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Cluster Analysis and Resistotyping of MDR *E. coli* Isolated from Poultry at Different Age Intervals

Femina Anjum* **and Sunil Maherchandani**

Department of Veterinary Microbiology and Biotechnology, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

**Corresponding author*

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A B S T R A C T

In poultry industry of many countries, antibiotics are usually administered to whole flocks abusively which leads to emergence of resistance in various bacteria which make the infections difficult to treat. So the objective was to evaluate differences in the composition of multidrug resistant (MDR) *Escherichia coli* population at different age intervals of various genetic lines of broilers. One hundred and twenty strains of *E. coli* isolated from broilers per 10 days of interval from 0day to 30 day of age from three different farms have been tested against 29 antibiotics. All the isolates were MDR and had MAR (Multiple Antibiotic Resistance) index greater than 0.2, showing abusive use of antimicrobials. Beside this the increased resistance from day0 to 30 day of age were observed for cefazolin, cefuroxime, cefotaxime, cefepime, ampicillin, ticarcillin, piperacillin, polymyxin-b, meropenem, kanamycin, amikacin, clindamycin, trimethoprim, co-trimoxazole, doxycycline, erythromycin, ciprofloxacin and levofloxacin. Resistotyping revealed increased richness in diversity of *E. coli* in broilers. Strains of *E. coli* showed temporal instability as unique strains were observed at each interval of age. So there is need to reduce the abusive use of antimicrobials to reduce the pressure of this global problem.

Introduction

MDR bacteria found in astonishingly wide range of environments (Chopra and Roberts, 2001). Being a major public health problem, antimicrobial resistance is a matter of concern especially in low income countries where there is reduced access to healthcare limits the options for the treatment of diseases (Bryce *et al.*, 2016). MDR bacteria have been documented in many parts of the world (Zinnah *et al.*, 2008; Yassin *et al.*, 2017). WHO reported that these resistant microbes bypass the effect of antimicrobial drugs and results in the persistence and spreading of untreatable infections (Tanwar *et al.*, 2014). Human health relies upon the pathogenicity of these resistant bacteria and their capacity to resist the conventional antibiotics (Nzima *et al.*, 2020).

In poultry industry of many countries, antibiotics are usually administered to whole flocks abusively. Studies have also been reported about the use of antibiotics in broilers as growth promoter and disease preventive measures (Osti *et al.,* 2017). This leads to many undesirable effects including emergence of resistance in various bacteria (Miles *et al.,* 2006). The transmission of these multiple antibiotic resistant microbes from animal to human renders the zoonotic diseases difficult to cure (Subedi *et al.,* 2018). Beside this the microbial load of every past flock maintained in the poultry litter and is assumed to be a reservoir of disease-causing microorganisms (Dumas *et al.,* 2011).

Being a normal inhabitant of gastrointestinal tract of human and animals, *Escherichia coli* is usually a harmless microbe but it can cause a number of considerable illnesses (Friedman *et al.,* 2002). In Birds it also constitutes the normal fraction of intestinal microflora (De Carli *et al.,* 2015). Some previous researches showed the similarity in phylogenetic backgrounds and virulence genes between avain extraintestinal pathogenic *E.coli* (ExPEC) and human *E. coli* that possesses zoonotic risk (Manges and Johnson, 2012). There were several reports about the antimicrobial resistance in *E. coli* (Hailu and Tefera, 2016) and increasing resistance to most first-line antimicrobial agents increasing the complications for treatment (Sabaté *et al.,* 2008). Spreading of Extended-spectrum β-Lactamases (ESBL) mainly involved in the increased resistance to cephalosproins among the members of enterobacteriaceae (Rasheed *et al.,* 2014). As commensal bacteria composed of a pool of resistance genes for potentially pathogenic bacteria, so their intensity of resistance intended to be a good indicator for selection pressure of the abusive use of antibiotics (Murray, 1992).

Elek and Leonie in 1970 described the principle of resistotyping. Resistotyping involved the discrimination of bacterial strains by the extent of their resistance to arbitrarily chosen chemicals that show discerning toxicity at a critical concentration (Elek and Moryson, 1974). Hence resistotyping is used for rational confirmation of identity between strains, which is supposed to describe isolates for epidemiological purposes (Elek *et al.,* 1973). There is scarcity of researches comparing the bacterial communities of animals at different age intervals. Therefore, our objective was to evaluate differences in the composition of MDR *E. coli* population at different age intervals of various genetic lines of broilers.

