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# Antibiotic Resistance Pattern and Phylogenetic Analysis of Commensal *Escherichia coli* Isolated from Poultry

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**In the present investigation a total of 40 isolates of *Escherichia coli* from healthy poultry birds were investigated for antibiotic resistance pattern and distribution of various phylogroups in different age groups of birds. All the isolates showed green metallic sheen on Eosin Methylene Blue agar plate and were confirmed by various biochemical test. Genotypic confirmation by 16S rRNA gene revealed an amplicon of 662bp in all *Escherichia coli* isolates. The antibiogram pattern showed high distribution of resistance to multiple drugs among all the isolates with at least each isolate being resistant to three or more antibiotics. The antibiogram results showed that 100% of the isolates were resistant to penicillin, oxacillin and nalidixic acid. Resistance level was more than 50% for doxycycline (97.5), tetracycline (90%), erythromycin (85%), rifampicin (80%) and trimethoprim (75%). The study revealed that amikacin and levofloxacin were the most effective drugs. Out of the four main phylogenetic groups (A, B1, B2, and D), only two phylogroups viz A and B1 were obtained by multiplex PCR with a prevalence of 62.5% and 37.5% respectively.**

**Key words**: *Escherichia coli*, poultry, PCR, Phylogroup, Antibiotic resistance.

Over the recent few decades the problem of bacteria becoming resistant to various antibiotics is on the rise globally especially in developing countries like India (Carlet *et al.*, 2014). The resistance mounting due to the selective pressure of antibiotics has become a somber concern and is presently most interesting area of research not only in pathogenic bacteria but also in commensals. Various commensal bacteria like *Escherichia coli* can obtain and transfer various resistance determinants easily to other virulent bacteria and can set hurdles for controlling disease (Kargar *et al*., 2014).

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Chickens are one of the important food animals which are reared under intense use of antibiotics. The imprudent usage of antibiotics as growth promoters and for therapeutic purpose has paved the way for the emergence of multidrug-resistant *E. coli*. These MDR *E. coli* can pass to humans via contaminated poultry carcasses. As a result, the resistance determinants originating from poultry MDR *E. coli* can get circulated among human population leading to intractable diseases (Smith *et al*., 2007).

*Escherichia coli* belong to four phylogroups viz A, B1, B2 and D which are mainly prevalent among human and animals (Herzer *et al.*, 1990; Clermont *et al.*, 2000). This categorization of clonal population of *E. coli* has helped in differentiating the strains and understanding the evolution of extra intestinal pathogenic *E. coli*. Even though, the phylogenetic structure of *E. coli*

has been extensively explored in several pathogenic and commensal isolates from human populations, very little literature is available about the distribution of clonal groups among poultry birds (Hiki *et al.*, 2014). Consequently, it becomes compulsory to determine the intraspecies phylogenetic relationships of *E. coli* isolates from the normal gut flora of healthy birds to create a background catalog for further studies on pathogenic strains.

## MATERIALS AND METHODS

### Sampling and Isolation

A total of 40 faecal samples were collected from different age groups (day old chick: 10 samples, ten day old: 10 samples, twenty day old: 10 samples and finisher: 10 samples) of healthy poultry birds. The samples were collected aseptically with the help of sterile cotton swabs soaked in nutrient broth from cloacae and placed in sterile test tubes, taking all precautions to avoid contamination. The samples were transported to the laboratory as soon as possible.

Fecal samples were immediately inoculated into nutrient broth (HiMedia, Mumbai, India). After overnight incubation at 37°C, all broth cultures were inoculated on MacConkey (HiMedia) plates for isolation of *E. coli*. After overnight incubation at 37°C, pink colonies were picked up and subcultured on Eosin Methylene Blue (EMB) agar plates to observe the characteristic metallic sheen of the *E. coli*. The well-separated colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests as described by Buchanan & Gibbon (1994).

### Genotypic characterization

DNA isolation for *E. coli* was carried out as per the method of Chin and Kou (1993) with some modifications. All the isolates were subjected to *16S rRNA* species specific PCR using forward primer 5' -GCTTGACACTGAACATTGAG-3' and reverse primer 5' -GCACTTATCTCTTCCGCATT-3' as per the method described by Khaled *et al.* (2010).

### Triplex PCR for identification of phylogenetic group of *E. coli*

Triplex PCR for the identification of various phylogenetic group of *E. coli* was done as per the method described by Clermont *et al.* (2000). The primers used in the study are shown in the table 1. Thermal cycling conditions included an initial 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final extension at 72°C for 10 min. PCR reaction products were resolved on 1.2% agarose gels stained with ethidium bromide.

