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PHYLOGENETIC GROUPING OF THE PATHOGENIC *E. COLI* ISOLATED FROM COMMERCIAL BROILER CHICKEN IN PAKISTAN

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ABSTRACT

E. coli isolates can be classified into four different phylogenetic groups on the basis of *chuA*, *yjaA* and TspE4.C2 biomarkers via multiplex PCR. Current study investigated the presence of various phylogenetic groups in clinical samples of collibacillosis from commercial broiler chicken in Pakistan. A total of 84 *E. coli* isolates out of 200 swab sample (n = 25 cloacal; n = 27 fibrinous layer of peri-hepatitis of clinically infected broiler chicken; and n = 32 cloacal swabs from clinically healthy chicken) were recovered. Out of 25 pathogenic *E. coli* isolates from cloacal swabs, 12%, 4%, 72% and 0% isolates belonged to group A, B1, D and B2, respectively. Out of 27 pathogenic *E. coli* isolates from swab of fibrinous layer,18.5%, 29.6%, 44.4% and 0% isolates belonged to group A, B2, D and B1, respectively. While out of 32 non pathogenic *E. coli* isolates from healthy birds, 21.8%, 9.3%, 46.8% and 18.7% isolates belonged to group A, B2, D, and B1, respectively. There were 06 uncategorized *E. coli* isolates, which amplified both *yjaA* and TspE4.C2 biomarkers. This study helped to categorize the *E. coli* isolates into intestinal and extra intestinal origin on the basis of phylogenetic grouping.

Key words: Collibacillosis, Fibrinous peri-hepatitis, Multiplex PCR.

INTRODUCTION

Escherichia coli (E. coli) is one of the most common Gram-negative, facultative anaerobic bacterium belonging to the family Enterobacteriaceae of the class Gamma Proteobacteria Williams *et al*. (2010). *E. coli* are the normal inhabitant of gastrointestinal microflora of various birds and animals and a versatile enteric microorganism, acting both as commensal and pathogenic organism. It is considered to be the major cause of morbidity as well as mortality both in birds and animals, worldwide (Miskinyte *et al*. (2013). Classification of *E. coli* on the basis of its DNA typing is now becoming very common and analysis of the genome mainly relied upon molecular based procedures. Genotyping is becoming more simplified due to the recent advances in polymerase chain reaction (PCR) and sequencing technique. In year 2000, Clermont along with his colleagues developed a method of triplex PCR based on the amplification of the *chuA*, *yjaA*, and the DNA fragment of the *TspE4.C2*. On the basis of amplification and presence or absence of these genes, *E. coli* is divided into four major phylogenetic groups A, B1, B2 and D (Clermont *et al*. (2000). It is observed by many researchers that the phylogenetic groups can differ on the basis of presence or absence of the virulence factor, life history and ecological niche and origin. They also hypothesized that some phylogenetic groups were specifically found in certain species (Clermont *et al*. (2013); Smati *et al*. (2015). Many researchers used the same technique for genotyping of *E. coli* using *chuA*, *yjaA*, and the DNA fragment of the *TspE4.C2* genetic marker (Martins *et al*. (2013). The present study has been undertaken to investigate the presence of various phylogenetic groups of *E. coli* in clinically healthy and *E. coli* infected samples of commercial broiler chicken populations in Pakistan using molecular markers.

MATERIALS AND METHODS

Samples Source: A total of 200 swab samples (n= 150 clinically infected and n = 50 clinically healthy broiler chicken) were collected from cases brought for diagnostic investigations to University Diagnostic lab, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan, as well as from various commercial broiler chicken flocks raised at 10 different districts (Okara, Toba Tek Singh, Narowal, Kasur, Layyah, Mianwali, Jhang, Sheikhupura, Gujrat and Lahore) of Punjab province. Out of 150 clinical samples, 75 swabs were collected from cloaca and 75 swabs were from fibrinous layer of peri-hepatitis mostly appearing in avian collibacillosis. While 50 cloacal swab samples were collected from clinically healthy chicken. The samples were initially enriched in modified tryptose soya broth (TSB) followed by culturing on selective media (McConkey's agar and eosin methylene blue agar. The isolated pure cultures were further confirmed by

microscopy and standard biochemical testing (IMViC: indole, methyl red, voges- proskauer and citrate test) and sugar fermentation tests as described by Bergey's Manual of Determinative Bacteriology. For differentiating pathogenic from non pathogenic *E. coli*, all the isolates (n=84) were cultured onto Congo red medium and incubated for 24 hours at 37ºC (Ionica *et al*. (2012).

The data collected were analyzed statistically through regression using SPSS 18.0 to see correlation of *E. coli* isolates of various sources and phylogenetic groups.

