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Detection and Characterization of *β-lactamases* and Biofilm-producing *Escherichia coli* and *Klebsiella pneumoniae* from Ducks and Duck Environments in West Bengal

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ABSTRACT

Background: *Escherichia coli* and *Klebsiella pneumoniae* are two important commensals with pathogenic potentiality in animals and birds, causing intestinal and extra-intestinal infections. These are somewhat zoonotic too via different routes. So, the present study was undertaken to investigate the prevalence of these two bacteria from cloaca and environment of ducks from different districts of West Bengal, followed by detailed characterization and antibiogram.

Methods: Around 197 duck and environmental samples were collected from different districts of West Bengal. *E. coli* and *K. pneumoniae* were isolated from collected samples as per standard methods followed by serotyping of the *E. coli* isolates. The detection of ESBL/ACBL producing isolates was done both phenotypically and genotypically by detecting the presence of antibiotic-resistant genes. Virulence property and biofilm-producing ability of these isolates were also studied. Antibiogram of the ESBL and biofilm positive isolates were done using common 12 antibiotics by disc diffusion method.

Result: A total of 134 (68.02%) *Escherichia coli* and 33 (16.75%) *Klebsiella pneumoniae* isolates were recovered and confirmed by PCR. The prevalence of *E. coli* and *K. pneumoniae* in cloacal swabs were less in comparison to the environmental samples (72.09% and 20.93%). Approx. 79.85% *E. coli* and 72.73% *K. pneumoniae* isolates were found to be the ESBL/ACBL producers *in vitro*. *E. coli* and *K. pneumoniae* isolates possessed all major antibiotic-resistant genes with the bla_{AmpC} (64.18% and 48.48%) being the most prevalent one. Twenty three (17.16%) *E. coli* isolates were found positive for the virulence genes, of which *stx1* (39.13%) was the most common one as detected by multiplex PCR. No virulence gene was found in *K. pneumoniae* isolates. Ninety one (67.91%) *E. coli* and 26 (78.79%) *K. pneumoniae* isolates possessed the biofilm-producing genes. All the ESBL and biofilms producing *E. coli* and *K. pneumoniae* isolates showed 100% - 95.24% resistance to ampicillin. A high percentage of resistances were also seen against cefotaxime, ticarcillin/ clavulanic acid, ertapenem, ceftazidime, norfloxacin and ciprofloxacin, which may be due to several reasons. Maximum overall sensitivity was detected against chloramphenicol, co-trimoxazole and gentamicin.

Key words: Antibiogram, Biofilm, Ducks, ESBL, *Escherichia coli*, *Klebsiella pneumoniae*.

INTRODUCTION

Rearing ducks is a source of income to the poor marginal farmers in India. Ducks grow faster, need less attention, simple housing and are resistant to common diseases (Rajput *et al*., 2014). The agricultural economy gets boosted up by the eggs and meat production in Indian scenario.

Ducks may get affected by a few pathogens like *Escherichia coli, Salmonella* spp., *Klebsiella pneumoniae, Pasteurella multocida, Reimerella anatipestifer* and *Streptococcus* spp., *etc.* Among these, *E. coli* and *K. pneumoniae* have virulence properties, biofilm production and antimicrobial resistance properties, which are a matter of concern. These can infect humans too *via* contaminated water and food. These gram-negative bacteria can produce β-lactamases (Wickens and Wade, 2005) which creates antimicrobial resistance in them. Most of them are biofilm producers too (Melo *et al*., 2012). Both can cause a wide variety of diseases causing up to 20-30% of mortality (Khelfa and Morsy, 2015). In this background, samples were collected from ducks and duck environments from different districts of West Bengal, to detect the prevalence of antibiotic-resistant and biofilm-producing *E. coli* and *K. pneumoniae* isolates to insight into the depth of the problem.