Material and Methods

Sample collections, bacterial isolation and identification

One hundred and twenty fresh faecal samples through cloacal swabs were collected from healthy broilers at different age intervals (0 day, 10 day, 20 day and 30 day) of three farms from two districts (Ajmer and Bikaner) of Rajasthan, India. First two farms were of Ajmer district of Rajasthan, India and were 50 km apart from each other. Firstly fecal samples were enriched in nutrient agar at 37°C for 16 hours. Culture were streaked on MacConkey agar and incubated at 37°C for 24 hours. Pure colonies were further streaked on Eosin Methylene Blue agar and incubated at 37°C for 24 hours. Bacterial colonies showing characteristic metallic sheen were further confirmed using Gram staining and MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry). *Escherichia coli* NCTC 12923 were used as reference strain.

Antibiotic resistant profiling of *E. coli*

Antibiotic susceptibility testing was done using the disc diffusion method following the guidelines of Clinical Laboratory Standard Institute (CLSI) against 29 antibiotics of different classes i.e. cell wall synthesis inhibitor (CZ, cefazolin; CXM, cefuroxime; CAZ, cetazidime; CTX, cefotaxime; CPM, cefepime; P, penicillin-G; OX, oxacillin; AMP, ampicillin; TI, ticarcillin; PI, piperacillin; PB, polymyxin-B; AT, aztreonam; MRP, meropenem; VA, vancomycin), protein synthesis inhibitor (K,

kanamycin; GEN, gentamicin; AK, amikacin; S, streptomycin; TE, tetracycline; DO, doxycycline; CD, clindamycin; E, erythromycin; C, chloramphenicol), DNA synthesis inhibitor (NA, nalidixic acid; CIP, ciprofloxacin; LE, levofloxacin), RNA synthesis inhibitor (RIF, rifampicin), antimetabolites (TR, trimethoprim; COT, co-trimoxazole [trimethoprim/sulphamethoxazole]). The antibiotic discs used in the present study were purchased from Himedia, India. MAR index were calculated for risk assessment of MDR isolates (Krumperman *et al.*, 1983). Bacteria that acquired non-susceptibility to at least one agent in three or more antimicrobial categories is defined as MDR (Magiorakos *et al.*, 2012). The MAR index is the ratio of number of antibiotics to which the isolate was resistant and the number of antibiotics to which the isolate was tested. If it is greater than 0.2 for the isolated bacteria, implies that several antibiotics are being used in the environment from where the bacteria is originated (Tambekar *et al.*, 2006).

Statistical analysis

The results of antibiotic resistant profiling were converted to binary matrix. For cluster analysis the results were exported to statistiXL (version 1.10) software (StatistiXL, Nedlands, Western Australia) add in within Microsoft Excel®. Subsequently similarity index was prepared using Jaccard similarity coefficient, which is defined as the size of the intersection divided by the size of the union of the sample sets. Agglomerative hierarchical clustering was performed using group Average method. Cluster analysis based on genetic similarities yielded a dendrogram and accuracy of the same calculated using Cophenetic Correlation (c), a measure of how faithfully a dendrogram preserves the pairwise distances between the original un-modeled data point.

Further the measure of richness of clonal groups (representing different genotypes or resistotypes) and their relative abundances were compared between farms at different age intervals using Shannon-Weiner Diversity Index (H).

Results and Discussion

All the 120 isolates were confirmed as *E. coli* using MALDI-TOF MS with the percent purity of 97-99%. Overall the antibiogram profile of *E. coli* isolates showed highest resistance to Cefazolin (99.5%), Cefuroxime (91.67%), Cefotaxime (92.5%), Penicillin-G (100%), Oxacillin (100%), Ticarcillin (92.5%), Vancomycin (91.67%), Tetracycline (97.5%), Clindamycin (98.34%), Erythromycin (90.84%), Nalidixic acid (100), Rifampicin (98.34%) and least resistance to Gentamicin (20.8%), Chloramphenicol (5%), Co-trimoxazole (38.34%). Beside this the increased resistance from day0 to 30 day of age in farm1 were observed for cefazolin, cefuroxime, cefotaxime, ampicillin, ticarcillin, piperacillin, polymyxin-b, meropenem, kanamycin, amikacin, clindamycin, trimethoprim, co-trimoxazole; in farm2 for meropenam, kanamycin and in farm3 for cefepime, meropenam, amikacin, doxycycline, erythromycin, ciprofloxacin, levofloxacin. All the *E. coli* isolates were resistant to three or more antimicrobial agents and had MAR index greater than 0.2. Isolates of farm 1 chicks had MAR index ranges from 0.41 to 0.76, isolates of farm 2 chicks had MAR index ranges from 0.62 to 0.97 and isolates of farm 3 chicks had MAR index ranges from 0.62 to 0.83. It was observed that some of the isolates of farm2 chicks were extreme drug resistant (Anjum *et al.*, 2021).

