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Detection of Antimicrobial Resistance in *Escherichia coli* and *Salmonella* Isolated from Flies Trapped at Animal and Poultry Farm Premises

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ABSTRACT

The aim of this study was to explore antimicrobial resistance in *Escherichia coli* and *Salmonella* species from flies trapped at livestock and poultry farm premises. A total of 36 pools of flies and 72 rectal/cloacae swabs were collected. All the flies were *Musca domestica* except one fly was *Calliphora erythrocephala*. *E. coli* were recovered from all the flies (100%) and fecal (100%) samples. Whereas, Salmonellae were obtained from 21 (58.33%) flies and 15 (20.83%) fecal samples. *E. coli* and *Salmonella* isolates were multi-drug resistant strains. *E. coli* exhibited resistance to amoxicillin-clavulanic acid (100%), cefotaxime (93.57%), aztreonam (59.63%), cefpodoxime (58.71%) and imipenem (48.62%). Salmonellae were also 100% resistant to ampicillin-clavulanic acid followed by cefotaxime (91.66%), cefpodoxime (94.44%) and imipenem (91.66%). Colistin resistance was recorded more in *Salmonella* (61.11%) than *E. coli* (12.84%) by phenotypic assays, however, *mcr1* to *mcr5* genes could not be detected in any of the *E. coli* and *Salmonella* isolates. Bacteria studied were ESBL (21.10%) and MBL positive. Present study is suggestive of the fact that flies harbor multidrug resistant, ESBL, MBL and colistin resistant *E. coli* and *Salmonella* strains. Extensive monitoring of indicators organisms of AMR in unconventional reservoirs like flies is needed.

HIGHLIGHTS

- Study focused on the detection of AMR in *E. coli* and *Salmonella* present in the flies.
- *Musca domestica* is highly prevalent at livestock and poultry farm premises.
- Flies harbor multidrug resistant, ESBL, MBL and colistin resistant *E. coli* and *Salmonella* strains in their gut.

Keywords: *E. coli*, *Salmonella*, AMR, flies, food animals, poultry

Antimicrobial resistance (AMR) is emerged as a global problem and today's situation is grave in the context of antimicrobial usage, AMR, and alternatives to the antimicrobial agents (Marshall and Levy, 2011). World Health Organization (WHO) published a list of priority bacterial pathogens belonging to 12 genera in 2017 and classified them as critical, high and medium priority, most of which are zoonotic bacterial pathogens. *Enterobacteriaceae*, carbapenem-resistant, ESBL producing organisms are grouped under critical priority.

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Colistin is now considered as very critical antibiotic for human treatment against Gram negative organisms. Colistin as animal growth promoter (AGP) was practiced extensively at global scale which is responsible for the emergence of mobile colistin resistance (*mcr*) genes. Emergence of colistin resistance in human and animal origin bacteria of *Enterobacteriaceae* family has been recently reported (Clemente *et al.,* 2019). MCR genes are located on the transferable plasmid and till date, *mcr-1 to mcr-10* genes are detected in bacterial species, principally *E. coli, Salmonella* and *Klebsiella pneumoniae* (Wang *et al.,* 2020). Colistin resistance is detected in bacteria associated with various sources viz. human, animals, food and invertebrates like flies (Zhang *et al.,* 2017; Principe *et al.,* 2018).

Studies on detection of colistin resistant in *E. coli* and *Salmonella* species are scanty from India except few recent studies on the *K. pneumoniae* (Singh *et al.,* 2018). In view of the increasing AMR situation, it becomes mandatory to investigate the environmental resistomes of AMR. Mechanism of AMR in the bacterial population is a complex but natural and therefore, timely information on the prevalence and molecular epidemiology of critical antibiotics like colistin is required. Recent study on the microbiota of the blowflies and house flies has gained importance in terms of microbial dispersion and AMR (Junqueira *et al.,* 2017). Houseflies can act as vector for harboring and spread of human enteric pathogens. They can even bio-enhance the transmission of AMR (Onwugamba *et al.,* 2018). In the present investigation, attempts have been made to explore the role of flies in harboring antimicrobial resistant *E. coli* and *Salmonella* strains with special reference to colistin.

