Explor Anim Med Res,
Vol.13, Issue - 1, 2023, p. 31-38
DOI : 10.52635/eamr/13.1.31-38

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

DETECTION OF DRUG-RESISTANT EXTENDED-SPECTRUM AND *AmpC*β-LACTAMASES PRODUCING *ESCHERICHIA COLI* FROM POULTRY FAECAL SAMPLES IN WEST BENGAL, INDIA

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Received27 September 2022, revised 23 March 2023

ABSTRACT: *Escherichia coli* is quite a common and sometimes pathogenic bacterium that can cause acute to severe infection in poultry birds affecting their performance. *E. coli* can also cause a few extra-intestinal infections too in poultry birds. Nowadays antimicrobial resistance is quite a menace which is reported from several gram-negative bacteria including *E. coli*. These traits in *E. coli* are governed by a few β-lactamases producing genes which may also be associated with plasmids in the pathogen. This study aimed to detect the presence of extended-spectrum (ESBL) and AmpC (ACBL) beta-lactamases producing *E. Coli* from fecal samples of diseased and apparently healthy poultry birds from different districts of West Bengal, India. A total of 177 (62.32%) *E. coli* isolates were detected and confirmed from the poultry fecal samples/ cloacal swabs (n=284) collected in this study. All isolates were typical in morphology. One hundred thirty-two (74.58%) bacterial isolates were positive for phenotypical beta-lactamases production, of which 83 (46.89%) were positive for phenotypical ACBL production. A total of 41 and 17 isolates were found to be positive for PCR detection of the *bla*CTXM and *bla*SHV genes respectively. No *bla*TEM gene was detected. Again 95 (53.67%) isolates possessed the *bla*AmpC gene as detected in this study. Antibiogram of the ESBL-producing *E. coli* isolates (49) revealed a significantly high level of resistance against most of the commonly used antimicrobials like ceftriaxone, ampicillin (both 100%), cefotaxime (97.96%), ceftazidime (91.84%) amoxicillin/clavulanic acid (85.71%), azithromycin, tetracycline, norfloxacin (@70-75%). These isolates were found to be sensitive (@ 96-81%) against a few antimicrobials, *viz.* amikacin, imipenem, gentamicin, and ampicillin/sulbactam which is a major point of concern.

Key words: Antibiogram, ACBL, Poultry, ESBL, *Escherichia coli*, Faeces.

INTRODUCTION

Poultry is one of India's fastest-growing segments of the agricultural sector, with around 8-10% growth per year (APEDA). Recently Indian poultry sector has developed to a large extent and now contributes significantly to the rural economy. Poultry farming operation has now been transformed from a mere backyard activity into a major commercial agri-based industry over a period of four decades. India ranks 3rd in egg production and 8th in meat production in the world (Economic Survey 2021-22). With this huge production, this industry now plays an important role in the rural economy and livelihood. The poultry industry is also in a high growth stage in the state of West Bengal. West Bengal is the 2nd largest contributor to chicken meat production and eggs within India (DAHD 2017).

Escherichia coli, the Gram-negative bacteria is one of the dreadful commensals found to affect a significant level of poultry production performance (Begum *et al*. 2018). The use of drugs, especially antibiotics, has increased tremendously in the last few decades leading to an increase in antimicrobial resistance among microbial populations (Ventola 2015). The incidence of extended-spectrum (ESBL) and *Ampicillinase* C (ACBL) beta-lactamases-producing *Escherichia coli* in poultry is quite significantly increasing nowadays all around the world causing the treatment of common bacterial infections very difficult (Olsen *et al*. 2004). ESBL/ ACBL production in bacteria is governed by the presence of *bla*CTX-M, *bla*SHV, *bla*TEM, and *bla*AmpC genes which can be easily transferred from one bacterium to another spreading the menace of antimicrobial resistance (Vaidya