Extraction and quantification of Genomic DNA: DNA from purified gram negative bacterial cultures was extracted by using Qiagen QIAamp DNA extraction kit (Qiagen, USA) spin method as per manufacturer's protocol. DNA quantification of the 1µL sample was done using Nanodrop equipment (Thermo Scientific spectrophotometer ND-2000, USA).

Multiplex PCR for Detection of Phylogenetic Groups: Multiplex PCR was performed to determine various phylogenetic groups of individual pathogenic *E. coli* isolate. Multiplex PCR was performed as previously described by Clermont *et al.* (2013) with minor modification. Three genetic markers were targeted simultaneously in this PCR reaction *chuA*, *yjaA* and TspE4.C2 (Primer sequences given in table. 1). Initially the multiplex PCR was optimized by using positive control of *E. coli* (ATCC 25922) in the thermo cycler (BioRad Corporation, USA, which amplify all three gene sequences. Each 30 µL of PCR reaction mixture for PCR contained 2µL of upstream primer and 2µL of downstream primer of each gene and DNA fragment, 3.5 µL of nuclease free water, 5µL of extracted DNA, 5 µL of PCR buffer (2mM), 2.5 µL of dNTPs (25mM), 1.5 µL $MgCl_2$ and 0.5 µL of Taq polymerase. Thermal cycling conditions which were optimized were as follows: Initial denaturation at 95ºC for 15 min followed by 35 repeated cycles at 94ºC for 30 sec, 60ºC for 90 sec, 72ºC for 90 sec and a final extension at 72ºC for 10 min. After performing PCR reaction the PCR product was run on 2% agarose gel and electrophoresis was performed. After electrophoresis the gel was visualized on UV transilluminator and was photographed under UV light on gel documentation system (BioRad Corporation, USA) (Fig. 2). The *E. coli* strains were assigned to different phylogenetic groups according to criteria as described in Table. 2.

RESULTS

Total 84 *E. coli* isolates were recovered out of 200 samples processed. Amongst those 27 isolates were recovered from swabs of fibrinous layer in peri-hepatitis, 25 isolates were recovered from cloacal swabs and 32 isolates were recovered from apparently healthy broiler chicken. *E. coli* isolates showed red colonies on Congo red medium were considered as Pathogenic (Fig. 1).

A total of 52 *E. coli* isolates recovered from swab samples collected from cloaca and from fibrinous layer of peri-hepatitis in clinically infected chicken were found to be pathogenic in nature, while the samples from clinically healthy chicken were found non pathogenic (commensals). All the 84 isolates analyzed via multiplex PCR for the presence or absence of various phylogenetic groups with respect to their origin of isolation. The results showed that out of 84 *E. coli* isolates, *chuA* marker was amplified in 56 swab samples (cloacal n=18, fibrinous layer n= 20 and commensals n=18). The marker *yjaA* was amplified in 32 swab samples (cloacal n=06, fibrinous layer n= 15 and commensals n=11). Whereas, TspE4.C2 marker were amplified in 13 samples (cloacal n=4, fibrinous layer n=2 and commensals n=07). The positive control of *E. coli* ATCC 25922 amplifies all three genetic markers (*chuA*, *yjaA*, TspE4.C2).

On the basis of the presence or absence of these three genetic markers (*chuA*, *yjaA* and TspE4.C2) in each isolates, the phylogenetic groups were assigned as per criteria given in table. 2. According to this criteria, out of 25 pathogenic *E. coli* isolates recovered from cloacal swabs of clinical cases, 03 isolates belonged to Group A (12%), 01 isolate belonged to Group B1 (4%) and 18 *E. coli* isolates belonged to Group D (72%). None of the isolate belonged to Group B2.

In case of *E. coli* isolates recovered from swabs of fibrinous layer from peri-hepatitis of clinical cases, 05 isolates belonged to Group A (18.5 %), 08 isolate belonged to Group B2 (29.6%) and 12 *E. coli* isolates belonged to Group D (44.4%). None of the isolates belonged to Group B1.

Among the commensals *E. coli* isolates recovered from apparently healthy chicken, 07 isolates belonged to Group A (21.8%), 03 isolate belonged to Group B2 (9.3%), 15 isolates belonged to Group D (46.8%) and 06 isolates belonged to Group B1 (18.7%). There were 06 uncategorized *E. coli* isolates (cloacal n=03, fibrinous layer n= 02 and commensals n=01), which amplified both *yjaA*, TspE4.C2 genetic markers. Distribution of *E. coli* phylogenetic groups amongst various sources analyzed is represented graphically (Fig. 3). Statistical analysis revealed that *E. coli* isolates from various sources has significant correlation ($p<0.05$) with phylogenetic groups B1 and B2. On the basis of phylogenetic group B1, commensal *E. coli* was significantly correlated ($p<0.05$) with *E. coli* isolates from cloacal swabs as well as organ swabs. Similarly on the basis of phylogenetic group B2, *E. coli* isolates from organ swabs were significantly correlated ($p<0.05$) with *E. coli* isolates from cloacal swabs and commensal *E. coli* with predominant correlation of Group B1 and Group B2 with intestinal and extra-intestinal *E. coli* respectively.

