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A surveillance and multi drug resistance profile study of extended spectrum beta lactamase producing *E. coli* in poultry

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Abstract
Antimicrobial resistance is recognized as a growing global threat. Emergence of antimicrobial resistance in bacteria may be transferred to humans, thereby reducing the effectiveness of antimicrobial drugs for treating human disease. The present study was conducted on extended spectrum beta lactamase producing *E. Coli* isolated from the caeca of freshly slaughtered healthy broilers. Surveillance in 10 poultry farms was also undertaken. A questionnaire was prepared to get the information about the usage of antibiotics for treatment /prophylaxis/both. Maximum farmers reported that even antibiotics given in the high doses in disease condition are ineffective, which itself reflect the problem of antibiotic resistance. Multi drug resistant profile of ESBL *E. coli* isolates were also observed against 15 antibioticsand found that ampicillin, cefixime, Ceftriaxone showed 100per cent resistance where as Cefoperazone, Cotrimoxazole, Cefuroxime showed resistance in more than 90 per cent of the isolates. Tetracycline, Ciprofloxacin, Norfloxacin, Netilmicin, Gentamicin, levofloxacin showed resistance of almost 50per cent of the isolates. Chloramphenicol and colistin minimum resistance.

Keywords: antibiotic resistance, extended spectrum beta lactamase, *E. coli*, poultry farms.

1. Introduction
Antimicrobial resistance, within a large range of infectious agents is a rising health risk of broad concern to countries and multiple sectors. It not only menaces the effective prevention and treatment of an ever-increasing range of infections but also results in reduced efficacy of antibacterial drugs. Intensively reared poultry, antibiotics are administered to whole flocks rather than individual animals. In addition to this poultry farmer also use low doses of antibiotics as growth-promoting substances, which result in the high antibiotic selection pressure for resistance with relatively high proportion of resistant bacteria in poultry faecal flora. Most resistant phenotypes present in animal populations are present in Escherichia coli therefore Commensal Escherichia coli can be used as indicators of the Gram-negative species. During the passage through the intestine, these bacteria may transfer their resistance genes to host-adapted bacteria or to pathogens. At slaughter, resistant strains from the gut readily soil poultry carcasses and as a result poultry meat are often contaminated with multi resistant Escherichia coli. All animals generally carry such indicator bacteria this is why trends in the occurrence of resistance, can be studied more accurately in indicator bacteria [4]. Beta-lactams (penicillins, cephalosporins, carbapenems & monobactams) constitute the therapy of choice for some well-established practices and infections in veterinary medicine [2].Extended spectrum beta lactamase -producing organisms are frequently co- or multi resistant, exhibiting resistance to other antimicrobial classes such as fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole due to associated resistance mechanisms, which may be either chromosomally- or plasmid-encoded [9, 11]. Extended spectrum beta lactamase production and multidrug resistance was studied in a total of 228 isolates of *Klebsiella pneumoniae* and *Escherichia coli* 100per cent resistance or decreased susceptibility to at least one of the third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone). Extended spectrum beta lactamase production was noted in 59.65percent of the isolates tested and was detected positive in 51.47percent strains of *Escherichia coli.* [14]. The aim of the present study was to study the prevalence and isolation of extended spectrum beta lactamase producing *E. Coli* in healthy broilers and multi drug resistance profile of the positive samples.

1. Material and Methods

1.1. Prevalence of extended spectrum beta lactamase *E. Coli*

Prevalence study was conducted on 400 caecal samples of freshly slaughtered healthy broilers of 38 various poultry sale outlets located in different parts of Jabalpur.

1.2. Phenotypic Characterization

Samples were collected from the caecal swab obtained from freshly slaughtered healthy birds at the poultry sale outlets. Sample were processed taking all the aseptic precautions. The sterile swab was then straight away transferred into Non-selective enrichment in buffered peptone water (@ 25 ml / 5 gm sample) and then selective enrichment in broth M.H. broth 10 ml/1 ml of the enriched sample containing a cephalosporin prior to plating on selective media. Selectively enriched samples were then inoculated into the selective agar media for preparing colonies of ESBL producing *E. coli*. Three methods were used:

a. Double Disc Synergy Test (DDST) – Discs containing cephalosporins (cefotaxime, ceftazidime, cefepime) were applied to plates next to a disc with clavulanic acid (amoxicillin-clavulanic acid).
b. Ezy MIC strip test – Ceftazidime and ceftazidime + clavulanic acid E strip were used and test was confirmed positive if MIC ratio ≥ 8 or deformed ellipse was present around ceftazidime + clavulanic acid.
c. Combination Disc Diffusion Test (CDDT) – Discs or tablets containing the cephalosporin alone (cefotaxime) and in combination with clavulanic acid were applied. If the inhibition zone diameter was 5 mm larger with clavulanic acid than without the test was considered to be positive [7].