MATERIALS AND METHODS

Sampling of flies

Flies were trapped from livestock (cattle, buffalo, sheep, goat, pigs) & poultry farm premises and Veterinary Clinical Complex (VCC) using manually prepared traditional traps. The unconventional fly-traps were made out of plastic bottles. Dried fish and chicken powder, in the ratio of 2:1, mixed with sugar syrup was used as bait. Sampling frequency for collection of flies was predetermined so as to collect pooled samples from farm premises. Traps were fixed at different places in the livestock/ poultry sheds where flies were abundant. For each food animal species, trapping was attempted six times, i.e. six pools of samples per species were collected (Table 1). Using no specific selection criteria, 40% of each catch was processed, provided that the numbers of flies caught are not ≤ 15, in such case, all the flies were processed for bacterial isolation.

Bacterial culture

Flies were first washed with 1x Phosphate Buffer Saline (PBS) to remove exterior debris and genus identification was done on the basis of wing pattern under 10x microscopic view (Sen and Fletcher, 1962). Identification at genus and species level was done by expert parasitologist. Identified flies were pooled and washing was carried out three times with 1× PBS to reduce surface contaminants. Washed pool samples were placed in a fresh tube and centrifuged for 10 min at 12000 rpm. Flies settled at the bottom of the centrifuge tubes were crushed by a sterile metal rod, and mixed with sterile PBS, again centrifuged for 10 min at

Table 1: Details of sample collection (flies and fecal samples)

Sl. No.	Site	No. of trappings	Actual flies trapped per sampling						Total flies
1	Cattle farm	6	31	25	18	40	71	49	234
2	Sheep farm	6	47	39	44	53	50	48	281
3	Goat farm	6	75	72	73	85	87	98	490
4	Pig farm	6	40	30	112	62	75	91	410
5	Broiler farm	6	45	22	96	62	124	97	446
6	TVCC	6	16	74	48	62	55	71	326
	Total								**2187**
7	Fecal swabs (2/visit)								72

6000 rpm. The supernatant was collected and used for isolation of *E. coli* and *Salmonella* species. Fecal samples randomly collected from cattle, buffalo, sheep, goat, pigs and poultry using rectal/cloacae swabs were also processed for bacterial isolation.

Isolation and identification of *E. coli* and *Salmonella* species

For obtaining pure culture of *E. coli* and *Salmonella*, enrichment followed by selective plating on specific medium was performed. For *E. coli*, 1 ml supernatant was inoculated into 5 ml *Enterobacteriaceae* Enrichment Broth (EEB) with incubation at 37 °C for 24 h. Similarly, rectal/ cloacae swabs were also enriched in 10 ml of EEB for 24 h at 37 °C. A loop full of enriched culture was streaked on Eosin Methylene Blue (EMB) agar and plates were again incubated at 37 °C for 24 h. For isolation of *Salmonella,* pre-enrichment in Buffered Peptone Water (BPW), enrichment in EEB and selective plating on Xylose Lysine Deoxycholate (XLD) agar was done. Fecal swabs and 1 ml supernatant of pooled flies were first inoculated in 9 ml BPW and incubated at 37 °C for 24 h. Then, 1 ml of pre-enriched culture was inoculated with 5 ml EEB and incubated overnight at 37 °C. A loop full of the enriched culture was used for selective plating on the XLD agar plates incubated at 37 °C for 24 h. Three to five representative *E. coli* and *Salmonella* colonies were picked up, purified and confirmed by biochemical tests namely, catalase, oxidase, indole, methyl red, Voges Proskaur, H_2S production and citrate utilization.

MALDI -TOF MS identification

Matrix-assisted laser desorption ionization time - of - flight (MALDI - TOF) mass spectrometry (MS) technique was used for confirmation of *E. coli* and *Salmonella*. Facilities available at Centre for Zoonoses, Department of Veterinary Public Health, Nagpur Veterinary College, MAFSU Nagpur were used for MALDI - TOF MS identification. Isolates were first grown on BHI agar and single colony from BHI agar plate was picked using sterile loop and placed in target plate. Colonies were dissolved in one to two micro liter of matrix solution. Matrix solution was composed of alpha-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile and 2.5% trifluoracetic acid. Target plates were allowed to dry at room temperature and placed into the plate chamber of the mass spectrometer. For calibration of the instrument known standard culture of *E. coli* was used during the assay. The further analysis of the spectra giving clear identification of the organisms was documented (Fig. 1, 2).