### Antibiotic sensitivity test

The method of disc diffusion (Bauer *et al.*, 1966) was used to determine the antibiogram pattern of the isolates against 27 different antibiotics (table 2) using CLSI guidelines.

## RESULTS AND DISCUSSION

A total of 40 isolates were recovered in the study which showed pink colonies on MCA and fermented lactose. All the isolates produced green metallic seen on EMB agar characteristic of *E. coli*. In species specific PCR, all tentatively positive isolates showed an amplicon of 662 bp confirming all isolates to be *E. coli* (Fig. 1).

Phylogenetic group analysis revealed that out of 40 isolates, 26 (65%) belonged to phylogenetic group A and 14 (35%) isolates were categorized in group B1. From 26 isolates of phylogroup A, 16 isolates produced an amplicon of 211bp whereas no band was obtained in 10 isolates (Fig. 2). Rest of the 14 isolates belonged

**Table 1.** The sequence of primers used in this study with the respective product sizes

| Type of Gene | Primer sequence 5' -3' | Product size (bp) |
|---|---|---|
| *ChuA* F | GACGAACCAACGGTCAGGAT | 279 |
| *chuA* R | TGCCGCCAGTACCAAAGACA | |
| *yjaA* F | TGAAGTGTCAGGAGACGCTG | 211 |
| *yjaA* R | ATGGAGAATGCGTTCCTCAAC | |
| *TspE4C2* F | GAGTAATGTCGGGGCATTCA | 152 |
| *TspE4C2* R | CGCGCCAACAAAGTATTACG | |

to phylogroup B produced an amplicon of 152 bp (Fig. 2).

In the back drop of great diversity among *E. coli* strains with most of the infections arising from fecal flora, this study recorded only two phylogroups (A and B1), out of the four phylogroups described. Among strains derived from day old chicks, 8 belonged to group B1 and 2 to the A group. The majority of strains derived from ten-day old birds showed higher number of group A similarity (n=9) compared to B1 (n=1) . Similar trend was found in twenty-day old chicks where 8 strains belonged to group A and only 2 isolates were of Group B1. However, distribution of phylogenetic groups was balanced in case of finisher birds in which 6 isolates were of phylogroup A and 4 isolates were recognized as phylogroup B members. In the present study, the results of phylogenetic group distribution revealed none of the isolates in the phylogroup B2 or D. Similar reports have been reported by different authors who have accounted that phylogroups A and B1 prevail among commensal *E. coli* strains (Chakraborty *et al.*, 2014; Bok *et al.*, 2013; Obeng *et al.*, 2011). Although, other studies conducted on commensal *E. coli* isolates have mentioned phylogroup A or D to be predominant phylogroups (Bailey *et al.*, 2010; Derakhshandeh *et al.*, 2013). The less diversity among isolates in our case can be attributed to the less number of isolates or avirulent strains in our study as extra-intestinal *E. coli* strains from fecal flora possess virulent genes which belong to phylogroup B2 or D (Duriez *et al.*, 2011).

*In vitro* antibiotic susceptibility assay revealed majority of the isolates were resistant to 3 or more antibiotics. Among the antibiotics which were ineffective in at least 50% or more isolates included oxacillin (100%), penicillin (100%) and nalidixic acid (100%) followed by doxycycline (97.5%), tetracycline (90%), erythromycin (80%),

**Table 2.** List of antibiotics used for antibiogram study against *E. coli*

| S. No. | Antibiotics | Concentration (mcg or unit/disc) |
|---|---|---|
| 1. | Kanamycin(K) | 30 |
| 2. | Gentamycin(GEN) | 10 |
| 3. | Amikacin(Ak) | 30 |
| 4. | Cefazolin(CZ) | 30 |
| 5. | Cefuroxime(CXM) | 30 |
| 6. | Ceftazidime(CAZ) | 30 |
| 7. | Cefotaxime(CTX) | 30 |
| 8. | Cefipime(CPM) | 30 |
| 9. | Erythromycin(E) | 15 |
| 10. | Streptomycin(S) | 10 |
| 11. | Penicillin-G(P) | 10 |
| 12. | Oxacillin(OX) | 1 |
| 13. | Ampicillin(AMP) | 10 |
| 14. | Rifampicin(RIF) | 5 |
| 15. | Pipracillin(PI) | 100 |
| 16. | Clavulanate(CIS) | 30 |
| 17. | Polymyxin-B(PB) | 300 |
| 18. | Nalidixic acid(NA) | 30 |
| 19. | Ciprofloxacin(CIP) | 5 |
| 20. | Levofloxacin(LE) | 5 |
| 21. | Cotrimoxazole(COT) | 25 |
| 22. | Tetracycline(TE) | 30 |
| 23. | Doxycycline(DO) | 30 |
| 24. | Chloramphenicol(C) | 30 |
| 25. | Aztreonam(AT) | 30 |
| 26. | Meropenam(MRP) | 10 |
| 27. | Trimethoprim(TR) | 5 |

