Characterization of avian pathogenic *E. coli* associated with Vent gleet in layer chickens

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Abstract

An investigation was made to identify and characterize the etiological agents associated with vent gleet in layer birds from a flock in Namakkal district. Cloacal swab samples were collected from the ailing birds and inoculated into nutrient broth, nutrient agar media followed by isolation on MacConkey agar and Eosin Methylene Blue Agar. The isolates were characterized by Gram's staining, cultural characteristics in the selective media and biochemical properties. The virulence of isolates was identified by Congo red binding ability. Further, the isolates were subjected to antibiotic sensitivity test (ABST) by disc diffusion (Kirby Bauer) method. The isolates phenotypically resistant to third generation cephalosporins were genotypically characterized by multiplex PCR targeting specific extended spectrum beta lactamase (ESBL) encoding genes viz, TEM, SHV and OXA. The isolates were identified as *E.coli* by gram-negative rods, lactose fermenters and by IMViC tests which produced green metallic sheen colonies on EMB agar. The Congo red binding indicated that the isolate was avian pathogenic *E.coli* (APEC). The antibiotic sensitivity test revealed that the isolate was sensitive only to Amikacin and Amoxycillin/Clavulanic acid and resistant to all other antibiotics that were used in the study. The molecular characterization showed that isolate harbored SHV-variants type of ESBL. The findings of this study highlight the involvement of APEC in Vent gleet infection and the isolate had multidrug resistance by producing SHV type of ESBL that inactivates beta-lactam antibiotics.

Key words: *E. coli,* Extended spectrum beta lactamase (ESBL), Layers chickens, Multiplex PCR, Vent gleet, Antibiotic sensitivity test

Introduction

Vent Gleet also known as Infected Cloaca, Cloacitis or Pasted vent is the inflammation of cloaca of chickens of all the age groups and is seasonal, increasing in spring and summer because of heat and humidity with mortality rates increasing to 0.5 to 1.25% weekly, and dead hens are typically in production. It can be caused by a number of different reasons including many types of organisms (fungi, yeast, bacteria, protozoa and parasites), ingestion of moldy or spoiled feed, drinking contaminated water or in unsanitary conditions, vitamin A deficiency, sour crop and imbalance of the normal flora in the digestive system. It can also occur due to the use of oral antibiotics or due to the mating with infected birds. Cloacitis can even be observed in stress conditions, often associated with egg laying, bowel infections or a hormonal-related issues. Stress causes an increase in pH which predisposes chickens to develop infection in cloaca and nearby organs including rectum and uterus. Symptoms most commonly observed in vent gleet infection are sticky yellow whiteish paste-like discharge from vent, hard white crusting on dirty tail feathers, strong unpleasant smell, depression, decreased appetite or increased appetite, loss of weight and swollen bloated abdomen. It is an economically important disease as there is drop in egg production and also vent pecking of the affected bird by normal healthy birds, which will latter turn into vice of the birds in the farm.

Escherichia coli a group of bacteria normally found in the flora of human and animal digestive tracts and symbionts participating in digestion and synthesis of certain vitamins (Sarowska *et al*., 2019). Avian pathogenic *E. coli* (APEC), member of the Extraintestinal pathogenic *E. coli* (ExPEC) is a serious threat to poultry businesses worldwide, generating infections such as colibacillosis, which can result in huge economic losses in terms of decreased egg and meat production, high mortality rates, hatching rates, live body weight, lower feed conversion efficiency, higher veterinary expenditures, and carcass condemnation at slaughterhouses (Guerra *et al*., 2018; Li *et al*., 2020). Virulence factors associated with APEC pathogenesis include possession of large virulence plasmids and the characteristics like resistance to phagocytosis and serum killing, acquisition of iron under the low-iron conditions within the poultry host and adherence to host structures (MSD, Veterinary Manual).