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MATERIALS AND METHODS

Sample background

A total of 197 samples (154 cloacal swabs and 43 environmental samples [soil 19, water collected from duck environment/sheds 13, duck feed 11]) were collected aseptically with sterilized cotton swabs (HiMedia, India) from ducks and their environments during the year 2020-21. The swab samples were transported under ice cover to the Department of Veterinary Microbiology laboratory, F/VAS, WBUAFS, Kolkata *via* the shortest route.

Isolation and characterization of *E. coli* and *Klebsiella pneumoniae* isolates

The enriched samples were streaked on sterilized MacConkey's agar plates (HiMedia, India) and overnight incubated at 37°C for the isolation of *E. coli*. The rose-pink colonies were selected and sub-cultured and incubated on EMB agar plates (HiMedia, India) for detection of *E. coli* isolates with metallic sheen'. All isolates were preserved in nutrient agar slants for further study.

The *Klebsiella* selective agar (HiMedia, India) plates were used for *Klebsiella pneumoniae* isolation. The characteristic magenta-coloured colonies were selected and subsequently preserved on nutrient agar (HiMedia, India) slants. Morphological and biochemical characterizations were performed as per Quinn *et al.* (1994) and Edward and Ewing (1972).

Molecular characterization of *E. coli* and *Klebsiella pneumoniae* isolates

The tentatively confirmed *E. coli* and *Klebsiella* spp. isolates were processed for molecular confirmation by PCR as per the methods described by Wang *et al.* (1996) and Brisse and Verhoef (2001) with some modifications. Then the positive *Klebsiella* spp. isolates were tested for species-specific PCR for the detection of *Klebsiella pneumoniae* (Liu *et al.*, 2008).

Serogrouping of *E. coli* isolates

The positive *E. coli* samples from ducks were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India for serotyping.

Phenotypic detection of ESBL/ACBL production in *E. coli* and *K. pneumoniae* isolates

Antibiotic discs containing cefotaxime (30 µg, Hi-Media) and ceftazidime (30 µg, Hi-Media) with and without clavulanate (10 µg, Hi-Media) were used in double-disc diffusion assay (DDSA) for phenotypic confirmation of the presence of ESBLs in all *E. coli* and *Klebsiella pneumoniae* isolates. A difference of ≥ 5 mm between the zone of inhibition in the presence and absence of clavulanate was considered for phenotypic confirmation of ESBL production (CLSI, 2012). All the *E. coli and Klebsiellae* spp. isolates were subjected to cefoxitin-cloxacillin double disc synergy (CC-DDS) test for phenotypic confirmation of AmpC *β-lactamase* production (Tan *et al.*, 2009).

Detection of ESBL and ACBL genes in *E. coli* and *K. pneumoniae* isolates

All the positive *E. coli* and *Klebsiella pneumoniae* isolates including negative control were subjected to PCR for detection of major antibiotic-resistant genes such as bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{AmpC} (Bert *et al.*, 2002; Colom *et al.*, 2003; Weill *et al.*, 2004 and Féria *et al.*, 2002).

Detection of Virulence genes in *E. coli* and *K. pneumoniae* isolates

All positive *E. coli* isolates were further subjected to multiplex PCR for detection of the Shiga-toxin gene (stx_1, stx_2) and *eaeA, hlyA* genes as described by Paton and Paton (1998) with some modifications. Likewise, in *K. pneumoniae* isolates, PCR detection of the *rmpA* gene was done (Nahavandinejad and Asadpour, 2017).

Molecular detection of biofilm-producing genes among *E. coli* and *K. pneumoniae* isolates

Detection of biofilm genes like *csgA*, *rpoS*, *rcsA* and *sdiA* was performed in isolated positive *E. coli* and *K. pneumoniae* isolates by PCR (Silva *et al.*, 2013; Adamus-Białek *et al.*, 2015).

