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Extended phylogrouping of pathogenic and non-pathogenic *Escherichia coli* isolates of avian origin

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Abstract
Avian Pathogenic *E. coli* (APEC) is the causative agent of avian colibacillosis, accounting for vast economic losses to the poultry industry in form of morbidity, mortality, and carcass condemnation The *E. coli* strains of the various phylogroups also differ in their genotypic and phenotypic characteristics, their ability to cause disease and their ecological niche. In the present study, 64 *E. coli* isolates comprising of APEC (n=50) and non-APEC (n=14) were phylogrouped. The combination of phylogenetic markers *viz.*, *chuA*, *yjaA*, DNA fragment TspE4.C2, *arpA* and *trpA* was used to amplify target specific fragments using PCR for phylogrouping. The results classified *E. coli* isolates (n=64) into A (28.1%), B1 (12.5%), D (10.9%), F (7.9%), B2 (6.2%), E (1.6%) and Clade I (1.6%). Sixteen *E. coli* isolates (25%) were untypable/unknown and none of *E. coli* isolates belonged to phylogroup C. A total of 6.2% *E. coli* isolates were either grouped to Clade I or Clade II. The phylogrouping of APEC isolates indicated that most of APEC isolates were untypable/unknown category (30%) followed by phylogroup A (24%). None of APEC isolates in the present study fall under phylogroup E and C. Similarly, among non-APEC isolates phylogroup A dominance was observed (6/14) followed by phylogroup B1 (3/14). The study revealed that phylogroup A is widely circulating among APEC as well as non-APEC pathotypes in Haryana. The circulation of unknown phylogroups warrants further investigation in phylogrouping protocols and methods, as there might be possibility of existence of newer phylogroups of APEC. The study further provided insight to the newer phylogroups of APEC as well as non-APEC isolates, generating valuable data which may be helpful in perceiving the origin and pathogenicity of APEC isolates on the basis of phylogrouping.

Keywords: APEC, avian, Haryana, phylogroup, non-APEC

1. Introduction

Avian pathogenic *E. coli* (APEC) are the causative agent for extraintestinal infections in poultry birds. APEC infections in poultry birds produce diverse array of lesions from localized to systemic, which results in huge economic losses to poultry industry in term of mortality, morbidity, treatment cost and carcass condemnation [1]. Also, APEC is recognized as an important zoonotic pathogen and can cause foodborne urinary tract infection in humans due to the consumption of contaminated poultry carcasses [2]. Traditionally APEC classification was based on serotyping with a limitation that it allows only for the classification of a limited number of APEC isolates because of overlapping serogroups among member of extraintestinal pathogenic *E. coli* (ExPEC) [3]. Virulence genotyping using PCR has enabled rapid classification of APEC and their differentiation from non-APEC isolates including commensal *E. coli*. However, owing to huge geographical variations and diversity among APEC isolates some studies failed to classify APEC on the basis of virulence genes [4]. The suitability of virulence markers for discriminating pathogenic and non-pathogenic groups within *E. coli* is questionable for certain regions of the world due to geographical variations and coinfections by otherwise commensal *E. coli* [4]. Additionally, the genome of *E. coli* strains frequently undergoes additions, deletions, and recombinations as a reaction to natural selective pressure, leading to divergence [5,6]. Consequently, there are diverse genetic substructure within the *E. coli*, comprised of at least eight phylogenetic groups segregated into three clusters: phylogroups B2, G, and F, phylogroups A, B1, C and E, and phylogroup D [7, 8, 9, 10, 11]. Each phylogenetic group is broadly associated to an ecological niche, for instance strains belonging to phylogroups B2 and D are commonly associated with virulent extra-intestinal infection, strains from phylogroup A are often categorized as of commensal origin, and phylogroup B1 associated with environmental reservoirs [8, 12, 13, 14].

The extended Clermont classification is an update to the previous classification of four phylogroups (A, B1, B2 and D) and has used a combination of phylogenetic markers *viz.*, *chuA*, *yjaA*, DNA fragment TspE4.C2, *arpA*, *trpA* and *arpA* (ArpAgpE) to amplify target specific fragments using PCR [7]. This phylotyping method, is a top-level, rapid, and inexpensive technique for classification of *E. coli* to phylogenetic groups and shows high correlation with other reference methods including multi-locus sequence type (MLST) analysis [7].