The chickens in the commercial farms are under enormous stress like vaccinations, biosecurity measures and frequent and unwarranted antibiotic usage to meet the global standards for export as well as to cater the protein demand at the domestic front especially after Covid (Poojitha *et al.*, 2024). The widespread misuse of antibiotics in the poultry industry has exacerbated the problem of antibiotic-resistant APEC strains (Nawaz *et al.*, 2024). Treatment of colibacillosis with antimicrobial agents is problematic due to widespread multidrug resistance among APEC isolates and restrictions on antimicrobial use in poultry imposed by regulation and public concern. If treatment is pursued, it should be based on antimicrobial susceptibility testing (MSD Veterinary Manual). Hence, the present study aims to identify the pathogens associated with the pated vent and its antibiogram profiling by phenotypic and genotypic characterization.

Materials and Methods

Sample collection

Cloacal swabs samples were collected from three different live layer chickens with pasty butt, swelling and discharge around cloaca (Fig.1) from the poultry farm in Namakkal district, Tamil Nadu (11° 13' 46.5312" N and 78° 10' 16.1688" E) during November 2023. The collected swabs were transported to laboratory and stored in refrigerator for further processing.

Isolation and identification of pathogens

The collected swabs were enriched in nutrient broth and incubated at 37℃ overnight. A small amount of inoculum from nutrient broth was streaked on nutrient agar to obtain pure colonies. The colonies were stained by Gram's staining procedure. Based on the morphology, a single colony from nutrient agar was inoculated on MacConkey agar. The lactose fermenting colonies grown on the MacConkey agar was inoculated on Eosin methylene blue (EMB) agar to confirm the isolates as *E.coli.* The colonies showing characteristic metallic sheen on the EMB agar were selected for further biochemical tests like catalase and IMViC for confirmatory identification of *E. coli.*

Congo Red Binding Assay

The Congo Red Binding (CRB) ability of the isolates were determined as described by Yadav *et al.,* 2014 to detect the Avian Pathogenic *E.coli* (APEC) isolates in Trypticase soy agar supplemented with 0.03% Congo red dye and 0.15% bile salt. The isolates were streaked onto the agar plates and incubated at 37°C for 24, 48 and 72 h and observed for the appearance of intense orange or brick red colored colonies as positive reaction while pale or white colonies were considered as negative. The results were interpreted as +++, ++ and + depending on their color intensity.

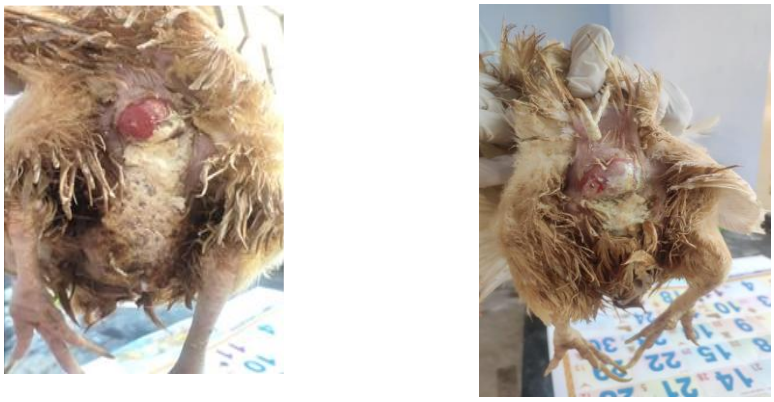


Fig.1 Affected birds showing white discharge, crusting on tail feathers

Table 1: Primers used for Multiplex PCR

β lactamase genes targeted	**Primer name**	**Primer sequence**	**Amplicon size (bp)**
TEM variants including TEM-1 and TEM-2	Multi TSO-T For	CATTTCCGTGTCGCCCTTATTC	800
	Multi TSO-T Rev	CGTTCATCCATAGTTGCCTGAC	
SHV variants including SHV-1	Multi TSO-S For	AGCCGCTTGAGCAAATTAAAC	713
	Multi TSO-S Rev	ATCCCGCAGATAAATCACCAC	
OXA-1, OXA-4 and OXA-30	Multi TSO-O For	GGCACCAGATTCAACTTTCAAG	564
	Multi TSO-O Rev	GACCCCAAGTTTCCTGTAAGTG	