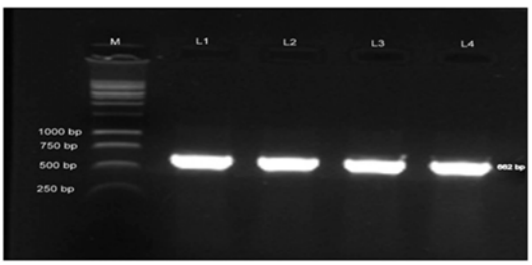


**Fig.1.** Amplified product (662bp) of *16 S r RNA* species specific PCR of *Escherichia coli*

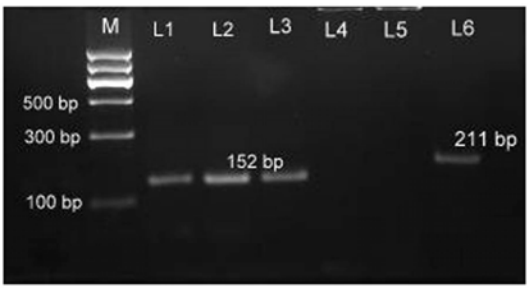


**Fig. 2.** Triplex PCR amplified product. L1 to L3: Phylogroup A specific PCR product (152bp); L4 & L5: no amplified product representing Phylogroup A; L6: Phylogroup B1 specific product (211 bp)

trimethoprim (75%) and meropenam (70%). The sensitivity pattern revealed maximum sensitivity to amikacin 97.5% followed by levofloxacin (95.0%), polymixin (92.5%) and gentamicin (92.5). The susceptibility to other antimicrobial was variable and is presented in the table 3.

The results determined that the resistance pattern was highest for β-lactam antibiotics (oxacillin and penicillin). This high resistance level can be explained due to the secretion of β- lactamase enzyme which is believed to be the most prevalent mechanism of resistance in *E. coli* against β-lactam antibiotics (Normark *et al.*, 1980). We also noticed absolute resistance against nalidixic acid which is a quinolone drug. Resistant *E coli* strains could have emerged due to the high exposure of quinolone drugs resulting in spontaneous mutations in target site of drug yielding such results. Higher trend of quinolone resistance has been also reported by other authors from poultry implying resistance can be of public health concern as they can be transferred to humans (Van den *et al.*, 2001; Wu *et al.*, 2013;). Higher resistance to tetracycline was in agreement to Adelowo *et al.* (2014) who recorded 83% of isolates resistant to tetracycline group. Tetracycline resistance has been ascribed to irrational usage of it in poultry feed as growth promoter. The resistance pattern of trimethoprim was very close to the observations of Wang *et al.* (2013) who recorded a resistance level of 66.7%. The resistance level was exactly similar to the findings of Akond *et al.* (2009) for rifampicin who also reported 80% resistance among their isolates recovered from poultry. High resistance pattern to erythromycin in our study is in agreement to the results of Salehi and Bonab (2006). Interestingly, high resistance which was noted against the carbapenam group (70%) has been rarely reported. More resistance level against such a broad spectrum β-lactam antibiotic draws serious attention in our case. The development of