The association between various phenotypic traits of APEC and non-APEC with reference to biofilm formation, antimicrobial resistance and virulence gene repertoire has been explored in our previous study [15] however, the potential of pathogenic traits associated with phylogenetic types warrants consideration into the potential role of virulence genes and their linkage with specific phylogenetic types. The purpose of the current study was to assess the usefulness of the phylogenetic typing tools in subtyping various APEC and non-APEC isolates.

2. Material and Methods

2.1 Source of samples

The samples were collected from avian colibacillosis affected broiler chicken farms in five districts in the state of Haryana, and isolation was carried out by standard methods using MacConkey agar, brain heart infusion broth, EMB agar, Gram's staining and further confirmation using Vitek 2 Compact and *uspA* gene amplification by PCR [15]. A total of 64 *E. coli* isolates differentiated as APEC (n=50) and non-APEC (n=14) were used in the study for phylogroup analysis.

2.2 Preparation of DNA Template

The genomic DNA extraction from isolated colonies was carried out using a heat lysis/snap-chill method [16]. Briefly, loopful of colonies were dispensed into 250 µl of nuclease free water in 0.6 ml eppendorf tube. The Eppendorf tubes with bacterial suspension were placed on a heating block at 98 °C for 10 minutes followed by snap chilling at -20 °C. Then after thawing at room temperature centrifugation was carried out at 12,000 rpm for 7 minutes. The supernatant was taken and stored as DNA for further use at -20 °C.

2.3 Phylogenetic Typing PCR Protocols

Samples of the DNA stock were subjected to phylogenetic typing using the revised protocols described [7]. The primer pairs used for the current study are summarized in Table 1. The PCR reaction used a 25 µl reaction volume with the following PCR conditions: denaturation for 4 min at 94 °C followed by 30 cycles of 5 s at 94 °C; 30 s at 52 °C (group E), or 60 °C (quadruplex) or 62 °C (group C) and 30 s at 72 °C with a final extension at 72 °C for 5 min. PCR products generated were subjected to electrophoresis in 1.5% (w/v) agarose gels in 1X TAE buffer and run at 120 V for 2 h. A 100 bp molecular weight marker (GeNei, India) was used as the size standard. Gels were stained with ethidium bromide, and bands corresponding to each gene present were digitized using a gel documentation system (Zenith, India).

Table 1: Primers used in the phylogenetic typing PCR assays

	Primer name	5'-3' sequence	Product size	Reference
Quadruplex	*chuA.1b*	ATGGTACCGGACGAACCAAC	288 bp	[3, 17]
	chuA.2	TGCCGCCAGTACCAAAGACA		
	yjaA.1b	CAAACGTGAAGTGTCAGGAG	211 bp	
	yjaA.2b	AATGCGTTCCTCAACCTGTG		
	TspE4C2.1b	CACTATTCGTAAGGTCATCC	152 bp	
	TspE4C2.2b	AGTTTATCGCTGCGGGTCGC		
	AceK F	AACGCTATTCGCCAGCTTGC	400 bp	
	ArpA1 R	TCTCCCCATACCGTACGCTA		
Group E	*ArpAgpE F*	GATTCCATCTTGTCAAAATATGCC	301 bp	[18]
	ArpAgpE R	GAAAAGAAAAAGAATTCCCAAGAG		
Group C	*trpA.1*	AGTTTTATGCCCAGTGCGAG	219 bp	
	trpA.2	TCTGCGCCGGTCACGCCCC		
Internal Control	*trpBA F*	CGGCGATAAAGACATCTTCAC	489 bp	[19]
	trpBA R	GCAACGCGGCCTGGCGGAAG		

2.4 Cluster analysis

A clustered heat map/double dendrogram was constructed using PCR results of phylogrouping analysis of APEC and non-APEC isolates. Data were processed in a binary matrix using the NCSS (trial version) (NCSS, LLC, USA) software package. Grouping of the isolates was made by agglomeration method, based on the unweighted average distance.