After cluster analysis and resistotyping a total of 10, 10 and 6 resistogroups were observed in farm1 chicks, farm2 chicks and farm3 chicks respectively with the distribution of 40 *E. coli* isolates in each farm. G3 Resistogroup of farm1 chick, G6 of farm2 chicks and G1 of farm3 chicks have maximum number of isolates. While 2 resistogroups in farm1 chicks, 2 in farm2 chicks and only one in farm 3 chicks had 1 isolate each (table 1-3).

Cluster analysis of *E. coli* based on antibiotic resistance indicates that there were uniqueness in resistogroup of 0day old farm1 chicks, farm2 chicks and farm3 chicks than any other day. In case of

farm1 chicks and farm2 chicks the other days also showed uniqueness in some of the resistogroups but farm3 chicks had same resistogroups for days except 0day of age (fig. 1-3).

The difference in resistogroup clustering may be due to the different feeding regimes of farms of Ajmer (Farm1 chicks and Farm2 chicks) and Bikaner (Farm3 chicks). Both richness and diversity of these resistotypes were observed as the three farms had a total of 26 resistogroups which may be an indicative of the widespread dynamic state of transfer of different R plasmids within the poultry.

Over all diversity of resistotypes was higher in farm1 chicks and farm 2 chicks compared to the farm3 chicks using Shannon-Weiner index (table 1-3), which calculates species diversity index using both species richness and relative abundance in a given population structure. Beside this the variation in farm1 chicks and farm2 chicks showed temporal instability in present study. Many environmental factors influence the intestinal bacteria among which GIT (Gastro Intestinal Tract) is the main contributor to the established bacterial population. The GIT of an animal serves as a specific niche for the intestinal bacterial communities to flourish and therefore considered as microecosystem (Apajalahti, 2005).

This microecosystem changes as the GIT grows affecting the intestinal bacterial communities and allowing optimal communities to flourish (Apajalahti *et al.,* 2004).

However the abusive use of antimicrobials in poultry as feed additive led to the emergence of multidrug resistant bacteria (Shecho *et al.,* 2017) which ultimately substitute the susceptible microgranisms (Miranda *et al.,* 2008). *Escherichia coli* is one such bacteria which have the capability to acquire and transfer antibiotic resistance determinants from other organisms within the gastrointestinal tract (Österblad *et al.,* 2000) and is the most common gram-negative pathogen in human (Salvadori *et al.,* 2004). Therefore the major concern of this was to assess differences in the composition of MDR *E. coli* population at different age intervals of various genetic lines of broilers.

Table.1 Shannon-Weiner Index for Farm1 chick Resistotype Diversity of *E. coli*.

Resistogroups	Name of isolates	Sample no.	Relative Abundance (Pi)	Ln (Pi)	Pi* LN(Pi)
G1	A4a	1	0.025	-3.68888	-0.09222
G2	A3a	1	0.025	-3.68888	-0.09222
G3	E5c, E2a, G8a, G3b, G1c	5	0.125	-2.07944	-0.25993
G4	G2a, E9c, G4a	3	0.075	-2.59027	-0.19427
G5	E3a, C10a, C5b, C3a, G10b, G9a, G7b, G5c, E10b, E7c	10	0.25	-1.38629	-0.34657
G6	G6a, E8a, E6b, E4b, E1c, C7b, A8c, A2a	8	0.2	-1.60944	-0.32189
G7	A9a, A10b, C6b, C1a, A10b	5	0.125	-2.07944	-0.25993
G8	C8a, C4b	2	0.05	-2.99573	-0.14979
G9	C9c, C2c	2	0.05	-2.99573	-0.14979
G10	A6a, A5b, A7c	3	0.075	-2.59027	-0.19427
Total		**40**	**1**		**-2.06088**
			Shannon-Weiner Index (H')		**2.060879**
			Linear log ($e^{H'}$)		**7.852868**

Table.2 Shannon-Weiner Index for Farm2 chick Resistotype Diversity of *E. coli*.