Table 2: PCR Primer sequences used to amplify *bla*$_{CTX\text{-}M}$ gene of *E.coli*

Primer name	**Primer Sequence**	**Amplicon size(bp)**
bla$_{CTX\text{-}M}$ For	ATGTGCACCAGTAARGT	593
bla$_{CTX\text{-}M}$ Rev	TGGGTRAARTARGTSACCAGA	

Detection of Antibiotic sensitivity of APEC

The isolates were subjected to antibiotic sensitivity test by disk diffusion method in accordance with CLSI guidelines in Mueller Hinton agar after adjusting the concentration of each isolate to 0.5 McFarland standard against twenty antimicrobial agents of different antibiotic groups viz., Azithromycin (AZM) 15 µg, Erythromycin (E) 15 µg, Tylosin (TL) 15 µg, Neomycin (N) 30 µg, Streptomycin(S) 10 µg, Gentamicin(GEN) 10 µg, Amikacin(AK) 30 µg, Ampicillin/Sulbactam(A/S) 10/10 µg, Amoxyclav (AMC) 30 (20/10) µg, Methicillin (MET) 5 µg, Tetracycline (TET) 30 µg, Oxytetracycline (O) 30 µg, Co-Trimoxazole (COT) 25 µg, and Ciprofloxacin (CIP) 5µg. Further, the production of extended spectrum beta lactamase (ESBL) by the isolates were determined by combination disc diffusion method (Matuschek *et al*., 2014) using the antibiotic discs like Ceftriaxone/ Sulbactam (CIS) 30/10 µg, Cefotaxime (CTX) 30 µg, Cefotaxime/Clavulanic acid (CEC) 30/10 µg, Ceftazidime (CAZ) 30 µg and Ceftazidime/Clavulanic acid (CAC) 30/10µg. The results were read after 24 h of incubation.

The MAR index of the APEC isolates were calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to (Rotchell and Paul, 2016).

Detection of ESBL Genes by Polymerase chain reaction (PCR)

The isolates identified as ESBL producers using the combination disc diffusion method were further characterized for genes encoding β-lactamases (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$) using multiplex PCR using primers listed in Table 1 (Dallenne *et al.,* 2010) and *bla*$_{CTX\text{-}M}$ by uniplex PCR using primer listed in Table 2 (Moghaddam *et al*., 2012). The template DNA for PCR was obtained from the isolates by boiling method (Shehata and Hershan, 2016).

The amplification was carried out using PCR master mix (2X) (Thermo scientific, USA), with an initial denaturation at 94℃ for 10 min, followed by 35 cycles at 94℃ for 30 sec, 60℃ for 40 sec, 72℃ for 1 min and a final extension at 72℃ for 7 min**. (**Dallenne *et al.,* 2010). The amplification was carried out using PCR Master Mix (2X) (Thermo scientific, USA), with an initial denaturation at 94℃ for 5 min, followed by 35 cycles at 94℃ for 30 sec, 48℃ for 30 sec, 72℃ for sec and a final extension at 72℃ for 3 min.

Results

Turbidity was observed in nutrient broth inoculated with cloacal swabs collected from all the three birds after overnight incubation at 37℃, indicating the presence of bacterial organism. A loopful amount of inoculum from nutrient broth streaked onto nutrient agar yielded pure colonies. A single colony from nutrient agar inoculated on MacConkey agar revealed the organism as lactose fermenting bacteria by producing small, round, smooth, and pink-colored colonies and appeared as Gram-negative, rod shaped organisms on Gram-staining. (Fig.2). The biochemical test further confirms that all the three isolates are *E.coli* and had similar reactions (++--) in IMViC test (Fig.5) and sugar fermentation tests as mentioned in Table.3. A colony of each isolate from MacConkey agar inoculated on Eosin methylene blue (EMB) agar further validate the isolates as *E. coli* (Fig.3).

