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Phenotypic colistin resistance in *E. coli* isolated from livestock and poultry of Patna district, Bihar

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Abstract
The present study was undertaken to determine the occurrence of *E. coli* in different livestock species and poultry in Patna, Bihar. A total of 254 samples comprising of cow milk, buffalo milk, poultry cloacal swab and goat rectal swab were processed for isolation of *E. coli*. Based on PCR 34.7% of cow milk samples, 48.4% of buffalo milk samples, 77.6% of goat rectal swab samples and 78.4% of poultry cloacal swab samples were found positive for *E. coli* which were tested for phenotypic resistance against colistin by MIC testing. 12% (3/25) cow milk isolate, 32.2% (10/31) buffalo milk isolate, 5.7% (3/52) isolate from goat rectal swab and 15% (6/40) isolates from poultry were phenotypically found resistant to colistin based on MIC value which need further genotypic confirmation.

Keywords: Phenotypic colistin, *E. coli*, livestock, poultry

Introduction
Escherichia coli (*E. coli*) is gram-negative bacterium belongs to the family Enterobacteriaceae, commonly found in the gastrointestinal tract of humans and animals. Most of the *E. coli* of humans are non-pathogenic in nature. However, the pathogenic *E. coli* is mainly transmitted to humans through consumption of contaminated animal foods, such as the raw meat, meat products, raw milk and milk products etc. (Barlaam *et al*., 2019) [3]. The pathogenic bacteria present in the animal foods, also carry the drug resistant genes of various classes of antibiotics. The extensive use of antimicrobials in animal production is associated with emergence and spread of antimicrobial resistance (AMR) in food borne bacteria (Van Boeckel *et al*., 2015) [28]. In recent past, due to emergence of carbapenem resistant *E. coli*, *Pseudomonas* and *Acinetobacter* strains has led to the re-introduction of colistin, *i.e*. a drug once considered to be inconvenient and too toxic for routine parenteral use, in to daily clinical application (Dhariwal *et al*., 2013) [7]. As for these pathogens colistin often represents the last resort of treatment options, resistance to it commonly leads to more severe complications and increased mortality (Capone *et al*., 2013) [4]. Therefore, the present study was design to assess the status of colistin resistant *E. coli* in different samples of animal origin from Patna region.

Material and Methods
Collection of samples
A total of 254 samples comprising of cow milk (72), buffalo milk (64), poultry cloacal swab (67) and goat rectal swab (51) were collected aseptically from Patna district, Bihar and transported to the laboratory under cold conditions.

Isolation and identification of *E. coli*
For isolation of *Escherichia coli* 2 ml of milk sample was taken and centrifuged at 3500 rpm for 10 min. A loopful of the pellet was inoculated in MacConkey broth for enrichment at 42 °C for 18-24 h. however rectal/cloacal swab were directly inoculated in Mac-Conkey broth for enrichment. Further a loopful of MacConkey broth incubated with samples were streaked on eosin methylene blue agar (EMB) plates and incubated overnight at 37 °C. The characteristic colonies with metallic sheen were further confirmed by biochemical test and PCR targeting *16SrRNA* gene with forward primer 5'GAAGAAGCTTGCTTCTTTGCTGAC3' and reverse primer 5' GCCCGGGGATTTCACATCTGACTTA 3' (Sabat *et al*., 2000) [20]. PCR amplification was performed in 25 µl reaction volume each containing 2.5 µl 10X PCR amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of

dNTPs (10 mM), 2.0 µl (10 pmol) of forward and reverse primers, 0.2 µl Taq DNA polymerase (5 unit/µl), 3.0 µl of bacterial lysate (DNA template) and 14.8 µl nuclease free water. The cycling condition was standardized with initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation (94 °C for 30 sec), annealing (72 °C for 45 sec) and extension (72 °C for 45 sec) with final extension at 72 °C for 10 min.

Determination of susceptibility of *E. coli to* colistin sulphate

The susceptibility to colistin was determined by broth microdilution method using 96 well culture plate. The antibiotic stock solution was prepared by dissolving 10 mg colistin sulphate powder in 1 ml of sterile distilled water. The working concentration was prepared by mixing 96 µl of antibiotic from stock solution and 14.904 ml of sterile distilled water, with final concentration of 64 µg/ml (A).