Resistogroups	Name of isolates	Sample no.	Relative Abundance (Pi)	Ln (Pi)	Pi* LN(Pi)
G1	H10b, H2a	2	0.05	-2.99573	-0.14979
G2	B10b	1	0.025	-3.68888	-0.09222
G3	H3b, F5a, F1c, D9a, D7a, B8b, H9b	7	0.175	-1.74297	-0.30502
G4	B6b, B1c	2	0.05	-2.99573	-0.14979
G5	B7c	1	0.025	-3.68888	-0.09222
G6	F9b, F7b, F3b, D10c, D8c, D3a, D1a, B5a, B4c, B2a, H8c, H6c, H4a	13	0.325	-1.12393	-0.36528
G7	B9a, B3c, H7a, H5a, F6c	5	0.125	-2.07944	-0.25993
G8	D6a, D5a, H1b, F10a, F4a	5	0.125	-2.07944	-0.25993
G9	F8a, F2b	2	0.05	-2.99573	-0.14979
G10	D4a, D2b	2	0.05	-2.99573	-0.14979
Total		**40**	**1**		**-1.97375**
			Shannon-Weiner Index (H')		**1.973748**
			Linear log ($e^{H'}$)		**7.197601**

Table.3 Shannon-Weiner Index for Farm3 chick Resistotype Diversity of *E. coli*.

Resistogroups	Name of isolates	Sample no.	Relative Abundance (Pi)	Ln (Pi)	Pi* LN(Pi)
G1	M1b, K10c, K9a, K3b, I3c, I2a, O9a, O5a, O3c, M10a, M3c	11	0.275	-1.29098	-0.35502
G2	O8c, O2b, M7c, M5b, K8a, K7c, O10b	7	0.175	-1.74297	-0.30502
G3	I8b	1	0.025	-3.68888	-0.09222
G4	M2b, K1a, I5b, I4c, O7a, O6b, O4b, O1b, M8a, M6a	10	0.25	-1.38629	-0.34657
G5	I9b, I6c, M9b, M4a, K6b, K5b, K4c, K2a, I10a	9	0.225	-1.49165	-0.33562
G6	I7a, I1a	2	0.05	-2.99573	-0.14979
Total		**40**	**1**		**-1.58424**
			Shannon-Weiner Index (H')		**1.584245**
			Linear log ($e^{H'}$)		**4.875608**

Fig.1 Cluster analysis of resistotypes of *E. coli* of Farm1 chicks (Similarity index at a scale of 0 to 1) with Cophenetic Correlation=1.000.

