ANTIMICROBIAL RESISTANCE PROFILING AND MALDI-TOF MS STRATEGY FOR IDENTIFICATION OF ENTERIC *ESCHERICHIA COLI* ISOLATED FROM POULTRY AT DIFFERENT AGE INTERVALS

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ABSTRACT

Poultry is considered as one of the major food animals which are reared by administering the antibiotics recklessly to whole flocks which in turn harbor high proportion of resistant bacteria in gastrointestinal tract. *Escherichia coli* is one such bacteria and is a prevalent commensal bacterium found in the gastrointestinal microflora of warm-blooded animals; the most common community-acquired pathogen among humans. In this study total 160 *E. coli* isolates of different age intervals (0, 10, 20 and 30 day) of poultry were identified using the MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry). Antibiotic profiling was done using 29 different antibiotics belonging to various groups *i.e.* cell wall synthesis inhibitor (cephalosporin, natural penicillin, penicillinae stable,aminopenicillin, carboxypenicillin, uredopenicillin, polypeptide, monobactums, carbapenems and glycopeptides); protein synthesis inhibitor (aminoglycosides, macrolides, lincosamides, phenicoles and tetracycline); DNA synthesis inhibitor (quinolone); RNA synthesis inhibitor and antimetabolites (sulphonamide and combination of sulpha + trimethoprim). All isolates were resistant to multiple antibiotics. Beside that number of isolates having drug resistance to maximum number of antibiotics increased from 0 day to 30 day of age. All the isolates had MAR (Multiple antibiotic resistance) index greater than 0.2 showing that the isolates have originated from an environment where several antibiotics have been used carelessly. Careful administration of antibiotics must be encouraged to only diseased animal and not to the whole flock.

***Key words*:** Chick, duck, MAR index, MALDI-TOF MS, antibiotic profiling, *E. coli*, MDR and commensal bacterium

Introduction

MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry) was introduced as a novel method for the rapid and direct identification of bacteria, even in the presence of other cellular components on the basis of protein spectra from whole cell (Claydon *et al.*, 1996; Lay and Liyanage, 2006 and Lotz *et al.*, 2010). Unlike the other techniques which are completely dependent on the known genetic sequences of the target bacteria (Pershing *et al.*, 2003), there is no need for such information about the organism to be identified using MALDI-TOF MS. This technique provides accurate and rapid identification of pathogenic micro-organisms which is of critical importance in disease treatment and public health (Hsieh *et al.*, 2008).

Every new antibiotic agent acts effectively against micro-organisms but later the antibiotic resistance emerged in those microbes (McClure and Day, 2014). The proportion of resistance strains increases over time and ultimately leads to the appearance of multi-drug resistant strains also called as superbugs (Fernández *et al.*, 2011). The abusive use of antibiotics further increases the risk by eliminating the sensitive strains and increasing the proportion of resistant strain in the host. Besides this the gut is the home to diverse microbiota (Sender *et al.*, 2016), which encompasses several essential functional roles for the body (Stecher and Hardt, 2008; Donaldson *et al.*, 2016). Drug resistance emerged in one bacteria *via* antibiotic treatment can favor the same in other bacteria especially in the gut and finally leading to the transmission, *e.g. via* the faecal-oral route which is of great concern as antibiotics are used worldwide. Moreover, the careless use of antibiotics to farm animals leads to drug resistant bacteria which may spread to humans (Dahms *et al.*, 2014; Bansept *et al.*, 2019). Globally the poultry industry represents the largest proportion of food animal production and there is a well known food safety challenge due to the frequent loads of antibiotic resistant bacteria in poultry and poultry products. As the antibiotic resistant bacteria rich faeces from poultry releases and contaminate the environment impacting environmental antibiotic resistant gene pool (Zhou *et al.*, 2016). Such multidrug resistant bacteria able to combat attack by antimicrobial drugs, which leads to untreatable presistant infections according to WHO. Studies from WHO have also reported very high rates of resistance in bacteria such as *Escherichia coli* against antibiotics as cephalosporin and fluoroquinolones (Nikaido, 2009; Tanwar *et al.*, 2014) mainly due to the spreading of extended-spectrum β-lactamases (ESBL).