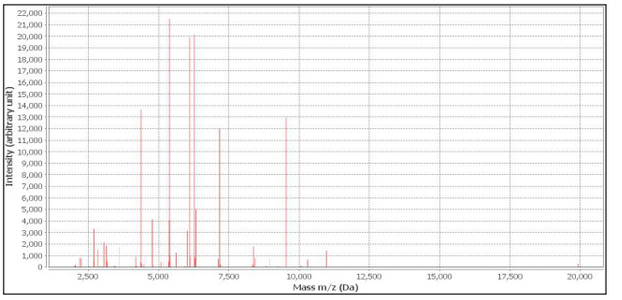

Fig. 1: MALDI-TOF MS spectra of *Escherichia coli* isolates

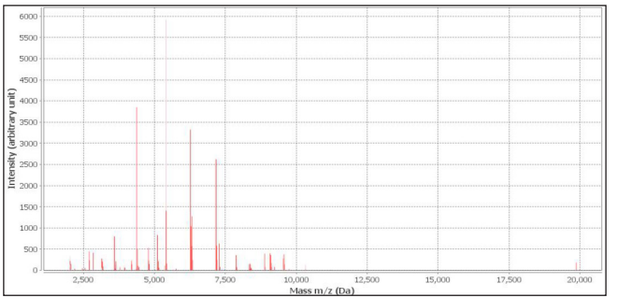

Fig. 2: MALDI-TOFMS spectra of *Salmonella enterica* subspecies *enterica* isolates

Antimicrobial susceptibility testing

Antimicrobial susceptibility and resistance patterns of *E. coli* and *Salmonella* species isolated from flies and fecal samples were studied using Kirby-Bauer disc diffusion method and interpreted using criteria suggested by the Clinical and Laboratory Standards Institute (CLSI, 2017). AMR studies were performed using 12 different antimicrobials as depicted in Table 3. Overnight grown bacterial cultures in BHI broth were smeared on Mueller Hinton agar (MHA) plates and antimicrobial discs were placed at suitable distance. After drying, plates were incubated at 37 °C for 24 h. Zones of inhibition were measured and interpreted in accordance to the manufacturer's instructions (HiMedia Laboratories, Mumbai).

Table 2: Oligonucleotide sequences used for multiplex PCR

Primer name	Sequence (5'-3')	Amplicon size (bp)
mcr-1 (F)	AGTCCGTTTGTTCTTGTGGC	320
mcr-1 (R)	AGATCCTTGGTCTCGGCTTG	
mcr-2 (F)	CAAGTGTGTTGGTCGCAGTT	715
mcr-2 (R)	TCTAGCCCGACAAGCATACC	
mcr-3 (F)	AAATAAAAATTGTTCCGCTTATG	929
mcr-3 (R)	AATGGAGATCCCCGTTTTT	
mcr-4 (F)	TCACTTTCATCACTGCGTTG	1116
mcr-4 (R)	TTGGTCCATGACTACCAATG	
mcr-5 (F)	ATGCGGTTGTCTGCATTTATC	1644
mcr-5 (R)	TCATTGTGGTTGTCCTTTTCTG	

Detection of ESBL production

Multidrug resistant strains were studied for ESBL production using double disk synergy test (DDST). The procedure involved initial screening followed ESBL confirmation. Cefotaxime (30 µg) and ceftazidime (30 µg) discs were used for initial screening, and cefotaxime/ clavulanic acid (30/10 µg) and ceftazidime/clavulanic acid (30/10 µg) discs for ESBL confirmation. BHI inoculated overnight grown cultures of *E. coli* and *Salmonella* were streaked on the MHA plates. Plates were incubated at 37 °C for 24 h and difference in the zone of inhibition was recorded. Isolates showing difference in zone of inhibition of ≥5 mm of cephalosporin discs and cephalosporin plus clavulanic acid containing disc were considered as potential ESBL producers.

