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Original Article

Pathogenicity Study on Extra-Intestinal Avian Pathogenic *Escherichia Coli* Isolated from Broiler Chickens

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Abstract

One of the major bacterial diseases that affect poultry industry is the Colisepticeamia, caused by *Escherichia coli.* The present study emphasise on understanding pathogenic potential of the extra-intestinal avian pathogenic *Escherichia coli* (APEC) isolated from heart blood of infected broiler chickens. The *in-vitro* pathogenicity studies carried on APEC isolates showed a clear haemolytic pattern on 10% Sheep blood agar and tested positive for biofilm formation. The multidrug resistant pattern of the APEC isolates along with biofilm formation made it difficult to control the disease. The *in-vivo* pathogenicity tests studied in day old chicks provided an understanding of the pathogenicity of the APEC isolates. The APEC isolates developed infection slowly with clear clinical signs that ends in 100% mortality of the infected chicks. The re-isolation of the injected APEC isolates from heart blood of the dead chicks clearly indicates the significance of virulence posed by the extra-intestinal avian pathogenic *E.coli* in developing Colisepticaemia.

Keywords: *Escherichia Coli;* APEC; Pathogenicity; Haemolysin; Biofilm.

Introduction

Escherichia coli, a member of the family Enterobacteriaceae is responsible for diverse diseases in humans, animals and birds. In poultry this Gram negative rod shaped bacterium causes a variety of conditions including Omphalitis, Coligranuloma, Cellulitis, Colibacillosis, Colisepticeamia etc. [13]. These infections are responsible for huge economic loses to the poultry industry. The avian pathogenic *E.coli* (APEC) strains may originate as extra-intestinal or intestinal *E.coli* [4, 6, 13]. The main economic losses are due to the extra-intestinal pathogenic *E.coli* [4, 13]. Generally, these bacterial infections can be controlled by antibiotic therapy. Instead, the property of acquisition of Multi-drug resistance by the APEC became problematic in the control of APEC infections [14, 15]. Many licensed vaccines are available for control of APEC infections in poultry, but they confer protection largely to the homologous strains [16]. Hence need exists for identifying and understanding pathogenic potential of the extra-intestinal avian pathogenic *E.coli.* In the present case the pathogenicity studies were carried out on the extra-intestinal APEC isolates isolated from an infected flock of 4-week old broiler chickens. Previous studies suggested that the extra-intestinal avian pathogenic *E.coli* also possess Zoonotic potential and cause infections in humans [11, 12].

Materials and Methods

In the infected flock the dying broiler chickens exhibit clinical symptoms like snoaring sounds and mild nervous signs. On post-mortem examination of the birds it reveals Pericarditis, Perihepatitis with slight enlargement of Liver, congested

gastrointestinal track (GIT) with catarrhal inflammation, catarrhal exudates, congestion and consolidation of Lungs, oedematous bursa and enlarged spleen were noticed. Aseptically swabs were collected from heart blood, liver and swabs were enriched in Brain-heart infusion broth for 6 hours at 37^{0}C followed by propagation on Eosin-Methylene blue (EMB) agar, Brain-Heart infusion (BHI) agar and incubated at 37^{0}C for 24 hrs. Greenish metallic sheen colonies on EMB agar were stained by Gram's Method and observed under 100X oil immersion objective. These colonies were processed by hanging drop method and observed for motility of the bacterium under 10X and 40X objectives. The greenish metallic sheen colonies on EMB agar were also subjected to Indole, Methyl Red, Voges-Proskauer and citrate (IMViC) tests.

The Antibiotic drug resistance pattern of APEC was studied by antibiotic disc diffusion method of Kirby and Bauer [2], by following the instructions of manufacturer (Hi-Media laboratories Pvt. Ltd.).

In-Vitro study of Virulence factor, Hemolysin of the APEC was analyzed qualitatively by the method described by Synder and Koch [18] with 10% Sheep blood agar and incubated at 37^{0}C for 48hrs. The results were recorded after 48hrs of incubation. The second virulence factor studied was biofilm forming ability of the pathogen, tested by Christensen tube method with 24 hrs. and 48 hrs. bacterial culture in nutrient broth [3]. After incubation, the culture was discarded aseptically and the biofilm was stained with crystal violet stain for 5 minutes followed by washing with distilled water. The test tubes were air dried and results were recorded.