Escherichia coli present in the gastrointestinal tract of humans and animals. It is the most prevalent and usually harmless facultative anaerobic species. But being medically important bacteria it can cause a number of major illnesses (Serefhanoglu *et al.*, 2009; Xu *et al.*, 2018) and were firstly detected as multidrug resistant enteric bacteria along with the *Shigella* and *Salmonella* in the late1950s to early 1960s (Levy and Marshall, 2004). For the past 60 years there is a spread of multidrug resistant avian *E. coli* which is usually credited due

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to the incorporation of antimicrobials in the broiler feed (Jones and Ricke, 2003) which is involved in the incidence of community acquired infections in many parts of the world. In developing countries where human antibiotics are easily available for non-therapeutic purposes leads to the indiscriminate use in healthy animals (Collignon, 2009). Therefore, there is the probability of augmentation of human strains by the resistant genes present in the multidrug resistant *E. coli* colonized in poultry all the way through the food chain (Lima-Filho *et al.,* 2012). Hence the aim of this study was to identify isolates using MALDI-TOF MS strategy and to determine the antimicrobial resistivity pattern of *E. coli* isolated from poultry at different age intervals.

Materials and Methods

Collection of faecal samples

Faecal samples of healthy poultry were collected through cloacal swabs. A total of 160 samples out of which 80 of broiler (Cobb), 40 of white leghorn (*Gallus gallus domesticus*) and 40 of mallard ducks (*Anas platyrhynchos*) were taken at different age intervals (0 day, 10 day, 20 day and 30 day) from three farms. Among the three farms two were of Ajmer district having broiler only and third having white leghorn and ducks which was of Bikaner districtof Rajasthan, India.The farms of Ajmer were approximately 50 km apart from each other. All the samples were collected aseptically using sterile cotton swabs from cloacae and stored in sterile container. The samples were transported on ice in sealed container.

Culturing and Isolation of *E. coli*

Isolation was done by following the standard protocol of Carter *et al.* (1994). After 16 hours enrichment of faecal samples at 37°C in nutrient agar, cultures were directly streaked on MacConkey agar and incubated at 37°C for 24 hours. The lactose fermenting pink colonies were then streaked on to Eosin Methylene Blue agar and incubated at 37°C for 24 hours. The characteristic metallic sheen showing bacterial isolates were transferred to paired slants and incubated at 37°C and assigned laboratory numbers. One of the slants was used for tests and the other was refrigerated at 4°C after proper sealing with paraffin wax.

Identification using MALDI-TOF MS

Identification of bacterial isolates was done using MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectromerty) system using SARAMIS premium software 2.0. The bacterial cultures and the standard culture of *E. coli* (NCTC 12923) were grown on nutrient agar and blood agar respectively over night at 37°C. The cultures were then smeared on the target plate along with the addition of 1µL of the matrix-organic solvent mixture (α-cyano-4- hydroxycinnamic acid (CHCA) (5 mg/ml) in a 50:48:2 acetonitrile (ACN):water:trifluoroacetic acid (TFA) matrix solution) to each spots.The standard culture was also spotted on the plate for reference and the target plate allowed to air dry. After drying the plate was inserted into the mass spectrometer and subjected to MALDI-TOF MS analysis. After completing the analysis the identified bacteria were shown along with the per cent purity.

Antibiotic resistant profiling of *E. coli*

Antibiotic susceptibility testing was done using the disc diffusion method (Bauer *et al*., 1966) following the guidelines of Clinical Laboratory Standard Institute (CLSI) against 29 antibiotics of different classes *i.e*. cell wall synthesis inhibitor (cephalosporin, natural penicillin, penicillinae stable, aminopenicillin, carboxypenicillin, uredopenicillin, polypeptide, monobactums, carbapenems and glycopeptides); protein synthesis inhibitor (aminoglycosides, macrolides, lincosamides, phenicoles and tetracycline); DNA synthesis inhibitor (quinolone); RNA synthesis inhibitor and antimetabolites (sulphonamide and combination of sulpha + trimethoprim). In brief, the isolates were inoculated in nutrient broth and incubated at 37°C for 18 h. After adjusting the opacity to 0.5 McFarland opacity standards, the culture were swabbed over the surface of Mueller-Hinton agar and then the antibiotic discs were placed carefully on the surface with adequate space around each disc for diffusion of the antibiotics.After incubating the plates for 24 h at 37°C, the zone of inhibition of growth around each disc of the organism was measured in millimetres and interpreted as per manufacturer (Himedia) recommendations. The antibiotic discs used for the experiment are given in Table 1.