The pure colonies of *E.coli* obtained from the three isolates streaked on to the plates containing Congo-red dye produced intense orange or brick red colored colonies within 48h of incubation indicating the presence of APEC organisms (Fig. 4).

The antibiotic sensitivity pattern of all the three isolates in the Muller Hinton agar plates against 20 different antibiotics belonging to 7 different groups are shown in Table.4. The tested isolates showed a varying degree of resistance towards these antibiotics. The antibiogram profiling of the current study revealed that all the three isolates of APEC were resistant to co-trimaxazole, tetracycline, oxytetracycline, ciprofloxacin, ceftriaxone, ceftazidime/clavulanic acid, ceftaxime/clavulanic acid, tylosin, neomycin and methicillin and sensitive to amoxicillin-clavulanic acid and amikacin. In addition, the isolate 3 APEC was also sensitive to erythromycin, azithromycin and gentamicin. The three APEC isolate were found to be multidrug resistant as they had the MAR index of 0.909, 0.909 and 0.772 respectively.

Further, the combination disc method used to detect the ESBL production reveals that the two of the three isolates as ESBL producers as they had a zone of inhibition around the combination disc having the clavulanic acid and cephalosporin of at least 5 mm larger than that of the cephalosporin alone.

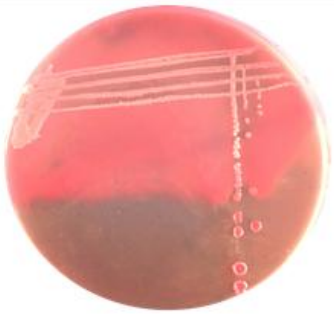


Fig. 2 Bright, pink colored transparent smooth raised colonies on MacConkey agar

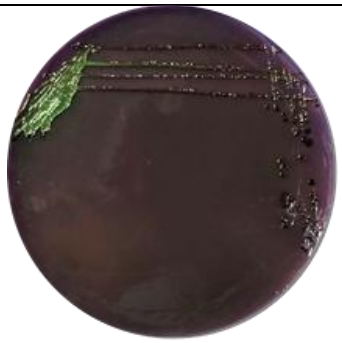


Fig. 3 Green Metallic Sheen Colonies on EMB agar

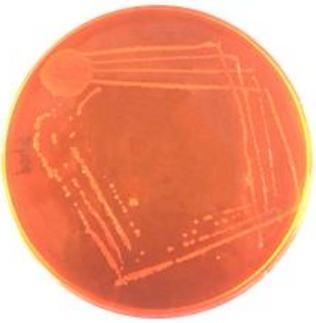


Fig. 4 Intense red colored, rough raised colonies of *E.coli* isolate in Trypticase soy agar supplemented with 0.03% Congo red dye and 0.15% bile salt