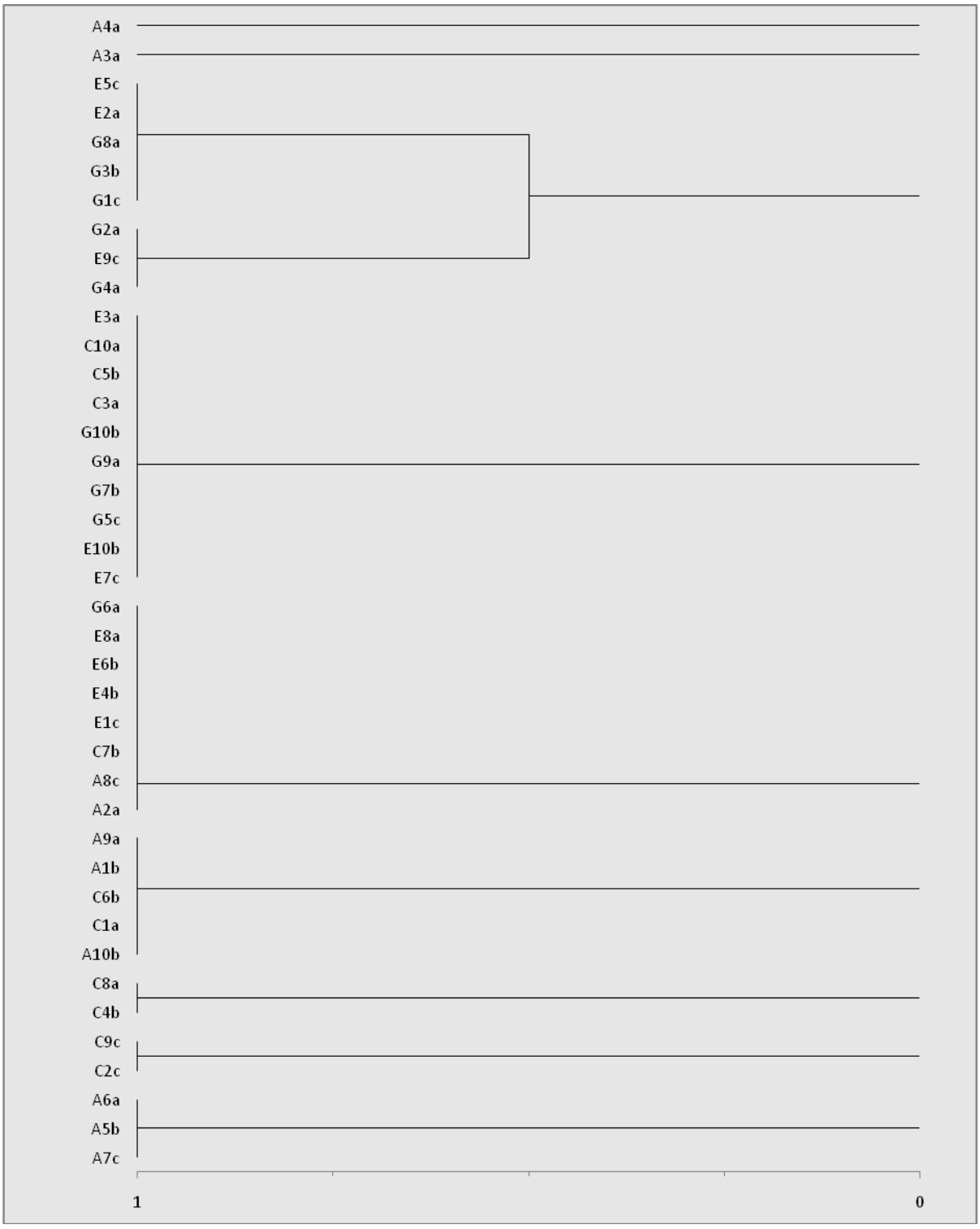


Fig.2 Cluster analysis of resistotypes of *E. coli* of Farm2 chicks (Similarity index at a scale of 0 to 1) with Cophenetic Correlation=0.990.