All *E coli* isolates had been evaluated for their multiple antibiotic resistance (MAR) index. This index was given by Krumperman *et al.* (1983) for risk assessment of MDR isolates. This index is an epidemiological tool which is used for risk analysis of environment through bacterial contamination and now a days it is used to assess whether or not the isolates has originated from an environment where several antibiotics had been used or not. The MAR index, is defined as a/b, where “a” represents the number of antibiotics to which the isolate was resistant, and “b” represents the number of antibiotics to which the isolate was tested. Index if greater than 0.2 for the isolated bacteria, implies that strains of such bacteria originate from an environment where several antibiotics are being used (Tambekar *et al*., 2006).

Results and Discussion

Isolation and Identification of *Escherichia coli* isolates

The study was done for a total of 160 isolates of *E. coli*. Following streaking on the MacConkey agar plates, pink colonies were selected (lactose fermenting type) and were additionally streaked on EMB agar plates. Three colonies were picked from each plate showing characteristic metallic sheen. One set of the isolates from MacConkey agar plates was preserved on nutrient agar slants and the other was used to study antimicrobial resistance.After the isolates were confirmed as medium sized Gram-negative rods by Gram staining the isolates were further confirmed by MALDI-TOF MS.

MALDI-TOF MS

MALDI-TOF MS is a quite simple mechanism for the identification of microorganisms. The prepared plate was inserted into the space provided in the machine where the ionization of crystallized smear occur and the resulting protein and peptide traveled towards detector in a vacuum tube and they get separated based on their mass to charge ratio (m/z). The mass spectrum generated was then compared with that of the other strains present in the reference database. The database includes biomarkers detected in MALDI spectra of

intracellular proteins primarily in the range of 2 to 20 kDa (Rahi *et al*, 2016). Most of the biomarkers detected in MALDI-TOF spectra of intact bacterial cells have a molecular mass below 15 kDa (Ryzhov and Fenselau, 2001; Suarez *et al*., 2013). All the 160 isolates were confirmed as *E. coli* using MALDI-TOF MS with the percent purity of 97-99%.

Antibiogram study for *Escherichia coli*

The resisto typing of 160 *E. coli* isolates belonging to 0, 10, 20- and 30-day old chicks and ducks were done using 29 different antibiotics belonging to different groups (Table 1). The organisms were interpreted according to their response as sensitive, intermediate and resistant based on the CLSI guidelines.

Multidrug resistance pattern was observed in 100% *E. coli* isolates as those were resistant to multiple antimicrobial agents.Overall in β-lactum group (cell wall synthesis inhibitors) 100% isolates were resistant to Penicillin-G and Oxacillin.

The other antibiotics studied in this group were representatives of all the four generations of Cephalosporins, Ampicillin, Ticarcillin, Pipracillin, Polymyxin-B, Aztreonam, Meropenam and Vancomycin. Of the above studied antibiotics there was comparatively more resistance observed for first (88%) and second generation (84%) cephalosporins followed by third generation of Cephalosporins (ceftazidime-76% and cefotaxime-79%). Ticarcillin (90%), Pipracillin (73%) and Vancomycin (93%) also showed more resistance. Complete resistance (100%) for first to third generation of Cephalosporins was shown by farm 2 chicks and farm 3 chicks followed by farm 1 chick (55%-77%) and then farm 3 duck (40%-75%). Similarly isolates of farm 2 chicks and farm 3 chicks was more resistant to ampicillin, pipracillin and polymyxin-B (92%, 97%, 75% and 100%, 72%, 100%, respectively) as compared to isolates of farm 1 chicks and farm 3 ducks (65%, 70%, 37% and 57%, 52%, 55%, respectively) while more resistance for ticarcillin was observed in isolates of farm 3 chicks (100%), farm 1 chicks (93%) and farm 2 chicks (85%) followed by farm 3 ducks (58%). There were 75% isolates of farm 1 chicks, 62% of farm 2 chicks, 25% of farm 3 chicks and 17% of farm 3 duck isolates were resistant to Meropenam.

For the present study among the aminoglycoside group, four antibiotics were taken namely: Streptomycin, Gentamicin, Kanamycin and Amikacin. The isolates from different farms and species showed variable resistivity patterns to all the antibiotics of this group. Only the isolates of farm 3 ducks were sensitive to streptomycin while resistance was shown by farm 1 chicks (22%), farm 2 chicks (87%) and farm 3 chicks (62%) for this antibiotic. But for gentamicin farm 1 chicks and farm 3 chicks were sensitive as compared to farm 2 chicks (50%) and farm 3 ducks (47%). In case of kanamycin more resistance was observed for isolates of farm 2 chicks (57%) and farm 3 chicks (52%) followed by farm 3 ducks (37%) and farm 1 chicks (20%). The high resistance for Amikacin observed in farm 2 chicks (77%) followed by farm 3 ducks (55%), farm 3 chicks (47%) and farm 1 chicks (17%).

