA and tetB gene. E. coli was detected in 64 per cent of the samples. The virulence genes, eaeA and aggR were detected in 52.2 and 3.9 per cent of the E. coli isolates, respectively. Tetracycline resistance by antibiotic susceptibility testing (ABST) was found in 30.84 per cent of the virulent isolates. The tetracycline efflux pump protein coding genes, tetA and tetB were detected in 56.67 and 25 per cent, respectively. The detection of drug resistant bacteria is a threat to public health as tetracycline is classified as a highly important antibiotic in human medicine.*

**Keywords**: *E. coli, tetracycline resistance, tetA, tetB*

Chicken is a cheap source of animal protein and it is relished because of its versatility in cuisine and because no religious or cultural taboos are associated with its consumption (Khara *et al.,* 2020). In order to meet the growing demand, chicken rearing in India has undergone a paradigm shift from mere backyard rearing to a commercial enterprise. In order to cater to the boost in demand, antibiotics are being rampantly used in metaphylaxis, therapy and also in feed as growth promoters. Promiscuous use of antibiotics leads to increase in the incidence of drug resistant bacteria.

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*Escherichia coli* are commensals which reside in the gut of poultry. Improper dressing practices leads to the contamination of chicken with faecal matter thereby contributing to the presence of *E. coli* in the meat. Most of the strains of *E. coli* are usually harmless but a few strains are highly pathogenic. *Escherichia coli* strains that cause enteric disease in their hosts are called diarrhoeagenic *E. coli* (DEC) and their pathogenesis is associated with a number of virulence attributes which varies with the pathotypes (Xia *et al.,* 2010). Intimin is a 94-kDa outer membrane protein encoded by the *eae*A gene which is required for the intimate attachment of bacteria with the enterocyte membranes (Trabulsi *et al*., 2002). The *agg*R gene is a transcriptional regulator which regulates the expression of aggregative adherence fimbriae in enteroaggregative *E. coli*. Penetration into the epithelial cells and dissemination from cell to cell in enteroinvasive *E. coli* are mediated by an invasion-associated locus (*ial*), located on a plasmid and the invasion plasmid antigen H (*ipaH*) genes present in both chromosome and plasmid (Farajzadeh-Sheikh *et al*., 2020).These organisms are notorious for their role in the transmission of antimicrobial resistance (AMR) as faecal microbes acts as reservoirs of AMR (Purohit *et al.,* 2019).These organisms are notorious for their role in the transmission of antimicrobial resistance (AMR) as faecal microbes acts as reservoirs of AMR (Purohit *et al.,* 2019).

Tetracyclines are broad spectrum antibiotic and are popular because of their efficacy and low cost (Zibandeh *et al*., 2016). They are used in therapy and also as growth promoters as they have been proven to increase the weight of the birds. The first generation tetracyclines such as oxytetracycline and chlortetracycline have been used as growth promoters for decades. Second generation tetracyclines such as minocycline and doxycyline have been used in metaphylaxis and therapy. The indiscriminate use of tetracycline has led to the selection of tetracycline resistant bacteria (Koo and Woo, 2011). The present study was envisaged with the objective of studying the prevalence of *E. coli* in retail chicken sold in central Kerala, occurrence of virulence genes, assess the susceptibility of the isolates to tetracycline and finally to check if the isolates harbour the efflux pump encoding genes, *tet*A and *tet*B.

**Materials and methods**

Hundred retail chicken samples each were collected from two central districts of Kerala *viz.*, Thrissur and Ernakulam. Each sample of chicken consisted of 250g and included portions of neck, breast and thigh (Bhandari *et al*., 2013). The chicken samples were brought to the laboratory immediately under refrigeration conditions. The isolation of *E. coli* was done according to the procedure of Meng *et al.* (2001). Briefly, from each pooled composite chicken meat sample, a 25 g portion was aseptically removed using sterile scissors and forceps. The sample was transferred to 225 mL volumes of buffered peptone water (Difco) in sterile stomacher bags, processed for 120 s. in a stomacher (Smasher, AES, France) and incubated at 37°C for 18 h. Following incubation, a loopful was transferred to Mac Conkey agar and incubated at 37°C for 24 h. At the end of incubation, lactose fermenting bright pink coloured colonies surrounded by bile precipitate was selected based on morphology and subjected to biochemical tests as described by Barrow and Feltham (1993). The isolates confirmed as *E. coli* by culture were subjected to snap chill method for the extraction of DNA (Swetha *et al*., 2015).