Detection of KPC (Carbapenemase) production

The isolates showing resistance to carbapenem discs (ertapenem, doripenem, meropenem and imipenem) during initial phenotypic screening were suspected as carbapenemase (KPC) positive. MIC was estimated using ertapenem/ertapenem plus boronic acid Ezy MIC strips (HiMedia). Ten randomly selected KPC positive isolates were cultured overnight in BHI broth and smeared on the MHA plates. The Ezy MIC strips were placed on the MHA plate carefully and incubated at 37 °C for 24 h. The ratio of ertapenem/ ertapenem + boronic acid (ETP/ETP+) more than 8 was considered positive for KPC.

MIC of AmpC (Ampicillin and Carbenicillin) producing isolates

Improved AmpC detection Ezy MIC strips (HiMedia) were used and all the isolates that were commonly resistant to amoxicillin-clavulanic acid, aztreonam and piperacillin-tazobactum were screened. The Ezy MIC strips containing MIX+ (ceftazidime, cefotaxime, cloxacillin & clavulanic acid- 0.032 - 4) and MIX (ceftazidime, cefotaxime & cloxacillin- 0.125 - 16) were placed on MHA plate and incubated at 37 °C for 24 h. If the ratio of the value of MIX and MIX in combination with clavulanic acid (MIX+) found more than 8 or no zone for MIX and zone obtained in MIX+, it was considered as ESBL+AmpC positive strain. When ratio of the value obtained for MIX and MIX+ was ≤ 8, it was considered as AmpC positive (AmpC present, but ESBL absent).

MIC of MBL (Metallo- β Lactamase) producing isolates

For the determination of MIC of suspected MBL (Metallo-β Lactamase) producing isolates, the common isolates showing ESBL and AmpC production were selected. Isolates were cultured in BHI broth and streaked on MHA plates as described above. The MBL plus ESBL Detection Ezy MIC™ strips containing ESBL+ (ceftazidime, cefotaxime, EDTA & clavulanic acid- 0.032 - 4) and ESBL (ceftazidime, cefotaxime & EDTA- 0.125 - 16) were placed on the agar plate and incubated. When the ratio of the value obtained for ESBL and ESBL+ found more than 8 or no zone obtained for ESBL and zone obtained for ESBL+, it was considered as MBL+ESBL positive strain.

When the ratio of the value obtained for ESBL and ESBL+ was ≤ 8, it was considered as MBL positive strain (ESBL is not present along with MBL).

MIC of colistin resistant isolates

Isolates resistant to ESBL, KPC, MBL and colistin were further screened for estimation of MIC for colistin using Ezy MIC strips (HiMedia). Thirty isolates were cultured in BHI broth and smeared on the MHA plates. The Ezy MIC strips were placed on MHA agar plates and incubated as described previously. Zone inhibition reading at the value of ≥ 2 µg/ml was considered as resistant, and a lesser value was termed as susceptible.

Detection of mobile colistin resistance (*mcr*) genes

Phenotypically confirmed colistin resistant isolates of *E. coli* and *Salmonella* were screened by multiplex PCR targeting mobile colistin resistance genes *viz*. *mcr1* to *mcr5* as per the method described previously (Rebelo *et al.,* 2018). Briefly, bacterial DNA was extracted by boiling and snap chilling method and 2 µl of supernatant was used as the DNA template for PCR. Multiplex PCR was performed in 25 µl volume containing 12.5 µl 2x PCR master mix (HiMedia), 0.5 µl of each forward and reverse primers (*mcr1* - *mcr5*), 2 µl DNA template and 5.5 µl nuclease free water to make final volume of 25 µl. Cycling conditions were set as: initial denaturation (94 ºC/15 min^{-1} cycle) followed by 35 cycles of denaturation (94 ºC/30 sec), annealing (58 ºC/90 sec), and extension (72 ºC/60 sec). Final extension was attained at 72 ºC/10 min and holding at 4 ºC. Five microliter amplified product was separated by electrophoresis in 1.5 % agarose gel dissolved in 0.5 × TBE stained by ethidium bromide. Oligonucleotide sequences used are depicted in Table 2.