**Table 3.** The Results of Antibiogram for *E. coli* isolates

| S. No. | Antibiotic | Sensitive (%) | Resistant (%) | Intermediate (%) |
|---|---|---|---|---|
| 1. | Kanamycin(K) | 72.5 | 7.5 | 20 |
| 2. | Gentamycin(GEN) | 92.5 | 2.5 | 5 |
| 3. | Amikacin(Ak) | 97.5 | 0 | 2.5 |
| 4. | Cefazolin(CZ) | 67.5 | 20 | 12.5 |
| 5. | Cefuroxime(CXM) | 75 | 10 | 15 |
| 6. | Ceftazidime(CAZ) | 82.5 | 10 | 7.5 |
| 7. | Cefotaxime(CTX) | 35 | 12.5 | 52.5 |
| 8. | Cefipime(CPM) | 65 | 25 | 10 |
| 9. | Erythromycin(E) | 0 | 85 | 15 |
| 10. | Streptomycin(S) | 45 | 17.5 | 37.5 |
| 11. | Penicillin-G(P) | 0 | 100 | 0 |
| 12. | Oxacillin(OX) | 0 | 100 | 0 |
| 13. | Ampicillin(AMP) | 90 | 0 | 10 |
| 14. | Rifampicin(RIF) | 2.5 | 80 | 17.5 |
| 15. | Pipracillin(PI) | 27.5 | 40 | 32.5 |
| 16. | Clavulanate(CIS) | 90 | 0 | 10 |
| 17. | Polymyxin-B(PB) | 92.5 | 7.5 | 0 |
| 18. | Nalidixic acid(NA) | 0 | 100 | 0 |
| 19. | Ciprofloxacin(CIP) | 30 | 10 | 60 |
| 20. | Levofloxacin(LE) | 95 | 0 | 5 |
| 21. | Cotrimoxazole(COT) | 62.5 | 25 | 7.5 |
| 22. | Tetracycline(TE) | 7.5 | 90 | 2.5 |
| 23. | Doxycycline(DO) | 0 | 97.5 | 2.5 |
| 24. | Chloramphenicol(C) | 42.5 | 32.5 | 25 |
| 25. | Aztreonam(AT) | 60 | 10 | 30 |
| 26. | Meropenam(MRP) | 7.5 | 70 | 22.5 |
| 27. | Trimethoprim(TR) | 25 | 75 | 0 |

resistance can be proposed due to production of metalo β-lactamase, mutation in penicillin binding protein or active efflux pumps.

On evaluating the results of susceptibility patterns, it was seen that aminoglycosides of higher generation were very effective which is supported by the observations of other reporters (Jana and Mondal, 2013; Plateel *et al.*, 2013). Next to them, levofloxacin which falls in fluoroquinolone group was highly effective. Contrary to our observations several authors have reported higher resistance against the levofloxacin (Xie *et al.*, 2014; Lima-Filho *et al.*, 2013) which can be attributed to high degree of exposure to the drug in their region putting more selective pressure on resistant ones.

## REFRENCES


1. Carlet J, C. Pulcini, L.J. Piddock. Antibiotic resistance: a geopolitical issue. *Clin Microbiol Infect,* 2014; **20**(10): 949-53.
2. Kargar M, Z. Mohammadalipour, A. Doosti, S. Lorzadeh, A. Japoni-Nejad. High Prevalence of Class 1 to 3 Integrons Among Multidrug-Resistant Diarrheagenic *Escherichia coli* in Southwest of Iran. *Osong Public Health Res Perspect,* 2014 **5**(4): 193-8.
3. Smith, D.P., J.K. Northcutt, J.A. Cason, J.A. Hinton, R.J. Buhr, and K.D. Ingram. Effect of external or internal fecal contamination on numbers of bacteria on prechilled broiler carcasses. *Poult. Sci.* 2007; **86:** 1241–1244.
4. Herzer, P. J., Inouye, S., Inouye, M. and Whittam, T. S. (1990). Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. *J. Bacteriol.* **172**:6175–6181
5. Clermont, O., Bonacorsi, S., and Bingen, E. Rapid and simple determination of *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 2000; **66**: 4555–4558.
6. Hiki, M., Usui, M., Akiyama, T., Kawanishi, M., Tsuyuki, M., Imamura, S., Sekiguchi, H., Kojima, A. and Asai, T. Phylogenetic grouping, epidemiological typing, analysis of virulence genes, and antimicrobial susceptibility of *Escherichia coli* isolated from healthy broilers in Japan. *Irish Veterinary Journal,* 2014; **67**:1
7. Buchanan, R.E. & Gibbon, N.E. Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore, MD 1994.
8. Chen, W.P. and Kuo, T. T. A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA. *Nucleic Acids Res.* 1993; **21:** 2260
9. Khalad, j., B. Jeremy, L. Pinyon, S. Anantham, M. Ruth. Distribution of Human Commensal *Escherichia coli* Phylogenetic Groups. *Journal of Clinical Microbiology,* 2010; **48**(9): 3455–3456.
10. Bauer, A., W. Kirby, J. Sherris, & M. Turck. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 1966; 493–496.
11. Chakraborty A, P. Adhikari, S. Shenoy. Characterization of plasmid mediated AmpC producing Escherichia coli clinical isolates from a tertiary care hospital in South India. *Indian J Pathol Microbiol,* 2014; **57**(2): 255-8.
12. Bok, E., M. Justyna, P. PaweB, S. MichaB and K. Baldy-Chudzik. Age as a Factor Influencing Diversity of Commensal *E. coli* Microflora in Pigs. *Polish Journal of Microbiology,* 2013**; 62**(2): 165–171.
13. Obeng, A.S., Rickard, H., Ndi, O., Sexton, M., Barton, M. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. *Vet Microbial.* 2011; **154**(3-4):305-15.
14. Derakhshandeh, A., R. Firouzi, M. Moatamedifar, A. Motamedi, M. Bahadori, Z. Naziri. Phylogenetic analysis of Escherichia coli strains isolated from human samples. *Molecular Biology Research Communications,* 2013; **2**(4): 143-149.
15. Duriez, P., O. Clermont, S. Bonacorsi, E. Bingen, A. Chaventre, J. Elion, B. Picard, and E. Denamur. Commensal Escherichia coli isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology,* 2001; **147:** 1671–1676.
16. Normark, S. T. Grundstrom, and S. Bergstrom. Susceptibility to penicillins and cephalosporins in β-lactamase producing strains of *E. coli* and relative amount of β-lactamase produced by these strains. Scand. *J. Infect. Dis,* 1980; **25:** 23–29.
17. Wu, Q., M. Xi, X. Lv , Y. Xu ,Y. Feng ,Q. Li, Q. Yang , X. Xia . Presence and antimicrobial susceptibility of *Escherichia coli* recovered from retail chicken in China. *J Food Prot,* 2014; **77**(10): 1773-7.
18. Van den Bogaard, A. E., N. London, C. Driessen and E. E. Stobberingh. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy,*2001; **47:** 763–771
19. Adelowo, O.O., O.E. Fagade, Y. Adelowo. Antibiotic resistance and resistance genes in