Preparation of test inoculums and protocol for MIC

The pure cultures of the test organism (3-5 colonies) were picked in sterile normal saline solution to have the inoculum of 0.5 McFarland (1.5 x 10^8 cfu/ml). Diluted @ 1:100 by adding 100 µl of the same in 9.9 ml of normal saline solution to have final inoculum of 1.5 x10^5 cfu/ml. 100 µl of solution A was added in first well of cell culture plate and 50 µl of MHA broth was added into the well from second to twelfth to prepare two-fold serial dilution of antibiotic. 50 µl of 1.5 x10^5 cfu/ml bacterial culture was added in each well except 11^{th} well which was used as control to check the sterility of MHA broth and 12^{th} well which was used as bacterial culture control and incubated at 37 °C for 18-20 hours.

Interpretation of MIC was made as the lowest concentration of antibiotic where bacterial growth was inhibited. The isolates were categorized as nonwild type at MIC ≥ 4 µg/ml and wild type at MIC ≤ 2 µg/ml (CLSI document M100-S25) [5].

Results and discussion

Out of 254 samples processed a total of 194 (76.3%) samples were found to produce characteristic flat, dark centred with metallic blue sheen colony on EMB agar. On further confirmation by biochemical profile and PCR 34.7% of cow milk samples, 48.4% of buffalo milk samples, 77.6% of goat rectal swab samples and 78.4% of poultry cloacal swab samples were found positive for *E. coli*. (Table-1, Fig.1)

The occurrence of *E. coli* in cow milk is in aggrements to the findings of Thaker *et al.*, (2012) [26]; Jyothi *et al.*, (2018) [13]; Mahanti *et al*. (2020) [17] who had reported 38%, 24.4% and 45.5% occurrence in cow milk samples, respectively. However, in contrast to the present findings Skockova *et al*. (2014) [23] and Paghdar *et al*., (2020) [19] reported higher rate of occurrence *i.e*. 92.4% and 62.66% respectively in cattle milk samples. However, the occurrence of *E. coli* in buffalo milk were comparatively higher than the cow milk which may be due to the natural wallowing habit of buffaloes that may results into more chances of milk contamination.

The higher rate of occurrence of *E. coli* in goat rectal swab (77.6%) and poultry cloacal swab (78.4%) corroborate with previous reports in goat (Hardik *et al.* 2017) [11]; (Shabana and Al-Enazib 2020) [21] and poultry (Doregiraee *et al*. 2016) [8]; (Gazal *et al*. 2021) [10]. The high occurrence of *E. coli* in poultry and goat in the present study indicates that they may act as a potential reservoir of *E. coli* and pose threat to farmer.

Susceptibility of *E. coli* to colistin sulphate by broth microdilution MIC testing

Out of 25 *E. coli* isolates from cow milk samples, 4% (1/25) isolate had MIC value of 8 µg/ml and 8% (2/25) isolates had MIC value of 32 µg/ml (Table-2). However, out of 31 *E. coli* isolates from buffalo milk 6.4% (2/31) isolates had MIC value of 4 µg/ml and 25.8% (8/31) isolates had MIC value of 32 µg/ml (Table-3).

Out of 52 *E. coli* isolates from goat rectal swab 1.9% (1/52) isolate have MIC value of 4 µg/ml and 3.8% (2/52) isolates have MIC value of 32 µg/ml (Table-4). Similarly out of 40 *E. coli* isolates from poultry cloacal swab 2.5% (1/40) isolate had MIC value of 4 µg/ml, 2.5% (1/40) isolate have MIC value of 8 µg/ml and 10% (4/40) isolates have MIC 32 µg/ml (Table-5).