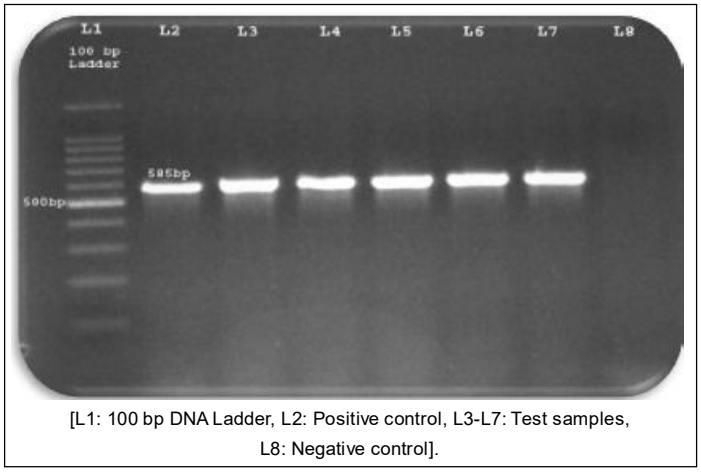
Antibiotic sensitivity assay

All the ESBL and biofilm-producing *E. coli* and *K. pneumoniae* isolates were tested for their sensitivity and resistance pattern to 12 different antibiotics by the disc diffusion method (CLSI, 2012). The antibiotics used were ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), tetracycline (30 µg), gentamicin (10 µg), amikacin (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), ticarcillin/clavulanic acid (30/10 µg) and ertapenem (30 µg).

RESULTS AND DISCUSSION

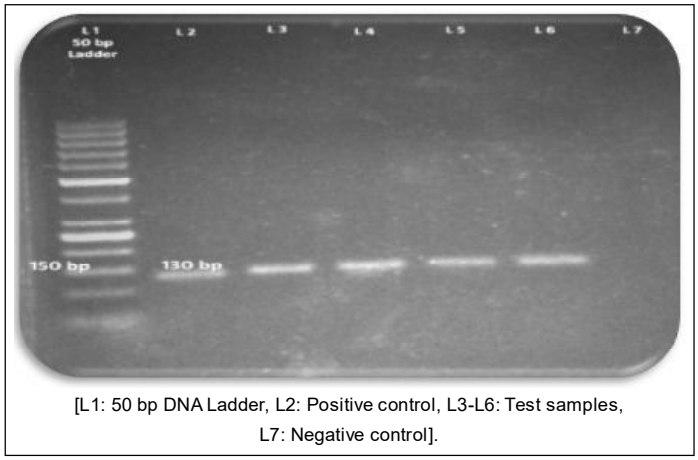
Out of 197 samples, a total of 134(68.02%) *Escherichia coli* and 33 (16.75%) *Klebsiella pneumoniae* were detected from the cloacal and environmental samples of the duck. The study showed a higher prevalence of *E. coli* (72.09%) and *Klebsiella pneumoniae* (20.93%) in environmental samples (Nnachi *et al.*, 2015). In Nigeria, a similar investigation by Adzitey *et al.* (2012) revealed 70.83% *E. coli* isolates from soil, confirming the present findings. Bariha *et al.* (2019) from India, reported 55% positivity of *E. coli* from different duck samples from ducks. Again Banerjee *et al.* (2019) found 53.96% *E. coli* and 14.80% *K. pneumoniae* from faecal samples of ducks in West Bengal, which were lower than the current findings. Both were gram-negative bacilli and typical in biochemical characterization (Quinn *et al.,* 1994).

All 134 nos. tentatively positive *E. coli* isolates were having the 16S *rRNA* gene (585 bp) specific for *E. coli* (Fig 1). Around 33 isolates were confirmed to be *Klebsiella pneumoniae* with 130 bp amplified product in PCR (Fig 2). A total of 12 different serogroups were found such as O98, O157, O135, O120, O63, O26, O121, O101, O8, O118, O119



[L1: 100 bp DNA Ladder, L2: Positive control, L3-L7: Test samples, L8: Negative control].