Table 2: Distribution of different phylogroups among APEC and non-APEC pathotypes

Pathotype	Phylogroup/Clade assigned									Total
	A	B1	B2	D	E	F	Clade I	Clade I/II	Unknown	
APEC	12	5	4	5	-	4	1	4	15	50
Non-APEC	6	3	-	2	1	1	-	-	1	14
Total	18	8	4	7	1	5	1	4	16	64

3. Results and Discussion

3.1 Phylogenetic Typing

All the *E. coli* isolates were analyzed using protocol described [7]. Table 2 summarized the assignments and distribution of different phylogroups among APEC and non-APEC isolates. Overall, phylogroup A (28.1%) was dominant among *E. coli* isolates used in the study followed by unknown/untypable group (25%), B1 (12.5%), D (10.9%), F (7.9%), B2, Clade I/II (6.2% each) and E and Clade I (1.6% each). None of the *E. coli* isolates belonged to phylogroup C in the current study.

On analyzing the results based on the individual pathotype (APEC and non-APEC), it was observed that most of the APEC isolates (30; 60.0%) were assigned to at least one phylogroup (A, B1, B2, D, E, F) and some others were assigned to either Clade I or II (8.0%) and Clade I (2.0%); whereas, significantly high number of APEC isolates remained untypable/unknown (15; 30.0%) based on the Clermont protocols. None of the studied APEC isolates get assigned under phylogroup E and C. Phylogroup A (24.0%) was dominant among APEC isolates followed by B1 and D

(10.0% each), B2 and F (8.0% each). In a study conducted [20] it was observed that majority of the APEC were assigned to groups A and D and less than 20% assigned to group B2, whereas dominance of phylogroup C followed by B1 among APEC isolates was observed in other [21]. The absence of phylogroup C and E in current study might be attributed to the huge diversity and geographical variations among these isolates [22,15]. Moreover, a larger number of samples used in other studies might also be the reason for the diverse phylogroups they have obtained and for absence of these phylogroups among *E. coli* isolates in our study. The studies conducted so far indicate that phylogroup A generally contained commensal strains and its dominance among APEC isolates point out the probable evolution of these pathogenic strains from commensal strains of *E. coli* in poultry [23]. The phylogrouping results indicated that most of the APEC were assigned to phylogroup A unlike *E. coli* of other animals and humans as also reported [20]. Phylogroups B2 and D are commonly associated with virulent extra-intestinal infection and their less occurrence among non-APEC as compared to APEC isolates in current study points this aspect evidently [8]. Phylogroup B1 is mainly associated with environmental *E. coli*, and its distribution among both APEC and non-APEC isolates indicates the possible acquisition of pathogenic traits by these *E. coli* and adoption to cause disease in poultry birds [14, 21]. Similarly, most of the non-APEC isolates (6; 42.8%) were of phylogroup A followed by B1 (21.4%), D (14.3%), E and F (7.1% each), and one isolate remained untypable/unknown. Phylogroup B2 was not detected among non-APEC isolates in the current study and similarly none of the non-APEC isolates were assigned under Clade I and Clade II. As discussed earlier, the occurrence of phylogroup A among non-APEC or commensal *E. coli* seems to be admissible. Similarly, absence of phylogroup B2 among non-APEC isolates indicates about specificity of B2 phylogroup towards APEC isolates [8]. As the *E. coli* isolates from colibacillosis affected birds as well as their environment were investigated for research, a higher occurrence of B1 phylogroup was expected among non-APEC isolates [14]. Phylogroup C is a group of strains closely related to, but distinct from, phylogroup B1 and was not found in our study as opined by [24]. Several novel lineages (new species) of *Escherichia* have been reported that are genetically distinct but phenotypically indistinguishable from *E. coli* [25]. At least one of these cryptic lineages, *Escherichia* clade I, should also be considered a phylogroup of *E. coli* based on the extent of recombination detected between strains belonging to clade I and *E. coli* [26]. Therefore, currently, there are eight recognized phylogroups of *E. coli*, belonging to *E. coli sensu stricto* (A, B1, B2, C, D, E, F and G) excluding *Escherichia* clade I, however most recently discovered phylogroup G was not investigated in present work. The protocol described by [7], assigned phylogroups to most of the *E. coli* isolates studied, however, few remained untypable or unknown. There may be various reasons for this *viz.* some *E. coli* strains cannot be get assigned to any phylogroup as these untypable strains either represent phylogroups that are extremely rare or, more likely, are the result of large-scale recombination events where the donor and recipient originated from two different phylogroups [7]. Another possibility of highly variable gene content of *E. coli* driven by the frequent addition and deletion of genes also exists [27]. Consequently, grouping of few *E. coli* isolates to unknown or untypable category is not surprising.