The results for MIC were interpreted as per CLSI document M100-S25. A MIC ≥ 4 µg/ml were categorized as non-wild type (resistant) and MIC ≤ 2 µg/ml were categorized as wild type (sensitive). Hence the MIC result for isolates under present study shows that out of 148 isolates of *E. coli*, 12% (3/25) isolates of cow milk samples, 32% (10/31) isolates of buffalo milk samples, 5.7% (3/52) isolates of goat rectal swab samples and 15% (6/40) isolates of poultry swab samples were showing phenotypic resistance to colistin sulphate having MIC between 4-32 µg/ml. The study revealed that 14.86% of the total isolates from different livestock and poultry sources were phenotypically resistant against colistin. As the study reveals isolates as phenotypic resistance to colistin, genotypic confirmation is needed further confirmation by amplification of *mcr* gene. In a study by Liu *et al*. (2020) [15] and Filioussis *et al*. (2020) [9] 2% and 6.7% isolates from bovine milk samples were reported as colistin resistant which is in concordance to the present finding. However, in a study by Shafiq *et al*. (2021) [22] and Tartor *et al*. (2021) [25] colistin resistance was reported in 70.23% and 57.14% isolates respectively, whereas Kamal *et al*. (2018) [14] reported moderately sensitive isolates against colistin from goat rectal swab samples.

In contrast to this study, Al Azad *et al*. (2019) [1] reported susceptibility against colistin sulphate *i.e* (73.5%) whereas Uddin *et al*. (2022) [27] and Dandachi *et al*. (2018) [6] reported 88.30% and 69% isolates resistance against colistin in poultry faecal sample respectively. In reference to this study, Assoumy *et al*. (2021) [2], Hassen *et al*. (2020) [12], Sobur *et al*. (2019) [24], Moawad *et al*. (2018) [18] and Maamar *et al*. (2018) [16] reported 26.42%, 53.1%, 2.1%, 7.9%, 4.1% isolates resistance against colistin respectively from poultry cloacal swab samples.

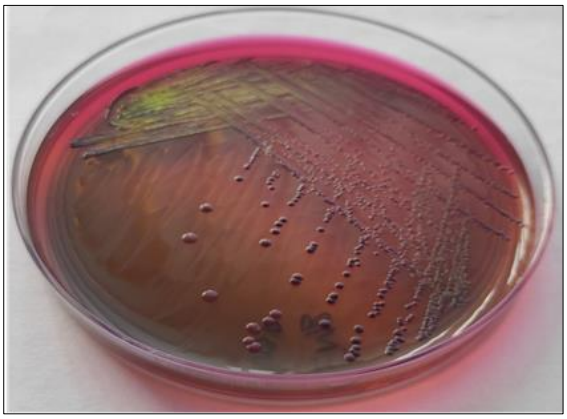

Fig 1: Metallic sheen on EMB plate

Table 1: Characteristics growth of isolates on EMB plate, confirmation of IMViC pattern and PCR assay targeting *16S-RNA* gene of *E. coli* and % occurrence of *E. coli* based on PCR

S. N	Animal species	Sample type	No. of sample	Growth on EMB plate	*E. coli* (*16srRNA*)	*%Occurrence based on PCR*
1.	Cow	Milk	72	35 (48.6%)	25	34.7
2.	Buffalo	Milk	64	48 (75%)	31	48.4
3.	Goat	Rectal swab	67	61 (91%)	52	77.6
4.	Poultry	Cloacal swab	51	50 (98%)	40	78.4
Total			254	194 (76.3%)	148	58.2

Table 2: Determination and interpretation of MIC of Colistin sulphate for *E. coli* isolates from cow milk samples

S.N	Isolates	MIC (µg/ml)	Interpretation	S.N	Isolates	MIC (µg/ml)	Interpretation
1.	C13D	32	**R**	14.	C19PS	1	S
2.	C15D	32	**R**	15.	C20PS	1	S
3.	C3PS	0.031	S	16.	C4PH	1	S
4.	C6PS	0.25	S	17.	C8PH	2	S
5.	C7PS	1	S	18.	C9PH	2	S
6.	C9PS	0.5	S	19.	C10PH	2	S
7.	C11PS	8	R	20.	C11PH	1	S
8.	C12PS	0.5	S	21.	C12PH	2	S
9.	C13PS	1	S	22.	C13PH	2	S
10.	C14PS	1	S	23.	C14PH	2	S
11.	C16PS	1	S	24.	C15PH	0.5	S
12.	C17PS	1	S	25.	C16PH	1	S
13.	C18PS	2	S				

Table 3: Determination and interpretation of MIC of Colistin sulphate for *E. coli* isolates from Buffalo milk samples