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2011). Among these resistance genes, the b*la*CTX-M gene is the most common gene associated with ESBL positivity and *bla*AmpC for ACBL production in *E. coli* isolates (Batabyal *et al*. 2020). AmpC beta-lactamase (ACBL) is the first bacterial enzyme reported to destroy penicillin in Gram-negative bacteria like *Escherichia coli* (Jacoby 2009). ACBL encoding gene *bla*AmpC is found in transmissible plasmids and also in bacterial chromosomes (Reich *et al.* 2013). These drug-resistant pathogens can create a major problem during the treatment of the infected poultry birds as well as can pass to the bird handlers/ consumers via food to spread AMR within humans too, forcing the clinicians to use newer and newer antibiotics (Manyi-Loh *et al.* 2018, Arun *et al.* 2022, Talib and Abdulrahman 2022). This is leading to the increased use of last-resort antimicrobials such as carbapenems even for non-life-threatening infections. These antimicrobial resistance genes of *E. coli* are easily transferrable to other pathogens transferring their resistance property (Vaidya 2011). In a study in Mexico, Castillo *et al.* (2018) revealed that *E. coli* associated with urinary tract infections (63%) of human beings were highly resistant (27-48%) to commonly used antibiotics. ACBL-producing *E. coli* strains are resistant to several broad-spectrum antibiotics but their resistance patterns are less expressed *in-vitro* than that of the ESBLs (Jacoby 2009) may be due to the plasmidic presence of the ACBL gene. ESBL-producing *E. coli* is frequently reported from poultry samples worldwide and may be pathogenic to humans causing urinary tract infections, septicemia, meningitis, etc. (Castillo *et al.* 2018, Nandanwar *et al.* 2014). A large quantity of commonly used antibiotics is also used in poultry farming, leading to the development of antimicrobial resistance in the bacteria associated with poultry as well as human beings. In addition, there are human health concerns about the presence of antimicrobial residues in meat, eggs, and other animal products (Sahoo *et al*. 2010, Darwish *et al*. 2013, Vineesha *et al.* 2021). In this perspective, the present research was aimed toward the detection and detailed characterization of ESBL and ACBL-producing *E. coli* isolates from poultry fecal samples from different districts of West Bengal, followed by an antibiotic sensitivity assay to know about their *in vitro* resistance patterns.

MATERIALS AND METHODS

Collection of poultry fecal samples

A total of 284 fecal samples were collected aseptically from poultry birds from a few poultry farms (11 in no.) in different districts of West Bengal (Table 3) from February to June 2022. Sterile swabs collected the cloacal swab samples from diseased and apparently healthy poultry birds irrespective of age. All the collected swabs were kept in sterile peptone water (M028-HiMedia, India) for transport. All the samples collected were placed on ice in a thermos flask with the proper label and were brought to the laboratory for further processing within 48 hrs of collection (Banerjee *et al.* 2019).

Isolation of *Escherichia coli* from fecal samples

The enriched swab samples were streaked onto sterile MacConkey's agar (MH081- HiMedia, India) plates followed by incubation at 370C overnight. The rose-pink lactose fermenting colonies were randomly picked and transferred to EMB agar (M317-HiMedia, India) plates followed by incubation at 37ºC for 24 hrs for selective isolation of *E. coli*. The colonies with metallic sheen were observed and streaked to nutrient agar (HiMedia, India) slants for further characterization. All these isolates were morphologically tested by Gram's method of staining (Quinn *et al.* 2011). Biochemical characterization of the isolates was done by the IMViC test as per Quinn *et al.* (2011).

Molecular confirmation of *E. coli* isolates by PCR

Bacterial DNA extraction

For genotypic detection of *Escherichia coli,* DNA was extracted (by simple boiling method) from all the *Escherichia coli* isolates as per Mahanti *et al.* (2013).

Detection of *16SrRNA* gene specific for *Escherichia coli* by PCR

All the tentatively positive *E. coli* isolates were subjected to PCR for the detection of the 16S *rRNA* gene (585bp) [Promega] specific for *E. coli* as described by Wang *et al*. (1996) with some modifications for confirmation. One *E. coli* and one *Staphylococcus* sp. isolate (both departmental isolates) were used as positive and negative controls in this study.

Detection of ESBL production in *Escherichia coli* isolates

Phenotypical detection of ESBL Production

The antibiotic discs containing cefotaxime (30 µg, HiMedia, SD040) and ceftazidime (30 µg, HiMedia, SD062) with or without clavulanate (10 µg, HiMedia) were used and a difference of ≥ 5 mm between the zone diameters of either of those single disks and their clavulanate combination disk was considered to be

phenotypically positive for ESBL property in the isolates (Bauer *et al.* 1966, Patel *et al.* 2015).

Detection of ESBL genes in *Escherichia coli* isolates by PCR (Bio-Rad)

All phenotypically ESBL-producing *Escherichia coli* isolates were screened for PCR detection of the *bla*CTX-M (540bp) and *bla*TEM (1080bp) (Table 1) as per the protocol of Weill *et al.* (2004), whereas the *bla*SHV (792bp) genes as per the protocol of Cao *et al.* (2002) with some modifications. All PCR chemicals and primers (Table 1) are procured from Promega, India (Table 2).