1.3. Genotypic Characterization

The phenotypically positive samples were further characterized genotypically by polymerase chain reaction. For the genotypic characterization of the positive samples, multiplex polymerase chain reaction was performed for the gene blatem blashv and blactx out of the total 76 samples screened for genotypic characterization 49 samples were positive for blaTEM gene and 15 samples were positive for bla CTX gene.Twelve samples showed the presence of both bla CTX and bla TEM gene. Two samples were found to be positive for the blaSHV gene and one was positive for bla TEM and blaSHV gene.

1.4. Multi drug resistance profile of extended spectrum beta lactamases *E. coli*.

Multi drug resistance profile was conducted on 15 antimicrobial agents /antibiotics considered critically important antibiotic listed in the EFSA and OIE (Table 1). The agar disc diffusion method was used which provides qualitative interpretive category results of susceptible, intermediate, and resistant microorganisms. AST using the agar disc diffusion method was based on the principle that antimicrobial agents of specified concentration in discs diffuse into media and inhibit the growth of susceptible organisms resulting in a zone of inhibition around the disc. Zones were measured and interpretation of the zones of inhibition was done as per the performance standard for Antimicrobial disc susceptibility test, CLSI [1]. Each culture to be tested was streaked onto a Mueller Hinton agar medium to obtain isolated colonies. The swab was streaked over the entire surface of the medium three times, rotating the plate 60 degrees after each application to ensure an even distribution of the inoculum. Finally, all around the edge of the agar surface was swabed. Discs were applied with sterile disc dispenser. After the discs were placed on the plate, incubated at 35°C for 16 to 18 hours. Zones were read from the back of the plate with rulers and were recorded in millimetres. [14]

Table 1: Standard Zone size interpretation chart as per CLSI.

S No.	Antimicrobial Agent	Symbol	Disc content (µgm)	Interpretative Criteria (mm)		
				Sensitive	Intermediate	Resistant
1	Ampicillin	AMP	10	17	14-16	13
2	Tetracycline	TE	30	15	12 to 14	11
3	Gentamicin	GEN	10	15	13-14	12
4	Cefuroxime	CXM	30	18	15-17	14
5	Ceftriaxone	CTR	30	23	20-22	19
6	Cotrimoxazole	COT	25	16	11 to 15	10
7	Colistin	CL	10	11		10
8	Norfloxacin	NX	10	17	13-16	12
9	Netilmicin	NET	30	15	13-14	12
10	Levofloxacin	LE	5	17	14-16	13
11	Ciprofloxacin	CIP	5	21	16-20	15
12	Cefoperazone	CPZ	75	21	16-20	15
13	Tobramycin	TOB	10	15	13-14	12
14	Cefixime	CFM	5	19	16-18	15
15	Chloramphenicol	C	30	18	13-17	12

1.5. Surveillance of various poultry farms

The surveillance study was conducted in ten organized poultry farms of Jabalpur to collect the information on usage of antibiotic and the problem of antibiotic resistance. The data was collected on the basis of a questionnaire prepared for the poultry farmers (Table: 1)

Table 2: Questionnaire for the farmers

Particulars	Remarks
Name of Poultry farm	
Name of poultry farmer/owner	
Number of birds	
Antibiotics used Yes/No	
Antibiotics used for prophylaxis /treatment	
Any herbal /alternative medicine used	
Problem of antibiotic resistance observed	

Results and Discussion

In the present study prevalence of extended spectrum beta lactamase producing *E. coli* was 33.5 per cent (135 samples positive among the 400 samples) through phenotypic characterization using standard protocols. Genotypic characterization revealed out of 76 samples 49 samples were positive for *bla*-TEM/ *bla*-CTX/*bla*-SHV resistant genes. Perusal of the results revealed that at some areas there were 100 per cent prevalence of ESBL E. coli isolates where as other areas exhibited lower range of prevalence (0 to 30 per cent). Our findings simulate with the results obtained by Hasan *et al*. (2011) in Bangladesh [8] and adjoining areas of India where they observed an overall prevalence of ESBL-producers as 30 per cent in poultry and domestic birds, 27 per cent in wild birds and 59 per cent prevalence was seen in hospitals and community people, they further concluded that ESBL- producing bacterial species diversity was highest in poultry and humans were the best ESBL carriers.