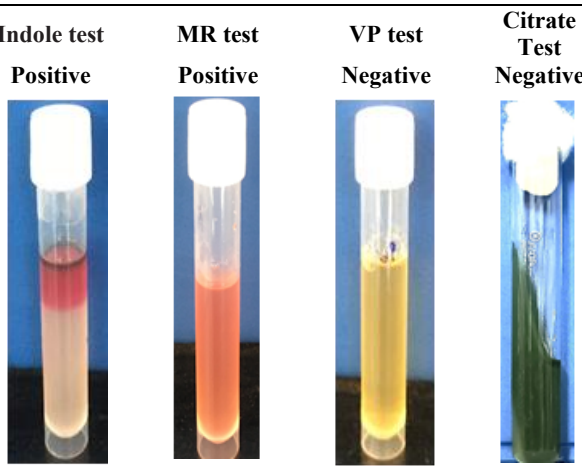


Fig. 5. IMViC test for the isolates

Table 3 Sugar fermentation test

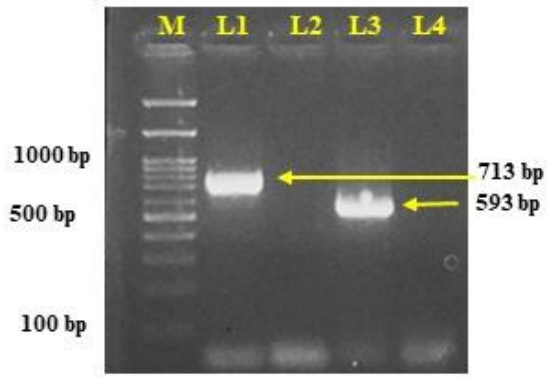
Sugar	*E.coli isolate*		
	1	2	3
Glucose	+	+	+
Adonitol	-	-	-
Arabinose	+	+	+
Lactose	+	+	+
Sorbitol	-	-	-
Mannitol	+	+	+
Rhamnose	-	-	-
Sucrose	+	+	+
+ = Positive	**- = Negative**		

Table 4: Antibiogram of *APEC* isolates

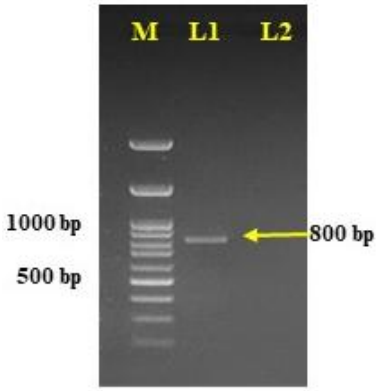
Antibiotic Discs *E.coli* isolates	AZM	E	TL	N	S	GEN	AK	A/S	AMC	MET	TET	O	COT	CIP	CIS	CTX	CAZ	MAR index
1	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	0.909
2	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	0.909
3	S	S	R	R	R	S	R	R	S	R	R	R	R	R	R	R	R	0.772
% of Resistance	66.6	66.6	100	100	100	66.6	33.3	100	0	100	100	100	100	100	100	100	100	

Table 5: Detection of ESBL production by Combination disc method

Antibiotic Discs / *E.coli* isolates	**CTX**	**CEC**	**Difference in ZOI**	**CAZ**	**CAC**	**Difference in ZOI**	**CTX**	**CIS**	**Difference in ZOI**
1	0	26	**26**	12	23	**13**	0	23	**23**
2	10	18	**8**	0	16	**16**	10	13	3
3	24	25	1	26	26	0	24	30	**6**
ZOI : Zone of inhibition									



M : 100bp DNA ladder
Lane 1: Isolates showing amplification of 713 bp amplicon indicating *bla SHV* type ESBL gene
Lane 2 & 4: NTC
Lane 3: Isolates showing amplification of 593 bp amplicon indicating *bla CTX-M* type ESBL gene



M: 100bp DNA ladder
Lane 1: Isolates having 800 bp amplicon indicates *bla TEM* type ESBL gene.
Lane 2: NTC

Fig.5. Molecular characterization of the ESBL producing APEC

The molecular characterization of APEC isolates identified phenotypically as ESBL producers were performed to identify the type of ESBL gene involved in conferring the resistance using multiplex and uniplex PCR. The multiplex PCR targeting the bla_{TEM}, bla_{SHV}, and bla_{OXA} genes identified the presence of bla_{SHV} and the uniplex PCR identified the bla_{CTX-M} gene in APEC isolate 1 . Hence, the bla_{SHV} and bla_{CTX-M} ESBL genes of APEC isolate 1 were responsible for the ESBL production whereas the APEC isolate 2 harbored bla_{TEM}, ESBL gene and the third isolate of APEC had none of the targeted ESBL genes.