Detection of ACBL production in *Escherichia coli* isolates

Phenotypical detection of AmpC beta-lactamase production in *Escherichia coli* isolates

All the *Escherichia coli* isolates were subjected to a cefoxitin-cloxacillin (HiMedia) double-disc synergy (CC-DDS) test for phenotypic detection of ACBL production as per the protocol of Tan *et al.* (2009).

Detection of AmpC gene in *Escherichia coli* isolates by PCR

All phenotypically positive ACBL-producing *Escherichia coli* isolates were tested for the presence of the *bla*AmpC gene (634bp) (Promega) by PCR assay as per the protocol of Féria *et al.* (2002) with some modifications. Gel electrophoresis was done in 1% gel (SRL, India) in a gel documentation system (Labnet).

Antimicrobial sensitivity test of the ESBL-producing *E. coli* isolates

The ESBL-producing *E. Coli* isolates were tested for their resistance patterns against 12 different antimicrobials [amikacin (30 µg), amoxicillin/ clavulanic acid (20/10 µg), ampicillin/ sulbactam (10/10 µg), ampicillin (10 µg), azithromycin (15 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), gentamicin (10 µg), imipenem (10 µg), norfloxacin (10 µg) and tetracycline (30 µg)] (HiMedia) by the disc diffusion method (Bauer *et al.* 1966, Patel *et al.* 2015).

RESULTS AND DISCUSSION

Isolation and characterization of *Escherichia coli* from fecal samples

In this study, 177 (62.32%) poultry cloacal swab samples showed the presence of *Escherichia coli* after detailed characterization (Abdelrahman *et al.* 2008). All the isolates yielded a characteristic pink-colored colony in MacConkey agar and a 'metallic sheen' on EMB agar plates. All were detected to be Gram-negative small rods in this study (Quinn *et al.* 2011) and showed typical results in the IMViC test [+ + - -] (Samanta 2013). All the tentatively positive isolates showed the presence of the 16S *rRNA* gene specific for *E. coli* in PCR which confirms the result (Fig. 1). The highest prevalence (71.21%) is seen in the samples collected from the Birbhum district (Table 3). Panchal *et al.* (2020) reported approx. 74% prevalence of *E. coli* from poultry samples from Gujarat, India in their study which is almost similar to the present findings. Scientists, namely Maciuca *et al.* (2015), Klimiene *et al.* (2018), and Ievy *et al.* (2020) also reported the prevalence of *E. coli* isolates from chicken (feces, cloacal swabs, postmortem samples, and chicken meat) samples @54%, 92%, and 68% respectively which almost match with the present study. Again Ibrahim *et al.* (2019) reported 53.4% positivity of *E. coli* isolates from poultry sources in their study in Jordan. The differences in prevalence rates may be due to many factors including geographical variation, environmental effects, and differences in sampling patterns.

Detection of ESBL/ACBL production in *E. coli* isolates detected from poultry samples

A total of 132 (74.58%) *E. coli* isolates were found to be positive for phenotypic detection of ESBL/ ACBL production in a combined disc diffusion assay (Table 4).

Different beta-lactamases-producing genes were detected in the isolates by PCR assay as detailed in Table 5, which showed the presence of *bla*CTX-M (Fig. 2), *bla*SHV (Fig. 3), and *bla*AmpC (Fig. 4) genes in different percentages (Table 5) with the *bla*AmpC gene having the highest prevalence (53.67%). No *bla*TEM gene was detected in this study. There is variation in the presence of ESBL/ACBL-producing genes in the isolates and their phenotypical expression *in vitro*. The expression of these genes may vary depending on several factors including the hosts' immune response, antimicrobials used, and other environmental factors (Beceiro *et al.* 2013). An earlier report by Geser *et al.* (2012) showed the prevalence of the *bla*CTX-M gene to be 94%, followed by the *bla*SHV gene (6%) and no *bla*TEM gene in *E. coli* isolates which almost matches the present findings. Kar *et al.* (2015) and Upadhyay *et al.* (2015) also reported the blaCTX-M gene to be the most prevalent among the ESBL genes. ESBL positivity in *E. coli* isolates was also reported in different countries by Klimiene *et al.* (2018) [54%], Casella *et al.* (2017) [92%], and Maamar *et al.* (2016) [35%].