RESULTS AND DISCUSSION

Prevalence

Out of 2187 flies trapped, all were identified as house fly (*Musca domestica*) and only one blow fly (*Calliphora erythrocephala*) could be trapped at VCC of the institute. *E. coli* was isolated from all the flies and fecal samples (100%). However, isolation rate of *Salmonella* species was comparatively less. It could be isolated from 21 (58.33%) out of 36 pools of flies and 15 (20.83%) fecal samples. Thus, total 109 *E. coli* and 36 *Salmonella*e species were confirmed. Similarly, 35 randomly selected isolates (15 *E. coli* and 20 *Salmonella*e) were also confirmed by MALDI-TOF MS identification system. All the 35 isolates were confirmed as *E. coli* and *Salmonella enterica* by MALDI -TOF MS system. Livestock, poultry farm and rural environment provides ideal environment for breeding and propagation of house flies and therefore *Muscidae* must be the most abundant species near human and animal dwellings. Abundance of house flies and blow flies in the animal farms were previously reported from Thailand (Fukuda *et al.,* 2018). *E. coli, Salmonella*, *Shigella* and helminth eggs were recently identified in *Musca domestica* collected from cattle byres, poultry farms and human houses (Issa, 2019). *Musca domestica* exposed to the chickens challenged with *Salmonella* Enteritidis get colonized with strain within 24 - 48 h and were able to transmit *Salmonella*e to naive chicken population (Holt *et al.,* 2007). Foodborne pathogens including bacteria, gastrointestinal parasites and viruses have been isolated from exterior body surface and gut of filth flies collected from human houses, livestock and poultry farms (Hald *et al.,* 2004; Lindeberg *et al.,* 2018). A study from Tamil Nadu, have established link between fly densities and diarrheal episodes in rural and urban households and 99.9% flies were from *Muscidae* family. Rotavirus, *Salmonella, Shigella* and *E. coli* were isolated from them (Collinet-Adler *et al.,* 2015).

AMR pattern

All the 145 isolates (109 *E. coli* and 36 *Salmonella)* were examined for antimicrobial resistance pattern by disc diffusion method (Table 3). Almost all the *E. coli* isolates expressed resistance to multiple antibiotics. Most of the *E. coli* strains irrespective of species/source of isolation has shown resistance to amoxicillin-clavulanic acid (100%), cefotaxime (93.57%), aztreonam (59.63%), cefpodoxime (58.71%) and imipenem (48.62%). Overall colistin resistance was recorded very low (12.84%) and only 14 isolates expressed resistance to colistin by disc diffusion method. Species/source wise prevalence of colistin resistance was recorded as: cattle-buffalo (66.6%), sheep (5.5%), goat (22.77%), poultry (11.11%), pigs (11.1%) and VCC isolates (5.26%). *E. coli* isolates were multi-

Table 3: Antibiogram of *E. coli* and *Salmonella* species (n =145)

Antimicrobials used	*E. coli* (n = 109)			*Salmonella* (n =36)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Amoxicillin Clavulanic acid (20/10 mcg)	0 (0%)	0 (0%)	109 (100%)	0 (0%)	0 (0%)	36 (100%)
Aztreonam (30 mcg)	26 (23.8%)	18 (16.51%)	65 (59.6%)	5 (13.8%)	11 (30.5%)	20 (55.5%)
Cefotaxime (30 mcg)	1 (0.91 %)	6 (5.50%)	102 (93.57%)	1 (2.77%)	2 (5.55%)	33 (91.6%)
Cefpodoxime (10 mcg)	32 (29.35%)	13 (11.9%)	64 (58.7%)	0 (0%)	2 (5.55%)	34 (94.44%)
Ceftazidime (30 mcg)	45 (41.28%)	29 (26.6%)	35 (32.1%)	5 (13.88%)	14 (38.8%)	17 (47.22%)
Ceftriaxone (30 mcg)	51 (46.78%)	26 (23.85%)	32 (29.35%)	14 (38.8%)	10 (27.7%)	12 (33.3%)
Colistin (10 mcg)	84 (77.06%)	11 (10.09%)	14 (12.8%)	11 (30.55%)	3 (8.33%)	22 (61.11%)
Doripenem (10 mcg)	36 (33.02%)	49 (44.9%)	24 (22.01%)	21 (58.3%)	11 (30.5%)	4 (11.11%)
Ertapenem (10 mcg)	60 (55.04%)	37 (33.94%)	12 (11%)	29 (80.55%)	6 (16.66%)	1 (2.77%)
Imipenem (10 mcg)	13 (11.9%)	43 (39.4%)	53 (48.62%)	0 (0%)	3 (8.33%)	33 (91.66%)
Meropenem (10 mcg)	22 (20%)	40 (36.69%)	47 (43.11%)	25 (69.44%)	10 (27.7%)	1 (2.77%)
Piperacillin- Tazobactam (100/10 mcg)	17 (15.59%)	43 (89.4%)	47 (43.11%)	7 (19.44%)	10 (27.7%)	19 (52.7%)