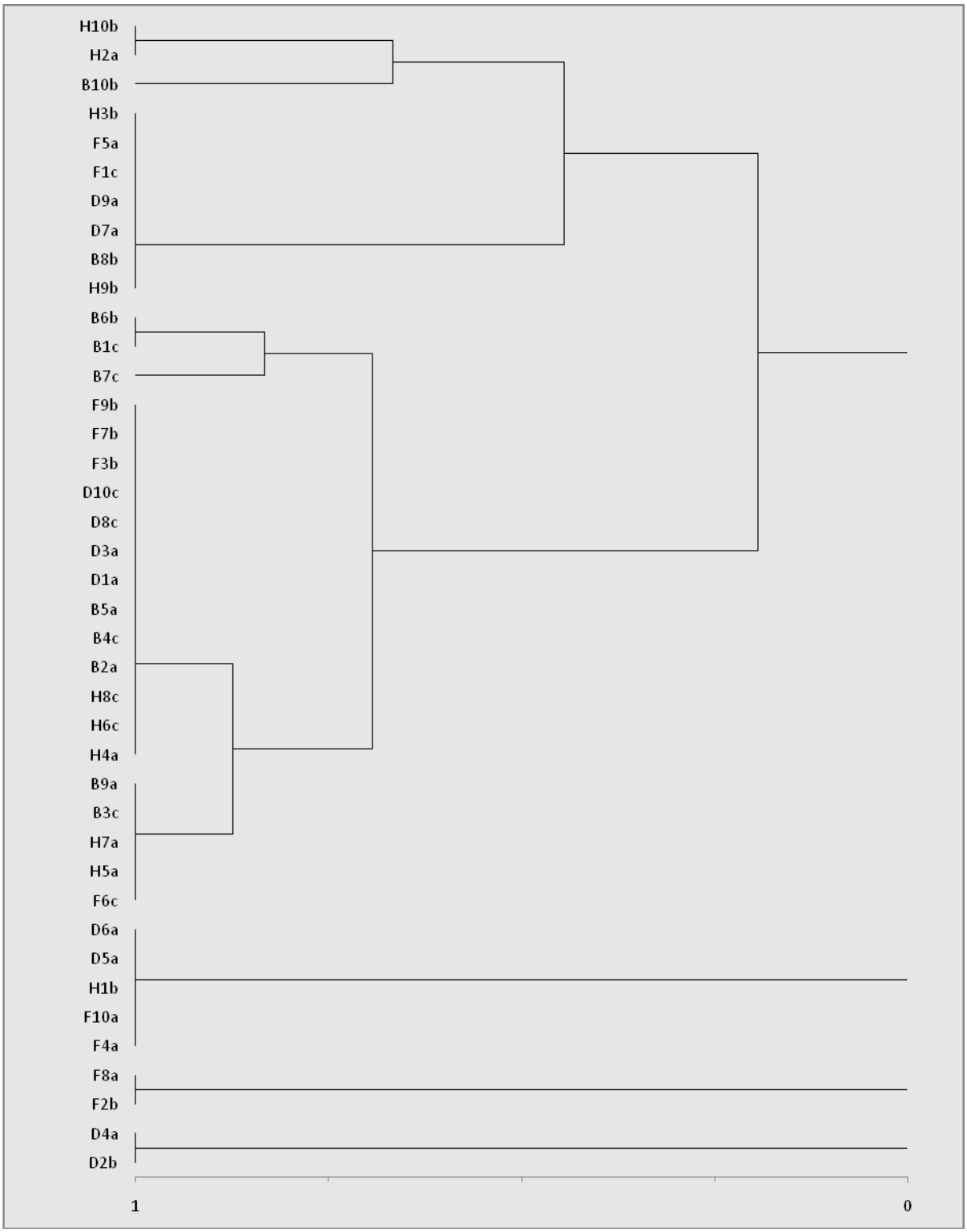
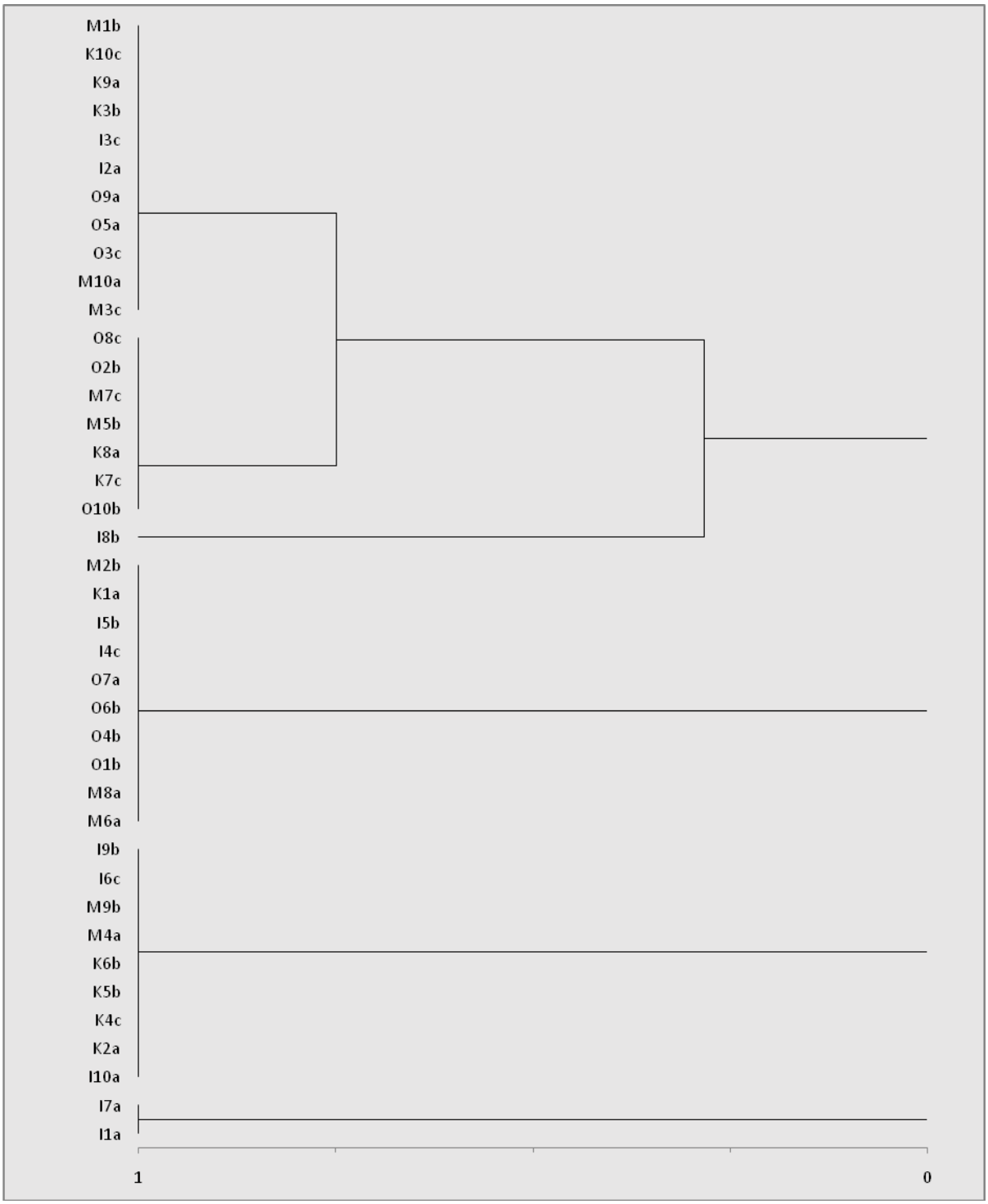