Fig 1: Confirmation of *Escherichia coli* strains by detection of 16S *rRNA* gene (585 bp).



[L1: 50 bp DNA Ladder, L2: Positive control, L3-L6: Test samples, L7: Negative control].

Fig 2: Confirmation of *Klebsiella pneumoniae* strains with the detection of the specific gene (130 bp).

and O22 among which, O120 was the most prevalent one, in serogrouping of *E. coli* isolates (Banerjee *et al.*, 2019; Wang *et al.*, 2010).

Out of 134, 107 (79.85%) *Escherichia coli* isolates (Fig 3) and out of 33, 24 (72.73%) *Klebsiella pneumoniae* isolates (Fig 4) were detected as phenotypical ESBL/ACBL producers based on double-disc synergy assays. During molecular detection of the resistance genes, a total of 36 (26.86%) *E. coli* and 6 (18.18%) *K. pneumoniae* isolates were found to possess the bla_{CTX-M} gene (540 bp) with other genes (bla_{TEM} and bla_{SHV}) too (Table 1). The presence of the bla_{AmpC} gene (634 bp) was quite high in both bacteria. Nalband *et al.* (2020), Ma *et al.* (2012) also reported much higher prevalence of ESBL genes in *E. coli* which is almost similar to the present study. The findings of Banerjee *et al.* (2019), Raza *et al.* (2017) also match this report and thus stand confirmed.

A total of 23 (17.16%) *Escherichia coli* isolates were found to be positive for different virulence genes *viz. stx1* (180 bp), *stx2* (255 bp), *eaeA* (384 bp) and *hlyA* (534 bp) in the multiplex PCR assay (Table 2, Fig 5). Majumder *et al.* (2017) and Banerjee and Acharyya (2020) reported approx. 20-24% STEC producing *E. coli* from cloacal samples of duck which was higher than the present findings.

No *Klebsiella pneumoniae* isolate was found positive for the *rmpA* gene (535bp) in this study. Yang *et al.* (2019) reported a very less (10.6%) prevalence of the *rmpA* gene in human origin *Klebsiella pneumoniae*. Animal origin *Klebsiella* spp. may possess this gene (Younis *et al.*, 2016) but the present variation might be due to variation in geographical variances.

Around 67.91% *Escherichia coli* and 78.79% *Klebsiella pneumoniae* isolates were found to possess at least one of these biofilm-producing genes *viz. rpoS* (120 bp), *csgA* (178 bp), *sdiA* (239 bp) and *rcsA* (306 bp) (Table 3). Approx. 27 *E. coli* and 13 *K. pneumoniae* isolates were found to possess more than one biofilm-producing gene in this study which was also supported by Bakhtiari and Javadmakoei (2017) and Banerjee and Acharyya (2020).

This study showed that 30.43% ESBL-*E. coli* and 47.82% biofilm-producing *E. coli* to possess virulence genes, but 56 ESBL-*E. coli* were positive for biofilm production too. Again only 02 isolates were positive for the ESBL, Biofilm

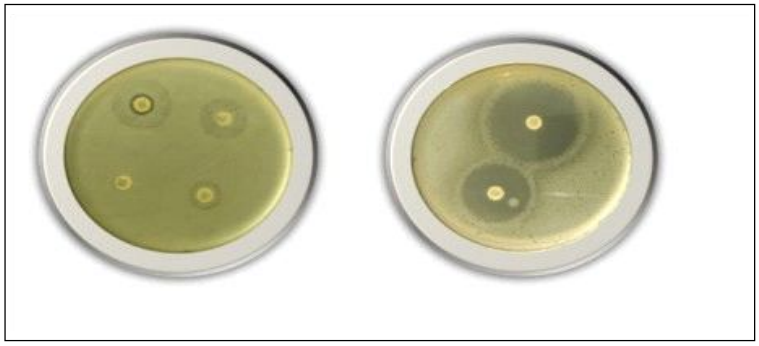


Fig 3: Phenotypic assay showing ESBL and ACBL production by *Escherichia coli* isolates.