The DNA was subjected to PCR for the detection of virulence genes, *eae*A, *agg*R and *ipa*H using the primers as shown in Table 1. Multiplex PCR was performed in a final volume of 25 µL reaction mixture using 3 µL of extracted DNA as template. The cyclic conditions were standardised in the study: initial denaturation at 95°C for 5 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C at 40 s followed by final extension at 72°C for 10 min.

All the isolates that were positive for virulence genes were subjected to antibiotic susceptibility test against tetracycline using standard disc diffusion method (Skočková *et al*., 2015) using tetracycline disc (HiMedia)

with a concentration of 30µg. The isolates that showed resistance to tetracycline by phenotypic method were subjected to two separate PCR for the detection of *tet*A and *tet*B genes. The reaction mixture included, 2.5 µL of 10X PCR buffer, 2 µL of 25m*M* $MgCl_2$, 0.50 µL of *Taq* DNA polymerase (5Units/µL), 0.50 µL of dNTP Mix (10m*M*), 10 pmoles/µL each of forward and reverse primers of *tet*A and *tet*B and nuclease free water made upto 25 µL total volume. The primers used for the detection of *tet*A and *tet*B are shown in Table 2.

The cyclic condition of PCR for *tet*A gene included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 59.5°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C at 5 min. The cyclic conditions followed for *tet*B gene was as follows; initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 66°C for 30 s, extension at 72°C for 45 s which was followed by final extension at 72°C for 5 min.

**Results and discussion**

The prevalence of *E. coli* by culture was found to be 67 and 61 per cent from Thrissur and Ernakulam, respectively, with an overall prevalence of the organism from central Kerala being 64 per cent. The results are similar to studies in Bangladesh, where a prevalence of 63.5 per cent was reported (Rahman *et al.*, 2020). However, a higher prevalence of 87.5 per cent was reported by Eyi and Arslan (2012) from Turkey and an occurrence of 76.5 per cent was reported from coacal swabs of chicken from Kollam district of Kerala (Afsal *et al*., 2021). A study from Wayanad in Kerala reported the occurrence of *E. coli* in 36.11 per cent of cloacal swabs from broiler chicken which is much lower than the present study (Sathya *et al*., 2019). There was no significant statistical difference ($p \geq 0.01$) in the prevalence of the organism between the two districts.

Of the three virulence genes, only *eae*A and *agg*R were detected. None of the isolates harboured *ipa*H gene. The amplicons were obtained at 209 and 254bp for *eae*A and *agg*R respectively (Fig. 1). The *eae*A gene was detected in 86.57 and 77.05 from Thrissur and Ernakulam, respectively. Totally, the gene was detected in 81.81 per cent of the isolates. This accounts for the presence of *eae*A in 52.2 per cent of the retail chicken tested. This is in accordance with the study by Wang *et al.* (2017), where *eae*A was detected in 50 per cent of the chicken tested in Japan. The *agg*R gene was detected from 2.99 and 4.92 per cent of the *E. coli* isolates from Thrissur and Ernakulam. Overall, *agg*R was detected in 3.9 per cent of the isolates. However, a higher per cent of occurrence of *agg*R (24.44) was detected by Kagambega *et al*. (2012) from West Africa. A study from Mumbai in India by Godambe *et al*. (2017) could not detect either *eae*A or *agg*R from any of the *E. coli* isolates from chicken.