Study of Multi drug resistance profile was undertaken against the obtained ESBL *E. coli* isolates. The selection of the antimicrobial /antibiotics were done as per the EFSA and OIE list. To provide continuity of monitoring data and allow epidemiological tracing of isolates with particular patterns of resistance it is recommended that these antimicrobials should remain in testing requirements.In the present study 100 per cent resistant was observed against Ampicillin, Ceftriaxone and Cefixime whereas almost 100per cent resistance was observed in Cefoperazone(97.8per cent), Cotrimoxazole (90.4per cent) and Cefuroxime (91.9per cent). And 53.3per cent isolates depicted resistance against Netilmicin. (Table 3).More than 75 per cent of the isolates were found to be resistant against Ciprofloxacin, levofloxacin was resistant for 51 per cent isolates and 57 per cent isolates showed resistance against norfloxacin.

The resistance per cent against these fluoroquinolones could have been due to various reasons. They are broad spectrum antibiotics, used to treat a wide variety of diseases on intensive farms. Secondly, when enrofloxacin/ciprofloxacin is added to the drinking water of chickens and turkeys, bacteria in the animals' intestines such as *Campylobacter, Salmonella* and *E. coli* can become resistant to fluoroquinolone antibiotics. Resistance can also pass to meat birds 'vertically' from the use of fluoroquinolones in breeding flocks [3].

In the present multi drug resistance profile study the alarming figure was obtained with tetracycline which showed 56.3per cent resistance against the ESBL *E.coli* isolates, however gentamicin, norfloxacin, tobramycin and levofloxacin exhibited 44.4per cent.,42.2per cent,38.5per cent and 37.8per cent resistance respectively. Minimum resistance percentage was seen with the drug chloramphenicol (20.7per cent) and colistin (7.9per cent). Colistin has been used for many years in livestock and is increasingly also used in human medicine, where it is one of the antimicrobials of last resort in extremely resistant Gram-negative bacterial infections. Mechanisms of acquired resistance have been described in *E. coli* [10]. It is therefore considered important to monitor resistance to colistin in food animals. Interestingly, in the present investigation resistance was even present against antibiotics like netilmicin, tobramycin, cefuroxime, cefixime and chloramphenicol, and it is a matter of concern that poultry pathogens have become resistant against these broad spectrum human antibiotics even though they are not used in veterinary medicine practices In India. Avoiding the use of cephalosporins such as ceftiofur in poultry hatcheries within the breeding pyramid is considered to be the most effective method of rapidly reducing the occurrence of ESBL producing *E. coli* in the poultry industry. [6]

On the basis of a short questionnaire a surveillance study of 10 poultry farms were also undertaken to find out the problem of antibiotic resistance at the farm level. Information were gathered from the poultry farmers regarding their day to day management practices and usage of the antibiotics. Almost 100per cent farmers found to be using antibiotics for their flock with 50per cent using both for prophylaxis and treatment (Table 4). The collection and reporting of antimicrobial resistance data at the isolate level enables more in-depth scientific analysis. In particular, it would be beneficial for detecting new multi-resistance patterns and performing analysis of the known co-resistance ones, evaluating geographical progression over time, conducting retrospective analysis and assisting in source attribution. Almost 50per cent of the farmers use herbs and other alternative medicines for their birds. Most of the poultry farmers has come across the problem of antibiotic resistance when they find that even stronger antibiotics like Ciprofloxacin, Enrofloxacin and Tetracyclines do not exhibit 100per cent results. This study reflects another aspect of the problem of resistance, reduction in prophylactic medication of poultry and improved husbandry to reduce the need for regular therapeutic treatment are desperately required to minimise selection pressure while at the same time ensuring that terminal hygiene of poultry houses is sufficient to prevent carryover of resistant organisms between flocks [5, 13]. It is also important to ensure that standards of cleaning and disinfection and pest control in hatcheries and on farms are sufficiently robust to avoid carryover and recycling of resistant organisms. This should be achieved by paying attention to optimum housing, nutrition and management so that the need for medication is reduced. [15]

Bacterial antibiotic resistance is transferable. If the resistant bacteria are allowed to distribute in the environment, they will most likely transfer resistance gene to other bacteria of same species or different. Dissemination of these resistant bacteria will not be restricted to a particular geographical area; drug resistance can be expected to spread steadily to all parts of the world. [12]