In-Vivo Pathogenecity Study was carried by inoculating a single metallic sheen colony of both the isolates into 1ml. sterile nutrient broth and incubated aerobically at 37^{0}C. At regular intervals of every 3 hours the OD at 600 nm was recorded in a spectrophotometer so as to find out the growth period at which the bacterium reaches a balanced growth state. The bacteria at a stage of balanced growth curve was diluted and adjusted the turbidity to 3x10^8 cells per ml, with the McFarland's turbidity standard tubes. This diluted bacterial culture was inoculated intra-peritonially into day old chicks at the rate of 100 µl. per chick. For each isolate two day old chicks were used for injection and one day old chick was kept as control. The control birds were injected with 100 µl. of sterile PBS (pH 7.2). All the chicks were maintained under similar conditions and the mortality pattern and post-mortem findings were recorded. From the dead chicks the pathogenic *E.coli* was re-isolated and biochemically characterized.

Results

Two extra-intestinal pathogenic *E.coli* isolates were isolated from the infected flock. On Grams staining, metallic sheen colonies from the EMB agar, revealed gram negative cocco-bacilli. The motility tested by Hanging drop method reveals motile rod shaped bacteria from the metallic sheen colonies. The biochemical characterization was done for all the bacteria isolated from two samples. The results (Table 1) confirmed the isolation of two extra-intestinal *E.coli* isolates.

In Antibiotic disc diffusion method, out of eight antibiotic groups studied, the extra-intestinal pathogenic *E.coli* was resistant to three antibiotics namely Amoxycillin, Pencillin-G and Tetracyclin. The antibiotics Amikacin, Chloramphenicol, Enrofloxacin, Ciprofloxacin and Streptomycin were shown to be effective in controlling the disease caused by extra-intestinal pathogenic *E.coli in-vitro* (Table 2).

In-Vitro pathogenicity study on Haemolysin reveals a clear zone of Haemolysis around the colonies on 10% Sheep blood agar of both the isolates of APEC was developed after 48 hrs. of incubation followed by refrigeration at 4^{0}C. The exogenous toxin, Haemolysin secreted by the APEC is one of the major virulence factors that determine the pathogenic potential of the bacteria in causing disease. The Christensen tube method for biofilm forming ability of the APEC isolates revealed that both the isolates were weakly positive for the biofilm production only after 48 hrs. of incubation.

The *In-vivo* pathogenicity studies of two APEC isolates were carried out in day old chicks by intra-peritoneal route. The chicks which receive extra-intestinal APEC-1 & 2 isolates were died in between 24-36 hrs. of incubation where as the control chicks which received only sterile PBS did not show any clinical abnormality even after 7 days (Table 3). Before death the chicks exhibit clinical symptoms like dullness, depression with mild nervous symptoms. Post-mortem examination of the four chicks revealed generalized

Table 1: Biochemical characterization of the isolates

Name of the biochemical test	Isolate 1	Isolate 2
Indole	+	+
Methyl red	+	+
Voges proskeur	-	-
Citrate	-	-
Triple sugar iron	Yellow slant with Gas production	Yellow slant with Gas production
Greenish Metallic sheen on EMB	+	+
Catalase	+	+
Motility	+	+
Capsule	-	-
Confirmation	*E.coli*	*E.coli*

+: Positive; -: Negative

Table 2: Antibiotic disc resistance pattern of the *E.coli*

Name of the antibiotic	Disc concentration used (mcg)	Result
Amikacin (Ak)	30	Susceptible
Amoxyillin (AM)	30	Resistant
Chloramphenicol (C)	25	Susceptible
Ciprofloxacin (CF)	5	Susceptible
Enrofloxacin (Ex)	10	Susceptible
Pencillin-G (P)	10	Resistant
Streptomycin (S)	25	Susceptible
Tetracyclins (T)	30	Resistant