Table 4: Resistance pattern of *E. coli* isolated from flies and food animals (n = 109)

Farm premise and species		Cattle/buffalo (18)		Sheep (18)		Goat (18)		Poultry (18)		Pig (18)		TVCC (19)		Overall resistance	
Antimicrobial	mcg/disc	No	%	No	%	No	%	No	%	No	%	No	%	No	%
AMC	20/10	18	100	18	100	18	100	18	100	18	100	19	100	109	100
AT	30	13	72.22	9	50	7	38.88	13	72.22	10	55.55	13	68.42	65	59.63
CTX	30	18	100	17	94.44	16	88.88	17	94.44	17	94.44	17	89.47	102	93.57
CPD	10	14	77.77	4	22.22	11	61.11	8	44.44	15	83.33	12	63.15	64	58.71
CAZ	30	6	33.33	3	16.66	6	33.33	7	38.88	6	33.33	7	36.84	35	32.11
CTR	30	6	33.33	2	11.11	6	33.33	9	50	4	22.22	5	26.31	32	29.35
CL	10	3	16.66	1	5.55	5	22.77	2	11.11	2	11.11	1	5.26	14	12.84
DOR	10	5	22.77	4	22.22	7	38.88	1	5.55	4	22.22	3	15.78	24	22.01
ETR	10	1	5.55	4	22.22	3	16.66	2	11.11	0	0	2	10.52	12	11.00
IMP	10	10	55.55	6	33.33	11	61.11	5	22.77	9	50	12	63.15	53	48.62
MRP	10	4	22.22	14	77.77	8	44.44	4	22.22	7	38.88	10	52.63	47	43.11
PIT	100/10	10	55.55	5	22.77	7	38.88	5	22.77	12	66.66	8	42.10	47	43.11

drug resistant strains and out of 109 isolates, 92 isolates (84.40%) expressed resistance to at least 3 out of 12 antibiotics. Multiple antibiotic resistance (MAR) index for the individual strains vary from 0.25 to 0.75. AMR pattern of *E. coli* and *Salmonella* species isolated from flies and fecal samples of food animals is presented in Table 4 and 5. Overall, *Salmonella* isolates showed 100% resistance to Ampicillin-clavulanic acid followed by cefotaxime (91.66%), cefpodoxime (94.44%), imipenem (91.66%), colistin (61.11%) and piperacillin-tazobactam (52.77%). Resistance to ertapenem, meropenem and doripenem was very low. Colistin resistant strains of *Salmonella* were isolated from flies as well as fecal samples and its prevalence was high (61.11%) as compared to colistin resistant *E. coli* (12.84%). Highest number of colistin resistant strains were recovered from pig farm (90%) and VCC (80%) premise. Resistance to multiple antimicrobials was also recorded in the *Enterobacteriaceae* including *E. coli* isolated from flies collected at dairy and poultry sites in Portugal (Barreiro *et al.,* 2013). Detection of AMR

Table 5: Resistance pattern of *Salmonella* isolated from flies and food animals (n=36)

Farm premise and species		Cattle/ buffalo (5)		Sheep (7)		Goat (1)		Poultry (8)		Pig (10)		TVCC (5)		Overall resistance	
Antimicrobial	mcg/disc	No	%	No	%	No	%	No	%	No	%	No	%	No	%
AMC	20/10	5	100	7	100	1	100	8	100	10	100	5	100	36	100
AT	30	3	60	5	71.42	1	100	5	62.5	4	40	2	40	20	55.55
CTX	30	4	80	7	100	1	100	7	87.5	9	90	5	100	33	91.66
CPD	10	5	100	6	85.71	1	100	7	87.5	10	100	5	100	34	94.44
CAZ	30	1	20	3	42.85	0	0	4	50	6	60	3	60	17	47.22
CTR	30	3	60	2	28.57	0	0	0	0	7	70	0	0	12	33.33
CL	10	2	40	3	42.85	1	100	3	37.5	9	90	4	80	22	61.11
DOR	10	1	20	1	14.28	1	100	0	0	1	10	0	0	4	11.11
ETR	10	1	20	0	0	0	0	0	0	0	0	0	0	1	2.77
IMP	10	4	80	5	71.42	1	100	8	100	10	100	5	100	33	91.66
MRP	10	0	0	0	0	0	0	0	0	0	0	1	20	1	2.77
PIT	100/10	3	60	4	57.14	1	100	3	37.5	4	40	4	80	19	52.77