DISCCUSION

Cloacal health is a strong indication of overall chicken health. A healthy bird with good cloacal health should produce tightly packed droppings with white caps on them and have a generally clean vent area. The vent is the common external opening of the urinary tract, reproductive tract and digestive tract of chickens often colonized by many commensal organisms. Vent gleet is a general term for the inflammation of the vent area. The present study determined that the organism isolated from the Vent gleet affected chickens as *E. coli*. as supported by Islam *et al*. (2014).

The *E. coli* isolated in the current study was further identified as APEC by Congo red binding assay and were found to be pathogenic as they produced bright pink coloured colonies as reported in the findings of Knöbl *et al.* (2011). Congo red binding was commonly used as markers of hydrophobicity and has been linked directly to virulence and pathogenicity. Congo red uptake has been considered a virulence factor responsible for pathogenicity in poultry, particularly in the avian pathogenic *E. coli* (APEC). A strong correlation between CRB

and pathogenic properties of APEC isolates was reported by several authors (Ahmad *et al*., 2009; Ezz *et al*., 2010; Amer *et al*., 2015; Zahid *et al*., 2016). Similarly, Yadav *et al*., (2014) reported that the Congo red binding ability can be used as a phenotypic marker of colisepticaemic (invasive) and non-coliseptecaemic *E. coli* in poultry and also as an epidemiological marker for discrimination of pathogenic strains from the commensals.

The APEC isolates could be the primary pathogen or a secondary etiological agent associated with other diseases and environmental stresses. The APEC primarily infects birds through the respiratory tract, although it can also enter via oral, nasal, or cloacal routes and typically colonizes the mucosal areas of the gastrointestinal, respiratory, and reproductive tracts without causing disease (Nawaz *et al*., 2024).

The current instance of vent gleet could be attributed to the laying stress or acute environmental stress factors such as change in the body temperature, lack of drinking water, hormonal fluctuations occurring during the onset of lay or due to an underlying bowel infection resulting in watery faeces and increase in pH favouring the intestinal bacteria to colonize and cause more severe clinical signs and symptoms in clinical infection in the hens.

The antibiogram profiling of the APEC isolates of this study exhibited resistance to most of the antibiotic classes (carbapenems, imipenem, streptomycin, and tetracycline) except macrolides, aminoglycosides and pencillins with beta lactamase inhibitor. These findings underscore the crucial need for comprehensive strategies to address antibiotic resistance in APEC and safeguard public health (Saha *et al.* 2020). It is noteworthy that the APEC isolates were resistant to three or more different class of antibiotics with a MAR index of 0.909, 0.909 and 0.772 respectively which is greater than 0.2 signifying that the isolates are multidrug resistant. A MAR greater than 0.2 means that the high risk source of contamination where antibiotics are frequently used (Rotchell and Paul, 2016). The MDR is a universal problem and its transmission from animal sources to humans, particularly through foodborne pathogens and zoonotic diseases, is a growing concern. Antibiotics are commonly used in veterinary field for treating and preventing infections. Overuse and misuse of antibiotics in animal agriculture contribute to the development and spread of MDR bacteria, which can then be transmitted to humans through direct contact with animals, consumption of contaminated food products, or environmental exposure. As MDR and ESBL incidence are considered as extreme public health issue, it needs to be addressed through a coordinated and collaborative approach to mitigate its impact on human and animal health, as well as preserve the effectiveness of existing antimicrobial treatments for future generations.

The multiplex PCR detected the type of ESBL gene involved in conferring resistance to the APEC isolates of the present study which were in analogous with the findings of Dallenne *et al*. (2010). The molecular characterization validates the phenotypic observations based on the disc diffusion method and provides further insights into the type of ESBL contributing to the resistance.

Conclusion

This investigation is a preliminary step in demonstrating the association of multidrug resistant APEC with a potential to produce ESBL in causing Vent gleet infection in layers. A detailed study in large numbers of cases would assist in identifying the other etiological factors in addition to potential role of APEC in causing Vent gleet infection in chickens.

Acknowledgment

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Conflict of interest

The authors declare that they have no conflict of interest.

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