Table 1. Primer sequences used in multiplex PCR for phylogenetic grouping.

Gene	Sequence(5'----------3')	Product size	Reference
chuA	GACGAACCAACGGTCAGGATTGCCGCCAGTACCAAAGACA	279 bp	Clermont *et al*. (2000)
yjaA	TGAAGTGTCAGGAGACGCTGATGGAGAATGCGTTCCTCAAC	211bp	
TSPE4.C2	GAGTAATGTCGGGGCATTCACGCGCCAACAAAGTATTACG	152bp	

Table-2. Criteria for assigning the phylogenetic groups

Phylogenetic Groups	Genes
B2	(*chuA*+, *yjaA*+, TspE4.C2+)
D	(*chuA*+, *yjaA*-)
B1	(*chuA*-, TspE4.C2+*)*
A	(*chuA*-, TspE4.C2-)

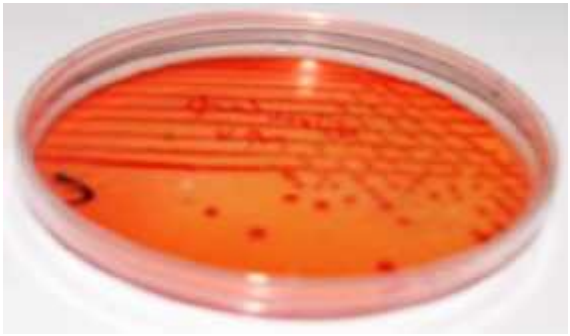

Fig. 1. Pathogenic *E. coli* isolates showing red colonies on Congo red medium

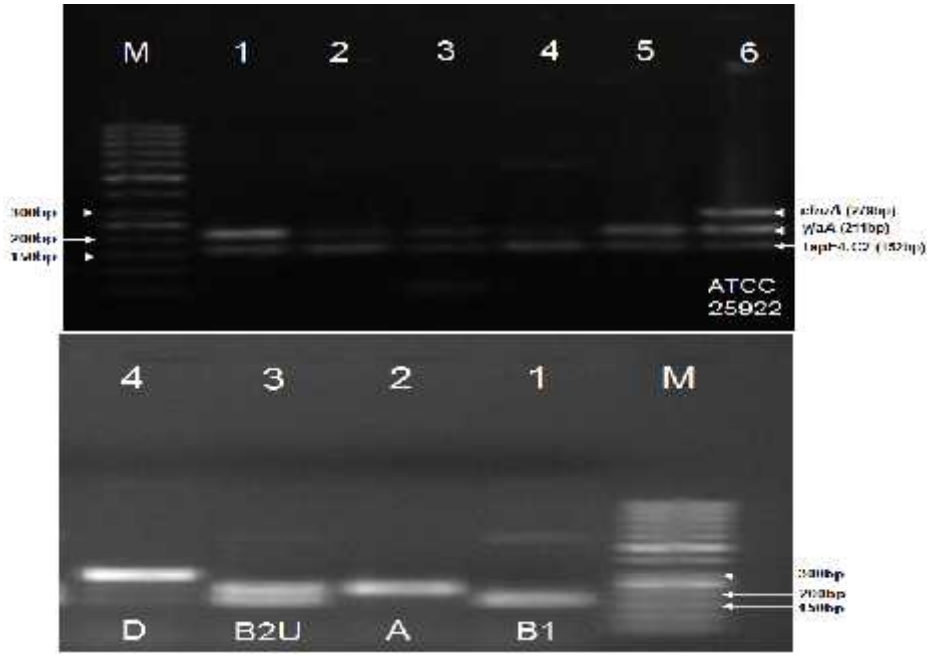

Fig. 2. Ethidium Bromide stained 1.5% agarose gel shows the PCR amplification of *yjaA* (211bp and *chuA* (279bp) and TSP.C2 (152 BP) gene for *E. coli*.