Table 3: Experimental design for in-vivo pathogenecity study

Parameter	Apec-1 Isolate			Apec-2 Isolate		
	Chick-A	Chick-B	Chick-C	Chick-D	Chick-E	Chick-F
BACTERIAL INOCULUM 3×10^8 cells/ml.	0.1 ml. of APEC-1	0.1 ml. of APEC-1	1 0.1ml. of PBS	0.1 ml. of APEC-2	0.1 ml. of APEC-2	CONTROL O.1ml. of PBS
Clinical symptoms before 24 hrs of incubation	NAD	NAD	NAD	NAD	NAD	NAD
Clinical symptoms after 24 hrs of incubation Dullness, Depression	++	++	NAD	++	+ Only inactiveness was noticed	NAD
Clinical symptoms 32-36 hrs of incubation	Dead	Dead	NAD	DEAD	Dull depression with mild nervous signs	NAD
Clinical symptoms after 36 hrs of incubation	NA	NA	NAD	NA	DEAD	NAD
Post-Mortem Findings	Congested heart, liver and lungs	Congested heart, liver and lungs	NAD	Congested heart, liver and lungs	Mild enlargement of liver, congested heart and lungs	NAD

NAD: No abnormality detected; NA: Not applicable; ++: severe symptoms; + = mild clinical signs.

congestion of viscera, hyperaemic heart, mild enlargement and congestion of liver, congested lungs were noticed. Microscopically septicaemic changes were noticed. Aseptically swabs were collected from the heart blood and re-isolation and its biochemical characterization of the extra-intestinal APEC isolates were successfully done.

Discussion

The *Escherichia coli*, a member of the Enterobacteriaceae family is associated with the most of clinical diseases in Poultry. The Extra-intestinal avian pathogenic *E.coli* alone is responsible for major diseases like Omphalitis, Coligranuloma, Cellulitis, Colibacillosis, Colisepticeamia etc. in poultry [4, 13]. These APEC isolates posses many kinds of virulence factors which includes adhesions, iron acquisition system, colicins, lethal and stable toxins, capsule, serum resistance offered by surface structures, temperature sensitive haemagglutinins and other virulence factors associated with genome etc.[6, 10, 12, 13]. The haemolysin, one of the major virulence factors of the APEC isolates, may contribute for septicaemia during the infections caused by extra-intestinal *E. coli*. The production of clear zone of haemolysis by these APEC isolates on 10% Sheep blood agar indicates the pathogenic potential of the haemolysin in disease production. The results of in-vitro study on haemolysin were correlates with the results of earlier studies [17]. The control of APEC infections will be achieved by both disinfecting the environment of poultry sheds and chemotherapy. In the environment of poultry sheds the control of the non-biofillm forming bacteria is much easier than

the biofilm forming bacteria. The biofilm itself protects the embedded bacteria from chemical disinfectants by preventing penetration of the drugs to their site of action [7]. The bio-film also offers certain antimicrobial resistance and protects bacteria from host innate immune defences such as lysozymes [20]. The lysozyme inhibitors protect the bacterium from the host innate immune system [19]. In the present case, two APEC isolates tested in-vitro were found to be moderately positive for the biofilm production. This should be of serious concern because if the biofilm producing APEC isolates were not controlled from the environment of poultry sheds, the problem of repeated infections by the Extra-intestinal APEC isolates will emerge. Moreover, the biofilms provides suitable environment for exchange of antibiotic resistant plasmids as the bacteria embedded in biofilms are very closely packed. The APEC isolates were also showed some resistance pattern towards the commonly used antibiotics Pencillins, Amoxycillins and Tetracyclins which may be due to irrespective use of antibiotics in the poultry feed results in development of resistance [10, 14, 15].

The day old chicks were one of the best models for understanding the pathogenic potential of APEC isolates by intra-peritoneal route [1, 7]. In all the four birds tested, the clinical findings were noticed only after 24 hrs. of incubation. The slow increase in the rate of severity of infection in the infected birds like dullness, depression, mild nervous signs followed by death was a good indication for slow progression of infection from site of injection to systemic infection. Moreover, the pathogenicity in Colisepticaemia was also associated with damage to the bone marrow cells [5]. The re-isolation and their biochemical characterization of APEC isolates from heart blood of experimentally injected dead birds, reveals the potential of APEC isolates in causing the septicaemic conditions there by death in the infected birds. The gross lesions appeared in dead birds includes congestion and mild enlargement of liver, congested heart and lungs which correlates with that of the earlier experimental studies suggested the death resulted from colisepticaemia [7, 9]. Further studies were required for serotyping, identifying strain variations and development of a good vaccine candidate for effective control of these of Extra-Intestinal Avian pathogenic *E.coli*.

The study of the virulence factors like haemolysin, biofilm production, motility in-vitro and in-vivo pathogenicity studies in day old chicks were an useful diagnostic tools for understanding the pathogenic potential of the of Extra-Intestinal Avian pathogenic *E.coli*.

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