On analyzing the combination pattern of four genes (*arpA*, *chuA, yjA,* TspE4.C2) and chi square analysis of phylogroup/clade assigned with the presence of genes it was observed that presence of *arpA* gene was significantly associated with phylogroup A, B1 and D ($p< 0.01$). Similarly, significant association was present between *chuA* gene and phylogroups B2, D and F ($p< 0.01$), *yjA* with Clade I/II, TspE4.C2 with the phylogroup B1, B2 and untypable isolates.

Table 3: Association of presence and absence of genes (*arpA*, *chuA, yjA,* TspE4.C2) and phylogroups

Phylogroup/Category/Clade	Genotype combination	Number of isolates
A (18)	*arpA* + *chuA* - *yjA* + TspE4.C2 -	13
	arpA + *chuA* - *yjA* - TspE4.C2 -	5
B1 (8)	*arpA* + *chuA* - *yjA* - TspE4.C2 +	8
B2 (4)	*arpA* - *chuA* + *yjA* + TspE4.C2 +	2
	arpA - *chuA* + *yjA* - TspE4.C2 +	2
Clade I (1)	*arpA* + *chuA* + *yjA* + TspE4.C2 -	1
Clade I/II (4)	*arpA* - *chuA* - *yjA* + TspE4.C2 -	4
D (7)	*arpA* + *chuA* + *yjA* - TspE4.C2 +	4
	arpA + *chuA* + *yjA* - TspE4.C2 -	3
E (1)	*arpA* + *chuA* + *yjA* + TspE4.C2 -	1
F (5)	*arpA* - *chuA* - *yjA* + TspE4.C2 -	5
Untypable (16)	*arpA* + *chuA* + *yjA* + TspE4.C2 +	10
	arpA + *chuA* - *yjA* - TspE4.C2 +	3
	arpA - *chuA* – *yjA* + TspE4.C2 +	3

3.2 Cluster analysis

The double dendrogram (Fig. 1) defined 2 distinct clusters and reflected the diversity of *E. coli* isolates in terms of phylogroup assigned to these isolates based on PCR results. Both the clusters (A and B) contained both APEC as well as non-APEC pathotypes. The gene *arpA*, *yja* and *chuA* were more consistently observed among isolates in cluster B as compared to cluster A. A large number of untypable isolates (13/16) were clustered in cluster B. These isolates consistently amplified four fragments (*arpA*, *yja, chuA* and TspE4.C2) indicating towards the virulence of these isolates. All the isolates with phylogroup B1, D and F clustered in cluster 'A' and maximum isolates with phylogroup A (15/18) got clustered in cluster 'B'. In summary, cluster 'A' comprised B1, D and F phylogroups and cluster 'B' comprised A and E phylogroup along with untypable, Clade I, Clade I/II isolates. B2 phylogroup was equally distributed among cluster 'A' and 'B'. Evolutionary, the closely related sister species of *E. coli*, phylogroups B2, F and D appear the most basal and their clustering together in current study implies the same (Fig. 1) [9, 28]. Subsequently, phylogroup E emerges, followed by most recently diverged phylogroups C, B1 and A [8]. The lifestyle or ecological niche of strains can be linked to phylogenetic history of the species. The most anciently diverged phylogroups B2, F and D comprises most of ExPEC strains, whereas the strains responsible for severe intestinal pathologies such as hemolytic and uremic syndrome (HUS) and dysentery belongs to the recently diverged phylogroups such as C, B1 and A [29, 9]. This suggests the role of the genetic background in virulence and emphasizes the need to identify and discover the novel *E. coli* phylogroups.