Fig.3 Cluster analysis of resistotypes of *E. coli* of Farm3 chicks (Similarity index at a scale of 0 to 1) with Cophenetic Correlation=1.000.



All the *E. coli* isolates in present study were found multidrug resistant (MDR) using 29 antimicrobial agents belonging to different groups, consistant with the previous studies around the globe (Khan *et al.*, 2002; Muhammad *et al.*, 2009). Increased resistance from day0 to 30 day of age in farm1 were observed

for cefazolin, cefuroxime, cefotaxime, ampicillin, ticar, pipracillin, polymyxin-b, meropenam, kanamycin, amikacin, clindamycin, trimethoprim, co-trimoxazole; in farm2 for meropenam, kanamycin and in farm3 for cefepime, meropenam, amikacin, doxycycline, erythromycin, ciprofloxacin, levofloxacin. Previous studies also reported increased rates of resistance to various antimicrobials conducted in other parts of the world (Kahlmeter, 2003; Kurutepe *et al.,* 2005) as was observed in this study.

In present study overall unique resistogroups were observed at every age interval except for farm 3 where only day old isolates showed uniqueness and resistogroup of rest of the isolates were subsumed with the other age groups. As previous studies observed that the rates of development of genetic lines of broilers are different and so as for the GIT which also attains absolute development at different ages (Corzo *et al.,* 2005). Therefore it would be assumed that the bacterial community would also be different because of the difference in the micro-ecosystem of the gut (Lumpkins *et al.,* 2010).

Both richness and diversity of these resistotypes were observed as the three farms had a total of 26 resistogroups which is an indicative of the widespread dynamic state of transfer of different R plasmids within the poultry. Beside this the variation in farm1 chicks and farm2 chicks showed temporal instability in present study. The similar study was done previously for the variable behavior of *E. coli* strains in temporal scale of persistence (Caugant *et al.,* 1981).

This study revealed that the broilers are the repository of multidrug and extreme drug resistant bacteria which can be a serious health risk to humans by cycling via food chain. Beside this there is richness in the diversity of *E. coli* in broilers and also showed temporal instability, as observed by resistotyping of *E. coli* isolates of three different farms at different age intervals.

Richness in diversity may be an indicative of the widespread dynamic state of transfer of different R plasmids within the poultry. So there is need to reduce the abusive use of antimicrobials to reduce the pressure of this global problem.

Ethics approval

The animal experiment in this study was approved via the Institutional Animal Ethics Committee (IAEC). All experimental procedures and animal care performed in the present study were approved according to the recommendations of the Institutional Animal Ethics Committee (IAEC). All efforts were made to minimize suffering.

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