Highest resistivity for Tetracycline was observed by farm 1 chick (100%) and farm 3 chick (100%) followed by farm 2 chick (92%) and farm 3 ducks (87%). The resistivity pattern to Doxycycline for farm 1 chick, farm 2 chick, farm 3 chick and farm 3 duck were 97%, 77%, 82% and 47%.

In protein synthesis inhibitor group high resistance was observed for clindamycin (98%) and erythromycin (93%). Isolates of farm 2 chicks and farm 3 chicks was 100% resistant to clindamycin followed by farm 3 ducks (98%) and farm 1 chicks (95%). In case of erythromycin 100% resistance was observed by the isolates of farm 1 chicks followed by farm 3 ducks (98%), farm 2 chicks (90%) and farm 3 chicks (83%).

Among the quinolone group of antibiotics (DNA synthesis inhibitors) Nalidixic acid, Ciprofloxacin and levofloxacin were used and the resistance observed to these antibiotics were

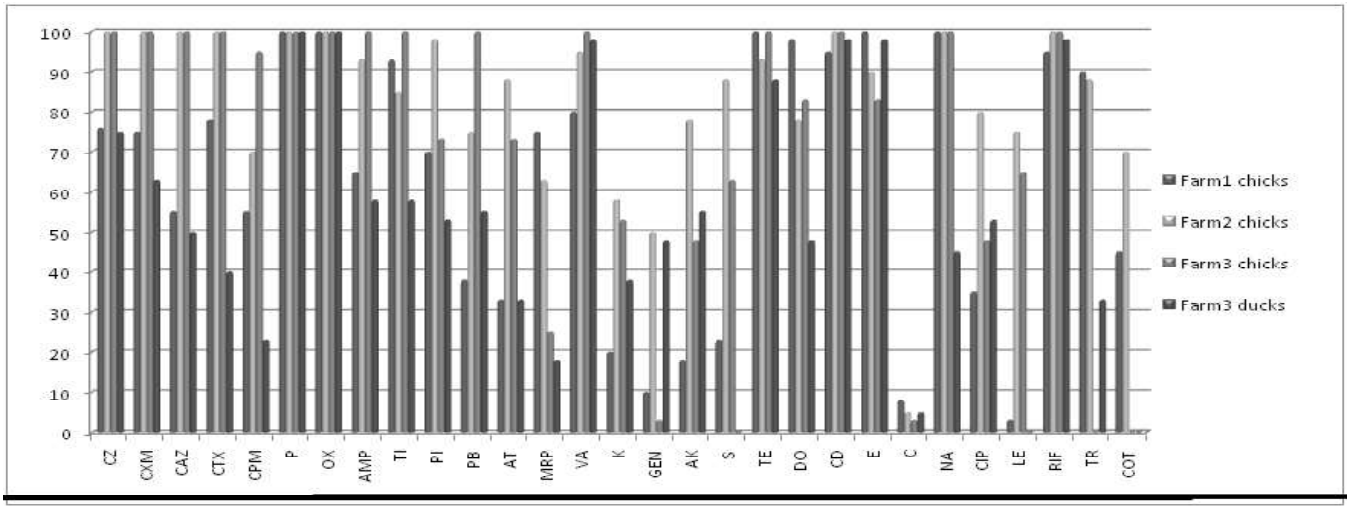

Fig.1: Antimicrobial resistance to 29 antibiotics of *E. coli* isolates of poultry from different farms. The columns denote the percentages of resistant isolates for individual farms (farm 1 chicks, farm 2 chicks, farm 3 chicks and farm 3 ducks). CZ, cefazolin; CXM, cefuroxime; CAZ, cetazidime; CTX, cefotaxime; CPM, cefepime; P, penicillin-G; OX, oxacillin; AMP, ampicillin; TI, ticarcillin; PI, pipracillin; PB, polymyxin-B; AT, aztreonam; MRP, meropenam; VA, vancomycin; K, kanamycin; GEN, gentamicin; AK, amikacin; S, streptomycin; TE, tetracycline; DO, doxycycline; CD, clindamycin; E, erythromycin; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; LE, levofloxacin; RIF, rifampicin; TR, trimethoprim; COT, co-trimoxazole (trimethoprim/ sulphamethoxazole).