*Escherichia coli* from poultry farms, southwest Nigeria. *J Infect Dev Ctries,* 2014; **8**(9): 1103-12.

20. Wang, C.G., J. C. Lv, and T. Zhang. Detection of resistance phenotype and genotype of avian *Escherichia coli* in Hebei Province. *Poultry Science,* 2013; **92**: 2326–2332.
21. Akond, M.A., S.M.R. Hassan, S. Alam and M. Shirin. Antibiotic Resistance of Escherichia Coli Isolated From Poultry and Poultry Environment of Bangladesh. *American Journal of Environmental Sciences,* 2009; **5**(1): 47-52.
22. Salehi, T.Z. and S.F. Bonab. Antibiotics susceptibility pattern of *Escherichia coli* strains isolated from chickens with colisepticemia in Tabriz Province, Iran. Department of microbiology and immunology, Faculty of Veterinary Medicine, Tehran University. *International Journal of Poultry Science,* 2006; **5:** 677-684
23. Jana, A., Mondal, A. Serotyping, pathogenicity and antibiogram of *Escherichia coli* isolated from raw poultry meat in West Bengal, India. *Vet Ital,* 2013; **49**(4): 361-5
24. Platteel, T.N., M.A. Leverstein-Van Hall , J.W. Cohen Stuart , G.M. Voets, M.P. Van den Munckhof , J. Scharringa, N. Van de Sande, A.C. Fluit , M.J. Bonten. Differences in the antibiotic susceptibility of human *Escherichia coli* with poultry-associated and non-poultry-associated extended-spectrum beta-lactamases. *Eur J Clin Microbiol Infect Dis,* 2013; **32**(8): 1091-5.
25. Xie, R.S. Y. Huo, L. Li, L.Chen, F. Zhang, F. X.Wu. Molecular epidemiological survey on quinolone resistance genotype and phenotype of *Escherichia coli* in septicemic broilers in Hebei, China. *Poult Sci,* 2014; **93**(2): 335-9.
26. Iran, T., S. Zahraei and S. Farashi Bonab. Antibiotics Susceptibility Pattern of *Escherichia coli* Strains Isolated from Chickens with Colisepticemia in Tabriz Province. *International Journal of Poultry Science,* 2006; **5**(7): 677-684.
27. Lima-Filho, J.V., L.V. Martins, D.C. Nascimento, R.F. Ventura, J.E. Batista , A.F. Silva, M.T. Ralph, R.V. Vaz, C.V. Rabello , M. Silva Ide, J. Evêncio-Neto. Zoonotic potential of multidrug-resistant extraintestinal pathogenic *Escherichia coli* obtained from healthy poultry carcasses in Salvador, Brazil. *Braz J Infect Dis,* 2013 Jan-Feb; **17**(1): 54-61.