Table 1. List of Primers used in this study.

Sl.No.	Target gene amplified	Primer sequence (5´→ 3´)	Annealing temp.	Product size (bp)	Reference
01	*E. coli* *16S rRNA*	F: GACCTCGGTTTAGTTCACAGA	58°C	585	Wang *et a1.* (1996)
		R: CACACGCTGACGCTGACCA			
02	bla_{TEM}	F: ATAAAATTCTTGAAGACGAAA	53°C	1080	Weill *et al.* (2004)
		R: GACAGTTACCAATGCTTAATC			
03	bla_{SHV}	F: TTA TCT CCC TGT TAG CCA CC	52°C	792	Cao *et al.* (2002)
		R: GAT TTG CTG ATT TCG CTC GG			
04	*bla* CTX-M consensus	F: CAATGTGCAGCACCAAGTAA	53°c	540	Weill *et al.* (2004)
		R: CGCGATATATCGTTGGTGGTTGGTG			
05	*bla*AmpC	F: CCCCGCTTATAGAGCAACAA	60°C	634	Féria *et al.* (2002)
		R: TCAATGGTCGACTTCACACC			

Table 2. List of Reagents used in this study for PCR.

Sl. No.	Reagents	Company Name	Catalogue No.
1.	dNTP	Promega	U1515
2.	Taq DNA Polymerase	Promega	M8295

The highest prevalence of the *bla*AmpC gene in the *E. coli* isolates was also reported earlier by Vinueza-Burgos *et al.* (2019) [94.3%], Banerjee and Acharyya (2020, 2021) [88.9%] but higher than the reports of Kar *et al.* (2015) [11.1%] and Casella *et al.* (2017) [4.2%].

Antimicrobial sensitivity test of ESBL-positive *Escherichia coli* isolates

All the ESBL-producing *E.coli* isolates showed almost similar resistance/ sensitivity patterns in their antibiogram (Dierikx *et al.* 2010). Most of the isolates were resistant to amoxicillin/ clavulanic acid, ceftriaxone, ampicillin, ceftazidime, norfloxacin, cefotaxime, azithromycin, and tetracycline in various degrees as detailed in Table 6. The isolates showed sensitivity to a few drugs *viz.* amikacin (89.79%), imipenem (95.91%), ampicillin/ sulbactam (73.47%), and gentamicin (79.59%) only. These patterns of sensitivity to a few selected antimicrobials and almost cent-percent resistance to all commonly used antimicrobials may be the effect of the presence of ESBL and ACBL genes in those isolates and that's quite an alarming fact.

Banerjee and Acharyya (2020, 2021) also reported high levels of antibiotic resistance in *E. coli* isolates, which

Table 3. District-wise description of sample collection with birds' details from broilers.

Name of Dist.	Name of Farms	Age (days)	No. of Samples	Total
Bardhhaman	Vada	36	38	64
	Bhota	38	26	
Nadia	Bishnu Poultry Farm	37	18	44
	Das Poultry Farm	38	14	
	Majumder Poultry Farm	42	12	
24 PGs (N)	Ramchandrapur	41	34	57
	Hamidpur	40	23	
Birbhum	Patharchapri	38	36	66
	Bhawanipur	44	30	
Medinipur	Jamirasholi	32	21	53
	Ledashal	35	32	
Total				284

almost matches the present report. The present study's findings are similar to the observations of Ibrahim *et al.* (2016) and Hinthong *et al.* (2017) who also reported a high percentage of drug resistance among their *E. coli* isolates. Ali *et al.* (2016) reported significant antimicrobial resistance against ampicillin (86.11%), amoxicillin-clavulanic acid (63.89%), cefotaxime (100%), ceftazidime (66.67%), tetracycline (72.22%) and gentamicin (61.11%) by ESBL producing *E. coli* pathogens in their study. Faruk *et al.* (2016) reported

Table 4. Detection of *E. coli* strains with phenotypical beta-lactamases production.

Name of the District	No. of samples screened	No. of *E. coli* strains Isolated (%)	Phenotypically positive isolates (ESBL+ACBL)
Bardhhaman	64	41 (64.06%)	34
Nadia	44	26 (59.09%)	21
24 PGs (N)	57	29 (50.87%)	17
Birbhum	66	47 (71.21%)	36
Medinipur	53	34 (64.15%)	24
Total	284	177 (62.32%)	132 (74.58%)

that ampicillin, cefotaxime, ceftazidime, and cefuroxime (all 100%), tetracycline (93.54%) were highly resistant but all the isolates were highly sensitive to but imipenem (100%). The resistance profile of the *E. coli* isolates in the present study may reflect the uncontrolled usage pattern of different antibiotics in the studied poultry birds.