Table 3: Multi drug resistance profile of extended spectrum beta lactamases *Escherichia coli* isolates

S No.	Antimicrobial Agent	Symbol	Disc content (µgm)	Observations (N=135)					
				Resistant	Sensitive	Intermediate	Per cent Resistant	Per cent Sensitive	Per cent Intermediate
1	Ampicillin	AMP	10	135	0	0	100	0	0
2	Tetracycline	TE	30	76	38	21	56.3	28.1	15.6
3	Gentamicin	GEN	10	60	58	17	44.4	43.0	12.6
4	Cefuroxime	CXM	30	124	0	11	91.9	0.0	8.1
5	Ceftriaxone	CTR	30	135	0	0	100.0	0.0	0.0
6	Cotrimoxazole	COT	25	122	13	0	90.4	9.6	0.0
7	Colistin	CL	10	10	125	0	7.4	92.6	0.0
8	Norfloxacin	NX	10	57	37	41	42.2	27.4	30.4
9	Netilmicin	NET	30	72	54	9	53.3	40.0	6.7
10	Levofloxacin	LE	5	51	54	30	37.8	40.0	22.2
11	Ciprofloxacin	CIP	5	102	6	27	75.6	4.4	20.0
12	Cefoperazone	CPZ	75	132	0	3	97.8	0.0	2.2
13	Tobramycin	TOB	10	52	75	8	38.5	55.6	5.9
14	Cefixime	CFM	5	135	0	0	100.0	0.0	0.0
15	Chloramphenicol	C	30	28	79	28	20.7	58.5	20.7

Table 4: Study of the surveillance of Poultry Farms

S No.	Name of the Poultry Farm	No. of birds	Antibiotics used Yes/No	Antibiotics used for prophylaxis /treatment	Any herbal /alternative medicine used	Problem of antibiotic resistance observed
1.	Poultry farm A	6000	Yes	Both	Yes	Yes
2.	Poultry Farm B	16000	Yes	Treatment	No	Yes
3.	Poultry Farm C	6000	Yes	Treatment	No	Yes
4.	Poultry Farm D	6000	Yes	Treatment	No	Yes
5.	Poultry Farm E	6000-10,000	Partially only for Treatment + Prebiotic &Probiotic	Treatment	Yes	No
6.	Poultry Farm G	6000	Yes	Both	Yes	Yes
7.	Poultry Farm H	12000	Yes	Both	Yes	Yes
8.	Broiler Farm I	16000	Yes	Both	Yes	Yes
9.	Poultry Farm J	6000	Yes	Both	Yes	Yes
10.	Poultry Farm K	6000	Yes	Treatment	No	Yes

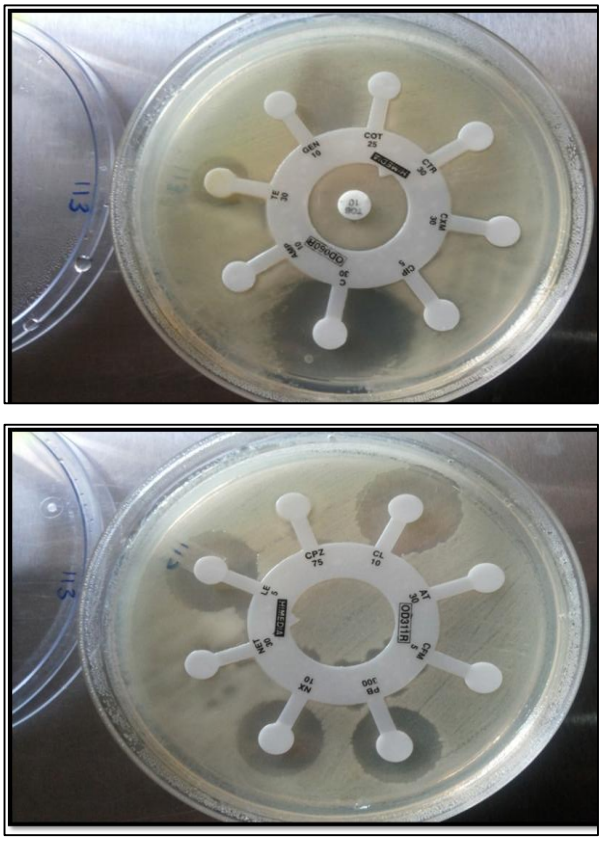

Fig 1: Multi drug resistance profile of ESBL *E.coli* positive isolates

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