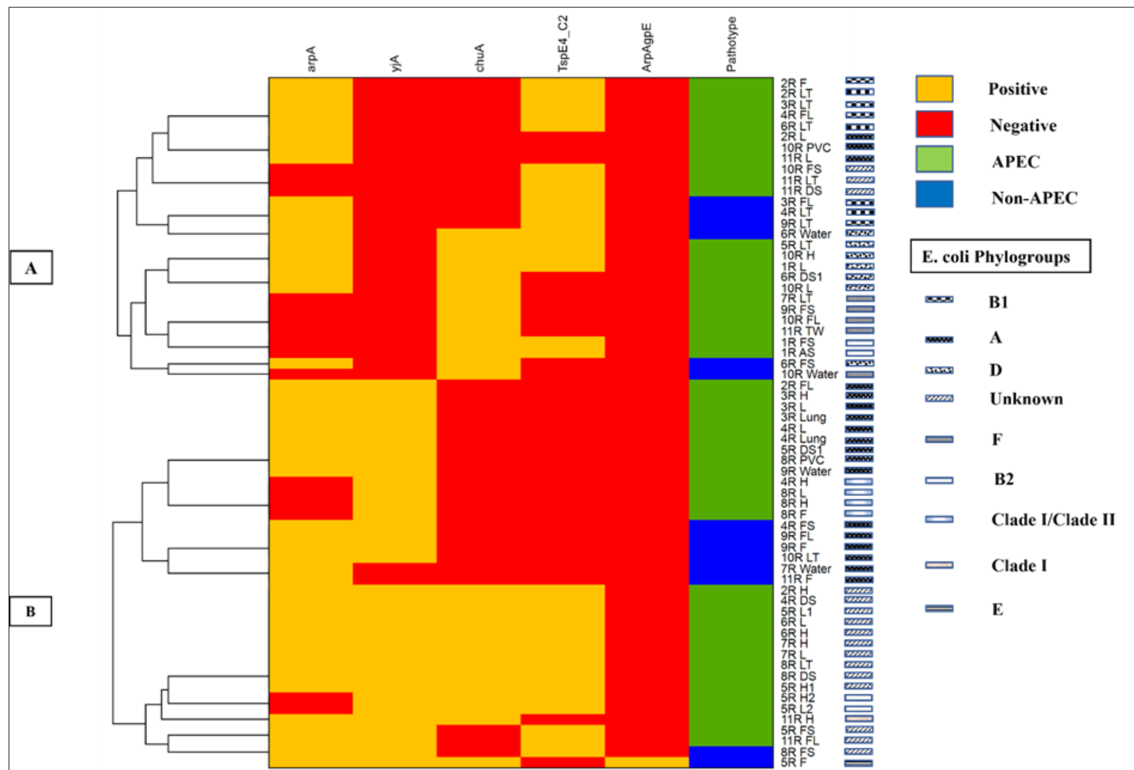

Fig 1: A double dendrogram on the basis of results of PCR analysis of APEC and non-APEC isolates. Left most portion of this figure is the dendrogram resulting from cluster analysis. There appear two clusters (A and B) of isolates. Cluster A contained *E. coli* isolates with B1, D and F phylogroup. Cluster B contained *E. coli* isolates of phylogroup A and E, untypable isolates, Clade I and Clade I/II *E. coli* isolates. Just to the right of the dendrogram are columns 1 to 5 which shows the genotype of each isolate tested. Each column in this group shows the results for a single gene amplification using PCR. The identity of each gene is shown on the top of diagram. Dark yellow color indicates that gene is present, and red indicates that a gene is absent. Column 6 indicates the pathotype of *E. coli* isolates (APEC or non-APEC), where green indicates APEC and dark blue indicates non-APEC pathotype. Column 7 contained the isolate number/ids, where source of isolation is: F; Feed, H; Heart, LT; Litter, L; Liver, Lung, FL; fecal/cloacal swab, FS; Feeder swab, Water, DS; Drinker swab, TW; Water source surface, PVC; pipe swab. Column 8 indicates the phylogroup assigned to individual isolate as described in the legends

4. Conclusions

In this study, attempt was made to classify APEC and non-APEC isolates of avian origin into six phylogroups *viz*., A, B1, B2, D, E and F. Phylogroup A was found as dominant group among *E. coli* isolates of avian origin. Despite of using recent method for phylogrouping, a significant number of *E. coli* isolates do not get assigned under any phylogroup and remained either as untypable or in *E. coli* clade I or clade I/II indicating that further investigations are required to explore the existence of more novel phylogroups.

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