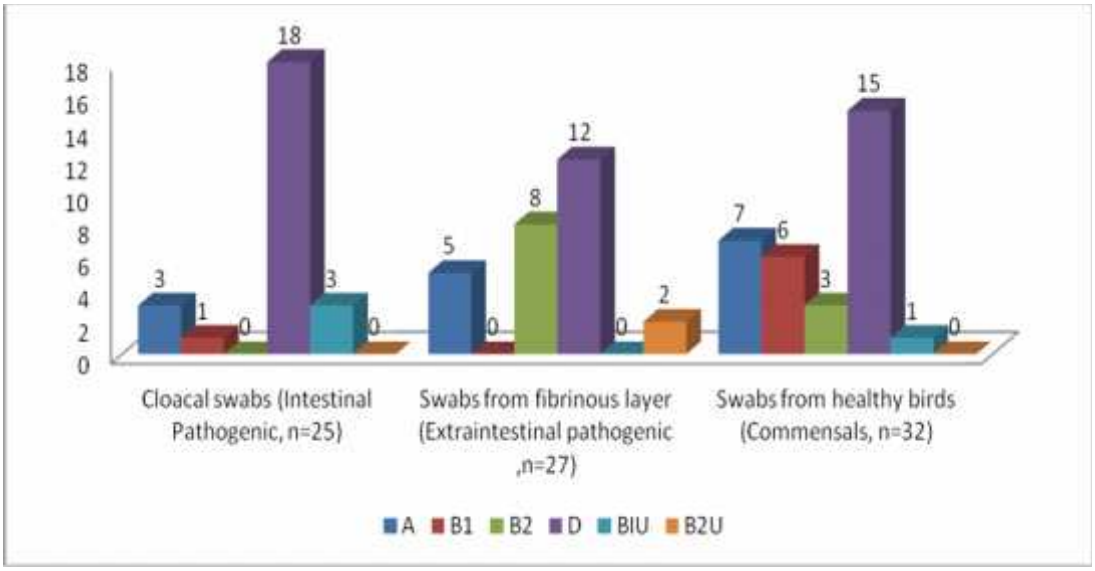

Fig. 3. Distribution of *E. coli* phylogenetic groups amongst various sources analyzed

DISCUSSION

This study was conducted to investigate the presence of various phylogenetic groups in clinical samples of commercial broiler chicken in Pakistan using molecular markers (*chuA*, *yjaA*, TspE4.C2). The findings of our study revealed the highest prevalence of phylogenetic group D amongst all samples irrespective of the site of collection. Phylogenetic Group D is the most common group which is prevailing in birds and is reported both in extra-intestinal as well as intestinal pathogenic *E. coli* as already reported by (Rodriguez-Siek *et al*. (2005; Higgins *et al*. (2007; Ishii *et al*. (2007); Lee (2011); Ciccozzi *et al*. (2013).

The second most common phylogenetic group reported in our study was Group A, which was present in all the swab samples including cloacal, fibrinous layer and commensals with percentage of 12%, 18.5% and 21.8% respectively. Previous reports also suggested that the phylogenetic group A is predominantly prevailing in the *E. coli* isolates of commensals as well as intestinal pathogenic category but in our study Phylogenetic group A is also present in extra-intestinal *E. coli* isolates. Similar types of results were reported by (Koo *et al*. (2012), who reported the 37.7% prevalence of phylogenetic group A from extra-intestinal *E. coli* in poultry. These findings suggest that phylogenetic group A can be found in extra-intestinal pathogenic *E. coli*. Neither of the *E. coli* isolate from intestinal pathogenic category showed the prevalence of phylogenetic group B2, nor the phylogenetic group B1 from swabs of fibrinous layer. These results suggests that the phylogenetic group B1 is more prevalent in intestinal *E. coli* isolates whereas B2 is predominantly present in extra-intestinal *E. coli* isolates or we can say that the *E. coli* strain involved in the formation of fibrinous peri-hepatitis is most probably belonged to extra-intestinal category. Similar types of results were observed in previous studies of the correlation of group B1 with intestinal commensals and pathogenic *E. coli* and group B2 correlation with extra-intestinal *E. coli* (Abdul-Razzaq and Abdul-Lateef (2013); Clermont *et al*. (2013).

We also observed 06 uncategorized *E. coli* isolates from swab samples of (cloacal n=03, fibrinous layer n= 02 and commensals n=01), which amplified both *yjaA*, TspE4.C2 genetic markers. This is novel information related to pathogenic *E. coli* isolated from clinical cases of collibacillosis in broiler chicken. Previous data is also available in which similar findings were observed in the extra-intestinal *E. coli*, isolated from clinical samples of human in which *E. coli* isolates were placed in B2 group due to their isolation from extra-intestinal sources but in our study as the *E. coli* isolates were recovered from both intestinal as well as extra-intestinal sources so we can relate our intestinal *E. coli* isolates with B1U (B1 unusual group) and extra-intestinal *E. coli* isolates with B2U (B2 unusual group) (Mendonça *et al*. (2011); Skjøt-Rasmussen *et al*. (2013). Although the genotyping technique by Clermont is simplest, easiest and rapid way of assigning the phylogenetic groups to *E. coli* isolates of different origins and sources and is helpful in categorizing the *E. coli* isolates into intestinal and extra-intestinal, but due to small sample size these results are not conclusive and there is need to utilize some advanced and modified methods so as to reduce the chances of those groups, which are still uncategorized via this method.

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