strains of *E. coli* and *Salmonella* isolated from flies and fecal samples of farm animals is worrisome as flies can carry antimicrobial resistant microbes mechanically from animals to humans. AMR in *E. coli* isolated from flies (Sobur *et al.,* 2019) and food animals (Malhotra-Kumar *et al.,* 2016) were studied earlier. In contrast to present findings ertapenem, imipenem and meropenem susceptible *E. coli* were found in healthy cattle from Spain (Hernández *et al.,* 2017). In another cross sectional study on houseflies collected from educational hospitals, vegetable centre and livestock slaughter house, *E. coli* resistant to gentamicin, cefotaxine, ciprofloxacin and other routine drugs were detected (Nazari *et al.,* 2017). *Salmonella* isolates under study showed very high resistance to ampicillin-clavulanic acid, cefotaxime, cefpodoxime, imipenem and colistin. However, resistance to ertapenem, meropenem and doripenem was very low. A recent study on AMR diversity of *Salmonella* Typhimurium exhibited high degree of AMR diversity in pig isolates as compared to chicken and cattle origin isolates (Mellor *et al.,* 2019). Isolation and characterization of *E. coli* and *Salmonella* from flies for AMR is not extensively investigated and there are no supporting reports from India with reference to AMR in bacteria associated with flies trapped at livestock and poultry farms. Present findings affirm that *Musca domestica* flies prevalent at livestock and poultry farms harbor multi-drug resistant strains of *E. coli* and *Salmonella.*

ESBL, MBL, AmpC, KPC and Colistin resistance

Out of 109 *E. coli,* 23 (21.10%) were ESBL positive strains. Among these, 8 (34.78%) isolates were from flies and 15 (65.21%) were from fecal samples. The distribution of ESBL positive *E. coli* revealed maximum isolates from broiler (6/23), followed by goat (4/23), sheep (4/23), cattle (4/23), pig (3/23) and VCC (2/23) origin samples. Similarly, prevalence of ESBL positive *Salmonella* was 19.44% and out of 36, seven strains were ESBL positive. Only one isolate was from fly source and other six strains were from fecal samples. Isolates resistant to amoxicillin-clavulanic acid, aztreonam and piperacillin- tazobactam during initial screening, were suspected as AmpC positive strains, however, all the isolates were negative for AmpC by Ezy MIC strips. Similarly, eight common ESBL and amoxicillin-clavulanic acid resistant isolates were screened for MBL type of resistance using Ezy MIC strips. Two isolates were positive for MBL, ESBL and AmpC production, while six were positive for MBL only. Further, 10 common isolates resistant to doripenem, meropenem, ertapenem and imipenem were screened for KPC using Ezy MIC strips and only one goat fecal origin *E. coli* was carbapenemase positive. Resistance to ESBLs, AmpC cephalosporinases, and carbapenem antibiotics are of greater public health significance. Thus, detection of ESBL, MBL, AmpC, KPC and colistin resistance in *E. coli* and *Salmonella* is highly significant in the recent context. ESBL positive *E. coli* strain was also detected in two fly

pools collected from the poultry farms in the Netherlands (Blaak *et al.,* 2014). In contrast to our findings, AmpC producing *E. coli* were highly prevalent in the broiler birds and farmers working at Dutch farms (Dierikx *et al.,* 2013).

Initial phenotypic screening by the disc diffusion method revealed 12.84% *E. coli* and 61.11% *Salmonella* resistant to colistin. Out of these, 30 isolates (13 *E. coli* and 17 *Salmonella*) were examined for MIC of colistin. Overall, 16 (53.33%) isolates found positive for colistin at a MIC level of ≥ 2 mg/L. Of these, 10 were *E. coli* and 6 were *Salmonella*. Maximum *E. coli* isolates showing MIC ≥ 2 mg/L were of goat origin (three), followed by cattle and pig (one each). Colistin positive *E. coli* with MIC ≥ 2 mg/L could be isolated from two fly pools trapped at poultry farm and one each from cattle farm, goat farm and VCC. Out of six positive *Salmonella* isolates showing MIC of ≥ 2 mg/L, two each, were from flies collected at sheep farm, pig farm and VCC (Fig. 3, 4, 5).