CONCLUSION

Approximately 62% of the poultry fecal samples screened in this study were found to be positive for *E. coli*. All isolates were typical in morphological and biochemical characters. A significantly high number of those (74.58%) bacterial isolates expressed the production of beta-lactamases phenotypically. Eighty-three (46.89%) and 49 (27.68%) *E. coli* isolates were positive for ACBL and ESBL production respectively. The frequency of the *bla*AmpC gene was the highest (53.67%) followed by others. No *bla*TEM gene was detected in this study. The ESBL-producing *E. coli* isolates exhibited a high level of resistance against antibiotics namely ceftriaxone, ampicillin, cefotaxime, ceftazidime, amoxicillin/ clavulanic acid, azithromycin, tetracycline, and norfloxacin whereas were sensitive to amikacin, imipenem, gentamicin, and ampicillin/ sulbactam. Further studies can be done in this area, to detect the prevalence of antibiotic-resistance

Table 5. Molecular detection of ESBL and ACBL genes in *E. coli* isolates.

E. coli isolates tested	Phenotypic Detection of ESBL production	Molecular Detection of				Phenotypic Detection of ACBL production	Molecular Detection of bla_{AmpC} gene
		bla_{CTXM} gene	bla_{SHV} gene	bla_{TEM} gene	bla_{CTXM} + bla_{SHV}		
177	49 (27.68%)	27	3	0	14	83 (46.89%)	95 (53.67%)

Table 6. *In vitro* resistance patterns of ESBL-producing *E. coli* strains (n=49) against 12 nos. antimicrobials used in this study.

Sl. No	Antimicrobials (Conc. in µg)	Isolates sensitive		Isolates intermediately sensitive		Isolates resistant	
		No.	%	No.	%	No.	%
1.	Amikacin (AK - 30)	44	89.79	5	10.20	0	0
2.	Amoxicillin / Clavulanic acid (AMC - 20/10)	0	0	7	14.29	42	85.71
3.	Ceftriaxone (CTR 30)	0	0	0	0	49	100
4.	Ampicillin/Sulbactam (*A/S* - 10/10 mcg)	36	73.47	13	26.53	0	0
5.	Ampicillin (AM - 10)	0	0	0	0	49	100
6.	Ceftazidime (CAZ - 30)	0	0	4	8.16	45	91.84
7.	Imipenem (IPM - 10)	47	95.91	2	4.08	0	0
8.	Gentamicin (GEN - 10)	39	79.59	10	20.40	0	0
9.	Norfloxacin (NX - 10)	0	0	15	30.61	34	69.38
10.	Cefotaxime (CTX - 30)	0	0	1	2.04	48	97.96
11.	Azithromycin (AZM - 15)	0	0	12	24.49	37	75.51
12.	Tetracycline (TE - 30)	0	0	14	28.57	35	71.42

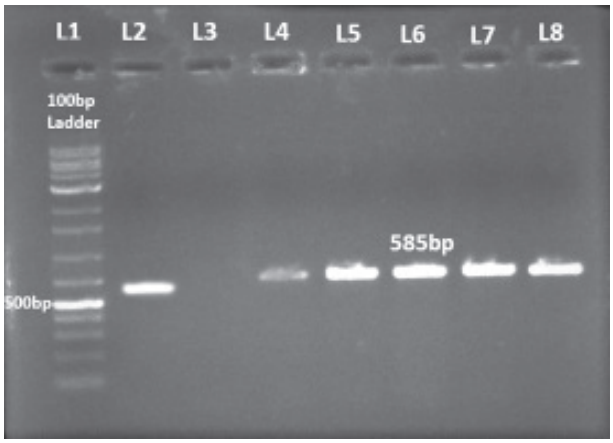

Fig. 1. The gel electrophoresis pattern exhibiting confirmation of *Escherichia coli* isolates possessing the *16S rRNA* gene (585 bp) [L1: 100 bp DNA Ladder (SRL, India), L2: Positive control (PC), L3: Negative control (NC), L4-L8: Test samples].