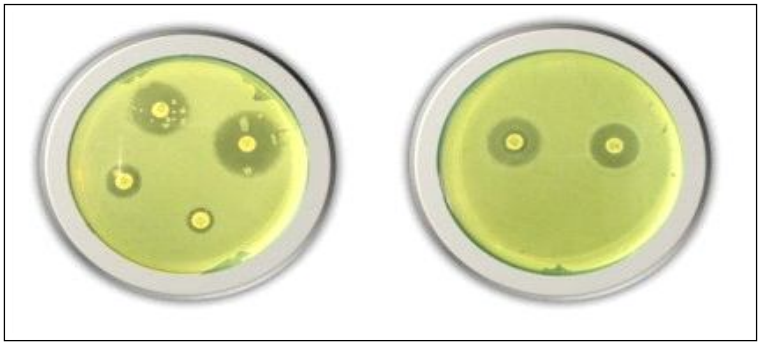
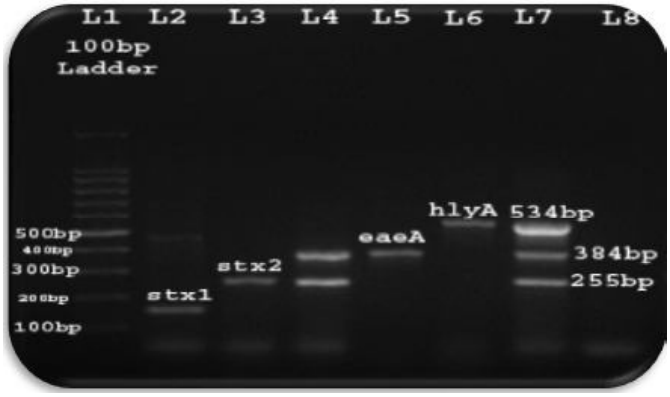


Fig 4: Phenotypic assay showing ESBL and ACBL production by *K. pneumoniae* isolates.



[L1: 100 bp Ladder, L2-L6: Test samples, L7: Positive control, L8: Negative control].

Fig 5: mPCR showing different STEC genes in *E. coli* isolates.

Table 1: Distribution of the ESBLs and ACBL genes in different isolates.

Types of bacteria	Positive in DDSA	PCR positive		
		Gene name	No. of isolates detected	Total
Escherichia coli (107)	ESBL (48)	bla_{CTX-M}	36	69
		bla_{TEM}	27	
		bla_{SHV}	06	
	ACBL (59)	bla_{AmpC}	86	86
K. pneumoniae (24)	ESBL (13)	bla_{CTX-M}	06	17
		bla_{TEM}	09	
		bla_{SHV}	02	
	ACBL (11)	bla_{AmpC}	16	16

producing and virulence genes as revealed in this study. This reveals a significant relationship were also reported by Jegadeesh Kumar *et al.* (2016) and Fattahi *et al.* (2015), which confirms these present findings too.

All the ESBL and biofilm-producing *E. coli* isolates were resistant to ampicillin and approx. 61-86% resistant to norfloxacin, ertapenem, ciprofloxacin, tetracycline and ceftazidime respectively (Table 4). Na *et al.* (2019) also reported resistance to tetracycline (59.3%) and other drugs of *E. coli* in their study.

A total of 95.24% of all ESBL and biofilm-producing *K. pneumoniae* isolates (n=21) were resistant to ampicillin

Table 2: Distribution of virulence genes in *Escherichia coli* isolates.

Type of Samples	No. of *Escherichia coli* isolates	Types of genes				
		Stx1	*Stx2*	*eaeA*	*hlyA*	*Stx2* and *eaeA*
Cloacal	103	07	04	04	01	01
Environmental	31	02	02	00	00	02
Total	134	09	06	04	01	03
Grand total				23		

Table 3: Distribution of the biofilm genes in *E. coli* and *K. pneumoniae* isolates.