Of the 107 isolates of virulent *E. coli* obtained from central Kerala, resistance to tetracycline was detected in 33 isolates (30.84 per cent), whereas intermediate susceptibility was noted in 27 isolates (25.23 per cent). The results are in accordance with that of Skočková *et al*. (2015), from Czech Republic, where the level of resistance to tetracycline was reported to be 34.5 per cent. However, studies by Ingram *et al*. (2013), Chakravarty *et al*. (2015) and Bhardwaj *et al*. (2021) from Western Australia and different parts of India, respectively reported cent per cent resistance to tetracycline. Both the tetracycline resistant and intermediate susceptible isolates were subjected to PCR for the detection of *tet*A and *tet*B genes. The amplicons for *tet*A and *tet*B were obtained at 209 and 169 bp, respectively (Fig. 2). The district-wise distribution of the tetracycline resistance genes *tet*A and *tet*B is shown in Table 3. The *tet*A gene was detected in 75.76 per cent and 33.33 per cent of the resistant and intermediate susceptible isolates, respectively. Whereas, *tet*B was detected in 39.39 and 7.40 per cent of resistant and intermediate isolates, respectively. Both *tet*A and *tet*B together were detected in 30.30 per cent of resistant and 3.70 per cent of intermediate susceptible isolates. Overall *tet*A was detected in 56.67 per cent which is in accordance with a study in Norway where *tet*A was detected in 55 per cent of resistant isolates (Sunde and Norström, 2006). Nevertheless, a higher level of detection of 89.5 and 74 per cent was reported by Van *et al*. (2008) and Bhardwaj

**Table 1.** Primers used for the virulence genes of *E. coli*

| Primer | Primer sequence | Size (bp) | Reference |
|---|---|---|---|
| *eae*A F | 5'- TCCTGGTTCCCTTATCAACG-3' | 209 | In-house designed |
| *eae*A R | 5'- GCGACCGCTACCAACATAG-3' | | |
| *agg*R F | 5'-GTATACACAAAAGAAGGAAGC-3' | 254 | Ratchtrachenchai *et al.* (1997) |
| *agg*R R | 5'-ACAGAATCGTCAGCATCAGC-3' | | |
| *ipa*H F | 5' -TCACATTGCCCATTTGTACG- 3' | 295 | In-house designed |
| *ipa*H R | 5' -GCAGAGACGGTATCGGAAAG-3' | | |

**Table 2.** Primers used for the identification of *tet*A and *tet*B genes

| Primer | Primer sequence | Size (bp) | Reference |
|---|---|---|---|
| *tet*A F | 5'- F GCTACATCCTGCTTGCCTTC-3' | 209 | In-house designed |
| *tet*A *R* | 5'- ATAGATCGCCGTGAAGAGGA -3' | | |
| *tet*B F | 5'- TCAGCGCAATTGATAGGCCA -3' | 169 | In-house designed |
| *tet*B R | 5'- TTTCGCCCCATTTAGTGGCT -3' | | |

**Table 3.** District – wise distribution of *tet* genes

| Sl. No. | District | Resistant isolates | | | | | | | Intermediate susceptibility | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of isolates | *tet*A | | *tet*B | | *tet*A and *tet*B | | No. of isolates | *tet*A | | *tet*B | | *tet*A and *tet*B | | |
| | | | No. | % | No. | % | No. | % | | No. | % | No. | % | No. | % | |
| 1 | Thrissur | 15 | 11 | 73.33 | 1 | 6.67 | 1 | 6.67 | 20 | 7 | 35.00 | 2 | 10.00 | 1 | 5.00 | |
| 2 | Ernakulam | 18 | 14 | 77.78 | 12 | 66.67 | 9 | 50.00 | 7 | 2 | 28.57 | 0 | 0.00 | 0 | 0.00 | |
| 3 | Total | 33 | 25 | 75.76 | 13 | 39.39 | 10 | 30.30 | 27 | 9 | 33.33 | 2 | 7.40 | 1 | 3.70 | |

*et al*. (2021), respectively. The *tet*B gene was detected in 25 per cent of the tetracycline resistant and intermediary isolates. However a study in Korea (Koo and Woo, 2011) reported a higher occurrence of *tet*B gene (41.3 per cent). Only 13 per cent of the *E. coli* isolates obtained from chicken in Karnataka, India harboured *tet*B gene (Bhardwaj *et al*., 2021). The combined presence of both *tet*A and *tet*B was detected in 18.33 per cent of the isolates. This is contrary to a study in Vietnam, where both the genes were present at the same level of 89.5 per cent (Van *et al*., 2008).