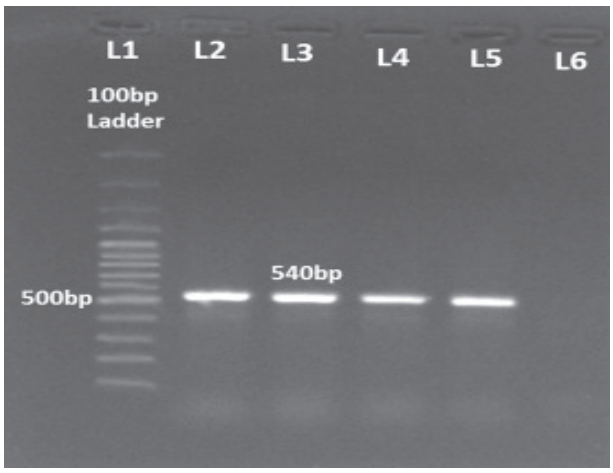

Fig. 2. The gel electrophoresis pattern exhibiting presence of bla_{CTXM} gene (540bp) in *E. coli* isolates [L1: 100 bp DNA Ladder (SRL, India), L2: PC, L3-L5: Test samples, L6: NC].

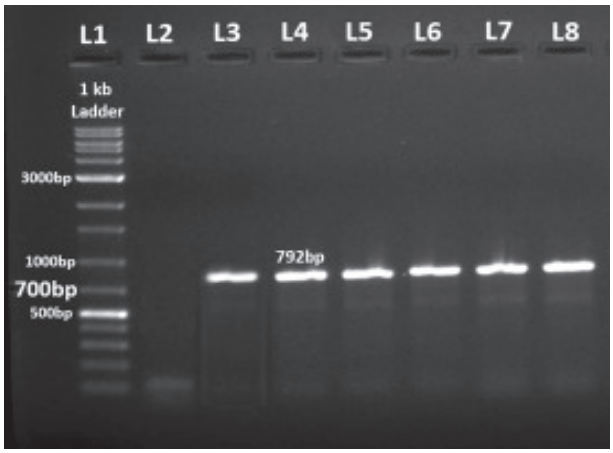

Fig. 3. The gel electrophoresis pattern with the detection of bla_{SHV} gene (792bp) in *Escherichia coli* isolates [L1: 1Kb DNA Ladder (SRL, India), L2: NC, L3: PC, L4-L8: Test samples].

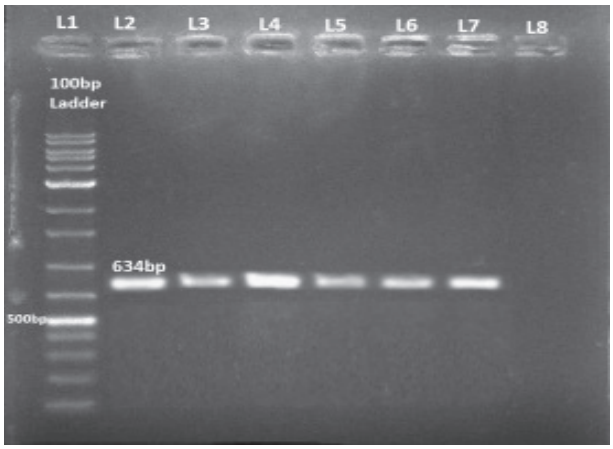

Fig. 4. The gel electrophoresis pattern exhibiting detection of bla_{AmpC} gene (634bp) in *Escherichia coli* isolates [L1: 100bp DNA Ladder (SRL, India), L2: PC, L3-L7: Test samples, L8:NC].

genes in Gram-negative bacteria isolates from duck samples and their correlation with human infections to establish a zoonotic point of view and possible routes of spread of AMR.

ACKNOWLEDGEMENT

The authors thank the Hon'ble Vice-Chancellor and DREF, WBUAFS for providing necessary funds and the Faculties of the Department of Veterinary Microbiology, WBUAFS, West Bengal, India for the research facilities and support for this study. The staff of the department, poultry farm owners and staff also deserve special thanks for their help.

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Cite this article as: Mondal T, Dey S, Isore DP, Samanta S, Banerjee A, Singh AD, Sharmila L, Hajarika ZH, Batabyal K (2023) Detection of drug-resistant extended-spectrum and *AmpCβ*-lactamases producing *Escherichia coli* from poultry faecal samples in West Bengal, India. Explor Anim Med Res 13(1): 31-38. DOI: 10.52635/eamr/13.1.31-38.