S.N	Isolates	MIC(µg/ml)	Interpretation	S.N	Isolates	MIC(µg/ml)	Interpretation
1.	B1BI	1	S	17.	B1PS	2	S
2.	B2BI	2	S	18.	B2PS	0.25	S
3.	B3BI	32	R	19.	B6PS	4	R
4.	B4BI	4	R	20.	B8PS	0.0125	S
5.	B7BI	2	S	21.	B9PS	1	S
6.	B10BI	1	S	22.	B10PS	2	S
7.	B13BI	32	R	23.	B13PS	2	S
8.	B14BI	1	S	24.	B14PS	1	S
9.	B15BI	2	S	25.	B15PS	2	S
10.	B16BI	32	R	26.	B1PH	0.5	S
11.	B4D	0.031	S	27.	B2PH	2	S
12.	B7D	32	R	28.	B5PH	32	R
13.	B8D	1	S	29.	B7PH	2	S
14.	B9D	0.5	S	30.	B8PH	2	S
15.	B12D	32	R	31.	B14PH	32	R
16.	B13D	32	R				

Table 4: Determination and interpretation of MIC of Colistin sulphate for *E. coli* isolates from goat rectal swab samples

S.N	Isolates	MIC(µg/ml)	Interpretation	S.N	Isolates	MIC(µg/ml)	Interpretation
1.	G1BI	1	S	27.	G10PS	2	S
2.	G5BI	32	**R**	28.	G11PS	1	S
3.	G6BI	32	**R**	29.	G12PS	2	S
4.	G7BI	1	S	30.	G13PS	1	S
5.	G8BI	1	S	31.	G14PS	2	S
6.	G9BI	1	S	32.	G15PS	1	S
7.	G10BI	1	S	33.	G16PS	1	S
8.	G11BI	1	S	34.	G17PS	2	S
9.	G13BI	2	S	35.	G18PS	1	S
10.	G14BI	1	S	36.	G19PS	2	S
11.	G15BI	0.5	S	37.	G1PH	1	S
12.	G16BI	0.5	S	38.	G2PH	1	S
13.	G3D	0.5	S	39.	G3PH	1	S
14.	G4D	2	S	40.	G4PH	2	S
15.	G5D	1	S	41.	G5PH	4	R
16.	G10D	0.125	S	42.	G6PH	2	S
17.	G12D	0.5	S	43.	G7PH	1	S
18.	G16D	0.5	S	44.	G8PH	1	S
19.	G1PS	0.5	S	45.	G9PH	1	S
20.	G2PS	2	S	46.	G10PH	1	S

21.	G3PS	0.5	S	47.	G11PH	1	S
22.	G4PS	1	S	48.	G12PH	2	S
23.	G6PS	0.25	S	49.	G13PH	1	S
24.	G7PS	0.5	S	50.	G14PH	1	S
25.	G8PS	1	S	51.	G15PH	1	S
26.	G9PS	0.5	S	52.	G16PH	2	S

Table 5: Determination and interpretation of MIC of Colistin sulphate for *E. coli* isolates from poultry cloacal swab samples

S.N	Isolates	MIC(µg/ml)	Interpretation	S.N	Isolates	MIC(µg/ml)	Interpretation
1.	P2BI	1	S	21.	P6PS	1	S
2.	P3BI	1	S	22.	P7PS	1	S
3.	P4BI	2	S	23.	P8PS	1	S
4.	P5BI	1	S	24.	P9PS	1	S
5.	P7BI	32	R	25.	P10PS	1	S
6.	P8BI	1	S	26.	P11PS	1	S
7.	P9BI	0.5	S	27.	P12PS	4	R
8.	P10BI	1	S	28.	P13PS	1	S
9.	P12BI	2	S	29.	P1PH	2	S
10.	P1D	1	S	30.	P2PH	1	S
11.	P5D	8	R	31.	P3PH	2	S
12.	P6D	2	S	32.	P4PH	1	S
13.	P7D	2	S	33.	P5PH	1	S
14.	P9D	0.25	S	34.	P6PH	2	S
15.	P11D	32	R	35.	P7PH	1	S
16.	P12D	2	S	36.	P8PH	1	S
17.	P2PS	1	S	37.	P9PH	2	S
18.	P3PS	0.25	S	38.	P10PH	2	S
19.	P4PS	1	S	39.	P11PH	32	R
20.	P5PS	1	S	40.	P12PH	32	R

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