Till date three resistance mechanisms to tetracycline have been recorded. The efflux pump proteins are one of the best studied mechanisms. These efflux pumps push the drug out of the bacterial cell using an energy dependent process. Twenty three different efflux pump genes are identified so far of which the most common ones being *tet*A and *tet*B. The *tet*A codes for resistance to tetracycline but not minocycline. On the other hand, *tet*B codes for resistance to both tetracycline and minocycline. Another mechanism of tetracycline resistance is by ribosomal protection coded by 11 genes. Yet another mechanism of resistance is by enzymatic inactivation of tetracycline by three genes (Roberts and Schwarz, 2017). The present study targeted the two most common genes coding for the efflux pump. Thus, not all the isolates that showed resistance to tetracycline by phenotypic methods were confirmed with the molecular tools.

The study highlights the presence of tetracycline resistant *E. coli* among healthy broiler chicken sold in central Kerala. The detection of drug resistant bacteria is a threat to public health as tetracycline especially chlortetracycline is classified as a highly important antibiotic to be reserved for use in humans and is listed as one of the critically important antibiotic published by the World Health Organisation (WHO, 2019). In order to

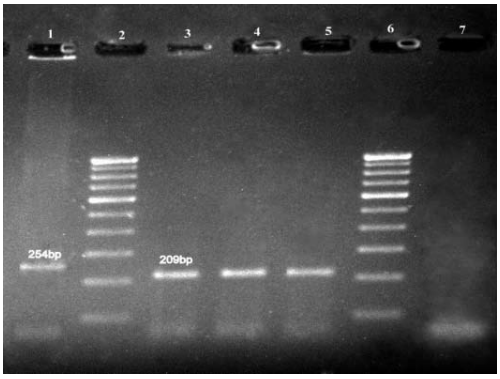


**Fig. 1.** Detection of virulence genes of *E. coli* by PCR.
Lane 1- *agg*R Lane 2 - 100 bp ladder Lane 3-5 – *eae*A Lane 6 - 100 bp ladder Lane 7 - Negative control

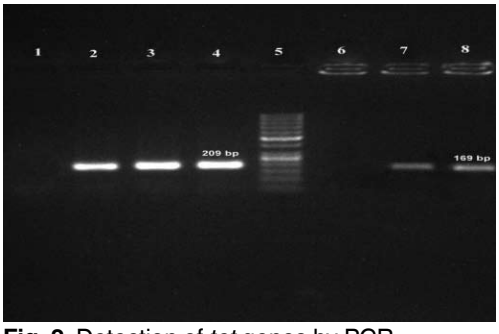


**Fig. 2.** Detection of *tet* genes by PCR

Lane 1 - Negative control *tet*A Lane 2 - 4 *tet*A Lane 5 - 50 bp ladder Lane 6 - negative control *tet*B Lane 7-8 – *tet*B

curtail the spread of antimicrobial resistance, a multisectoral and multifaceted one health approach must be adopted in addition to antibiotic stewardship, infection control and strict regulations on the use of antimicrobials.

**Conclusion**

Tetracycline is listed as an important antibiotic, because of its significance in human medicine. Confirmation of resistance against tetracycline in *E. coli* isolated from chicken is a great threat to public health. Furthermore, the organism is also known for the transfer of drug resistance genes not only within the same genera but also among other bacterial genera which again complicates the issue. Hence the results of the present study emphasises the need to use tetracycline with caution in poultry production so as to curb the spread of AMR through the food chain.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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