Table 1: List of antibiotics and their susceptibility as per manufacturer (Himedia) used for antibiogram study against *E. coli* with their mechanism.

Cell wall synthesis inhibitor			Zone of inhibition(mm)		
Class of antibiotics	Antibiotics	Conc. (mcg)	R	I	S
1st gen. Cephalosporin	Cefazolin (CZ)	30	≤14	15-17	≥18
2nd gen. Cephalosporin	Cefuroxime (CXM)	30	≤14	15-17	≥18
3rdgen.cephaloantipseudo	Ceftazidime (CAZ)	30	≤14	15-17	≥18
3rd gen. Cephalosporin	Cefotaxime (CTX)	30	≤14	15-22	≥23
4th gen. Cephalosporin	Cefipime (CPM)	30	≤14	15-17	≥18
Natural penicillin	Penicillin-G(P)	10	-	-	-
Penicillinase stable	Oxacillin (OX)	1	-	-	-
Aminopenicillin	Ampicillin (AMP)	10	≤13	14-16	≥17
Carboxypenicillin	Ticarcillin (TI)	10	≤14	15-19	≥20
Ureidopenicillin	Pipracillin (PI)	100	≤17	18-20	≥21
Polypeptide	Polymyxin-B (PB)	300	≤11	-	≥12
Monobactums	Aztreonam (AT)	30	≤15	16-21	≥22
Carbapenems	Meropenam (MRP)	10	≤13	14-15	≥16
Glycopeptides	Vancomycin (VA)	30	-	-	-
Protein synthesis inhibitor (30-S)					
Aminoglycoside	Kanamycin (K)	30	≤13	14-17	≥18
Aminoglycoside	Gentamicin (GEN)	10	≤12	13-14	≥15
Aminoglycoside	Amikacin (Ak)	30	≤14	15-16	≥17
Aminoglycoside	Streptomycin (S)	10	≤11	12-14	≥15
Natural tetracycline	Tetracycline (TE)	30	≤14	15-18	≥19
Semisynthetic tet.	Doxycycline (DO)	30	≤12	13-15	≥16
Protein synthesis inhibitor (50-S)					
Lincosamides	Clindamycin (CD)	2	-	-	-
Macrolides(50-S)	Erythromycin (E)	15	-	-	-
Phenicoles(50-S)	Chloramphenicol (C)	30	≤12	13-17	≥18
DNA synthesis inhibitor					
1st gen quinolone	Nalidixic acid (NA)	30	≤13	14-18	≥19
2nd gen quinolone	Ciprofloxacin (CIP)	5	≤15	16-20	≥21
3rd gen quinolone	Levofloxacin (LE)	5	≤13	14-16	≥17
RNA synthesis inhibitor					
RNA synthesis inhibitor	Rifampicin (RIF)	5	-	-	-
Anti metabolite antibiotics (folic acid synthesis inhibitors)					
Sulphonamide	Trimethoprim (TR)	5	≤10	11-15	≥16
Combination of sulpha + trimethoprim	Cotrimoxazole (COT)	25	≤10	11-15	≥16

*R= Resistant, I=Intermediate, S=Sensitive

86%, 54% and 36%, respectively. High resistance to ciprofloxacin was observed in the isolates of farm 2 chicks (80%) while low level of resistance were shown by the isolates of farm1 chicks (35%), farm 3 chicks (47%) and farm 3 ducks (52%). Seventy five per cent and 65% resistance were observed in the isolates of farm 2 chicks and farm 3 chicks and the isolates of rest of the farms (farm 1 chicks and farm 3 ducks) were sensitive to this antibiotic.

Overall 98% isolates were resistant to rifampicin. For Trimethoprim, 90%, 87% and 32% resistance were observed in isolates of farm 1 chicks, farm 2 chicks and farm 3 ducks,

Table 2: Multiple antibiotic resistance (MAR) index of different isolates of *Escherichia coli*

S. No.	Species	MAR value Range
0 day old		
1	Farm 1 Chick	0.41-0.68
2	Farm 2 Chick	0.72-0.96
3	Farm 3 Chick	0.62-0.83
4	Farm 3 Duck	0.37-0.62
10 day old		
5	Farm 1 Chick	0.52-0.69
6	Farm 2 Chick	0.66-0.97
7	Farm 3 Chick	0.62-0.83
8	Farm 3 Duck	0.38-0.62
20 day old		
9	Farm 1 Chick	0.66-0.76
10	Farm 2 Chick	0.66-0.97
11	Farm 3 Chick	0.62-0.83
12	Farm 3 Duck	0.41-0.62
30 day old		
13	Farm 1 Chick	0.66-0.76
14	Farm 2 Chick	0.76-0.97
15	Farm 3 Chick	0.66-0.83
16	Farm 3 Duck	0.51-0.62

respectively while farm 3 chicks were sensitive to this antibiotic.