Bacterial isolates tested	No. of biofilm gene positive isolates	PCR			
		rpoS	*csgA*	*sdiA*	*rcsA*
Escherichia Coli (134)	91	42	37	23	16
Percentages (%)		31.34	27.61	17.16	11.94
Kleb. pneumoniae (33)	26	11	13	07	08
Percentages (%)		33.3	39.4	21.2	24.2

Table 4: Antibiogram of ESBL and biofilm-producing *E. coli* isolates (n=56).

Used antibiotics (Concentration)	Sensitive (%)	Intermediate (%)	Resistant (%)
Ampicillin (10 µg)	00	00	100
Chloramphenicol (30 µg)	85.71	00	14.28
Co-Trimoxazole (25 µg)	46.42	3.57	50
Tetracycline (30 µg)	25	3.57	71.43
Gentamicin (10 µg)	28.57	57.14	14.28
Amikacin (30 µg)	17.85	42.85	39.28
Norfloxacin (10 µg)	10.71	3.57	85.71
Ciprofloxacin (5 µg)	17.85	7.14	75
Cefotaxime (30 µg)	7.14	35.71	57.14
Ceftazidime (30 µg)	3.57	35.71	60.71
Ticarcillin/Clavulanic acid (75/10 µg)	3.57	46.42	50
Ertapenem (10 µg)	7.14	7.14	85.71

Table 5: Antibiogram of ESBL and biofilm-producing *Klebsiella pneumoniae* isolates (n=21).

Used antibiotics (Concentration)	Sensitive (%)	Intermediate (%)	Resistant (%)
Ampicillin (10 µg)	00	4.76	95.24
Chloramphenicol (30 µg)	90.47	00	9.52
Co-Trimoxazole (25 µg)	76.19	00	23.81
Tetracycline (30 µg)	42.85	9.52	47.61
Gentamicin (10 µg)	61.90	4.76	33.33
Amikacin (30 µg)	38.09	23.81	38.09
Norfloxacin (10 µg)	28.57	14.29	57.14
Ciprofloxacin (5 µg)	42.85	4.76	52.38
Cefotaxime (30 µg)	00	14.29	85.71
Ceftazidime (30 µg)	4.76	19.04	76.19
Ticarcillin/Clavulanic acid (75/10 µg)	00	14.29	85.71
Ertapenem (10 µg)	19.04	00	80.95

followed by 85.71%, 85.71%, 80.95%, 76.19%, 57.14% and 52.38% resistant to cefotaxime, ticarcillin/ clavulanic acid, ertapenem, ceftazidime, norfloxacin and ciprofloxacin respectively. Sensitivity was detected against chloramphenicol, co-trimoxazole and gentamicin (@61-92%) (Table 5). Almost similar reports were shown by Nnachi *et al.* (2015) and Kumar *et al.* (2022), thus the current result stands confirmed.

CONCLUSION

The prevalence of *Escherichia coli* and *Klebsiella pneumoniae* was quite significant in duck samples and environmental samples. Twelve different *E. coli* serogroups were found in this study and O120 was the most prevalent one. The frequency of the bla_{AmpC} gene was the highest among all the ESBL/ACBL producing *E. coli* and *K. pneumoniae* isolates. Approx. 65% and 18% of all the *E. coli* isolates were positive for biofilm production and the presence of virulence genes. Most of the ESBL and biofilms producing *Escherichia coli* and *Klebsiella pneumoniae* isolates showed resistance towards ampicillin, cefotaxime, ticarcillin/clavulanic acid, ertapenem, ceftazidime, norfloxacin and ciprofloxacin in antibiotic sensitivity assay.

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Conflict of interest

All authors declare that they have no conflict of interest.

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