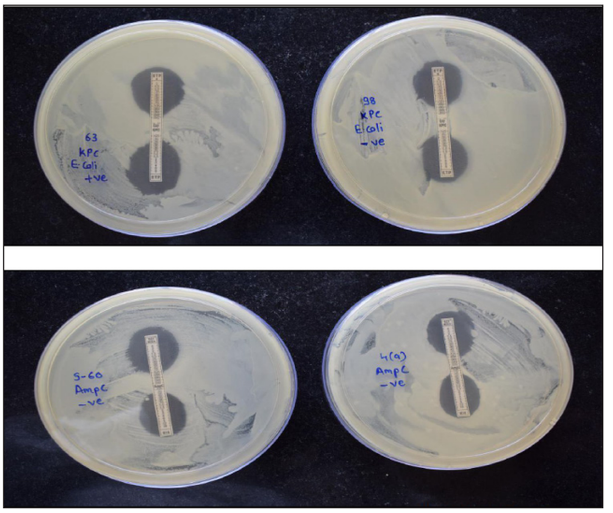

Fig. 3: Detection of carbapenemase and AmpC producing isolates using Ezy MIC strips

Multiplex PCR was performed on all the 34 colistin positive isolates as per the standard protocol, however, we could not detected *mcr* genes in any of the phenotypically colistin resistant *E. coli* and *Salmonella* isolates. Use of colistin (polymyxin E) has been re-introduces in human medicine and now it is a last resort antibiotic to treat infections from multi-drug resistant bacteria. As compared to our study, less prevalence of colistin resistant *E. coli* isolated from livestock and food was noted in Germany (Irrgang *et al.,* 2016).

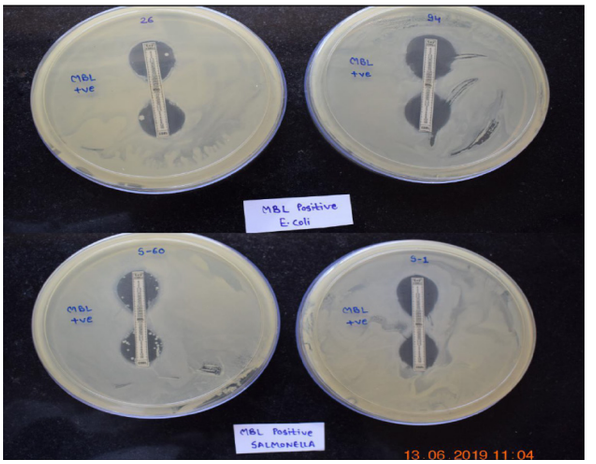

Fig. 4: Detection of MBL production using Ezy MIC strips

Fig. 5: Detection of colistin resistance using Ezy MIC strips

Similar to the present observations high frequency of colistin resistance in pigs (23.75%) and poultry (7.92%) was recorded in a study from China (Huang *et al.,* 2017). Sporadic reports are available on detection of *mcr1* and *mcr3* genes in *E. coli* isolated from flies (Fukuda *et al.,* 2018; Sobur *et al.,* 2019). Other than India, prevalence of *mcr* genes in *E. coli* and *Salmonella* isolated from food animals has been reported globally (El Garch *et al.,* 2018). As colistin is used as last resort antibiotic, development of resistance against this important antibiotic is great cause of concern.

CONCLUSION

Present findings highlighted the significance of flies in harboring multi-drug resistant *E. coli* and *Salmonella* species. Although, colistin resistance could not be confirmed by PCR during this study, detection of ESBL, MBL, and colistin resistance by phenotypic assays warrants the need for undertaking extensive monitoring of AMR in bacterial pathogens present in the invertebrate species associated with animal and human dwellings. It is assumed that variety of invertebrate species living in close proximity to animals may be a source of gene pool for antimicrobial resistance and their role in AMR transmission from animals to humans cannot be ruled out.

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