In the present study all the isolates had an MAR index of more than 0.2 (Table 2). Poultry isolates of farm1 chicks had MAR index ranges from 0.41 to 0.76; isolates of farm 2 chicks had MAR index ranges from 0.66 to 0.97. Similarly the isolates of farm 3 chicks and farm 3 ducks were also greater than 0.2 and ranges from 0.62 to 0.83 and 0.37 to 0.62, respectively. It was also found that antimicrobial resistance increased from 0 day to 30 day of age in all farms (Fig.1).

MALDI-TOF MS is an ideal protocol for the identification and classification of different bacteria at genus, species and subspecies level with accuracy. It can achieve a higher bacterial identification rate by analyzing the m/z values instead of the whole spectra, so it is a reliable method for protein profiling of bacterial colony (De Bruyne *et al*., 2011). In this study all the *E. coli* isolates identified accurately and rapidly by using this protocol with the per cent purity of 97-99%.

Both developed and developing countries are facing the problem of antimicrobial resistance (Cohen, 2000; Miles *et al*., 2006). The uncontrolled use of antimicrobial agents in veterinary played a major role for the emergence of multidrug resistance in gram-negative bacteria (Simonsen *et al*., 2004). The major concern of this study is the antibiotic resistance in *E. coli*, as it is the most common gram-negative pathogen in human (Salvadori *et al*., 2004). *Escherichia coli* have the capability to acquire and transfer antibiotic resistance determinants from other organisms within the gastrointestinal tract (Österblad *et al*., 2000).

In this study the prevalence of antimicrobial resistance in 160 *E. coli* isolates of poultry at different age intervals were observed using 29 antibiotics belonging to different groups.The isolates (100%) were found resistant to more

than two antimicrobial agents similar to the observations of other studies (Fig. 1) (Meena *et al*., 2016; Yassin *et al*., 2017).

The high rates of resistance was observed to penicillin-G (100%), oxacillin (100%), clindamycin (98%), rifampicin (98%), tetracycline (95%), vancomycin (94%), erythromycin (93%), ticarcillin (90%) and nalidixic acid (86%) followed by ampicillin (79%), doxycycline (76%) and pipracillin (73%). Previous studies also reported multidrug resistance in *E. coli* strains from different regions of the world (Zhao *et al*., 2005; Muhammad *et al*., 2009; Hailu and Tefera, 2016). High resistance to cephalosporins mainly cefazolin, cefuroxime, ceftazidime and cefotaxime were observed; similarly, high resistance to cephalosporins were reported in food animals (Zhang *et al*., 2017). Previous studies also reported increased rates of resistance to various antimicrobials conducted around the globe (Kahlmeter, 2003; Kurutepe *et al*., 2005) as was observed in this study.

In this study the multidrug resistance *E. coli* strains were observed even from the day old chicks in all farms. The similar findings have also been reported in other study (Kobe *et al*.,1995) which was influenced by the management and hatchery related factors (Persoons *et al*., 2011).

The prevalence of multidrug resistance among *E. coli* isolates of different farms in this study was greater and is consistence with the other studies.The reason behind this may be due to the irresponsible use of antimicrobial agents which eventually substitute the susceptible microorganisms (Miranda *et al*., 2008). Same is the case of poultry where antimicrobials used carelessly as feed additive which is the main reason behind the emergence of multidrug resistance (Shecho *et al*., 2017).

In conclusion,MALDI-TOF MS allows a rapid and low-cost identification of microorganisms. In this study accurately identified *E. coli* isolates than subjected to antibiogram analysis. All the isolates had MAR index greater than 0.2. Beside that number of isolates having drug resistance to maximum number of antibiotics increased from 0 day to 30 day of age. The present study revealed that the multidrug resistant *E. coli* strains were common in broilers and ducks which were due to the indiscriminate use of antimicrobials in the animal production.These multidrug resistant strains may pose a serious public health issues *via* the cycling through the food chain.Therefore, care should be taken while using the antimicrobials and minimize the haphazard use of available antimicrobials.Beside this there is a need of good hygiene practices in the farms for food animal production.

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