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Genome based phylogeny and virulence factor analysis of mastitis causing *Escherichia coli* isolated from Indian cattle

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ABSTRACT

Mastitis is a highly infectious disease prevalent in dairy cattle and it is majorly caused by *Escherichia coli* (*E. coli*). The objective of present study is to investigate the occurrence of virulence genes, antimicrobial susceptibility and comparative analysis of *E. coli* (IVRI KOL CP4 and CM IVRI KOL-1) isolates from mastitis infected animal. Whole-genome sequencing (WGS) was performed using a PacBio RS II system and *de novo* assembled using Hierarchical Genome Assembly Process (HGAP3). Bacterial Pan Genome Analysis Pipeline (BPGA) was used for pangenome analysis. A set of 50 *E. coli* isolates were used for comparative analysis (48 collected from the database and 2 reference sequences). Core genes were further concatenated for phylogenetic analyses. In silico analysis was performed for antibiotic resistance and virulence gene identification. Both of the *E. coli* isolates carried many resistance genes including, b-lactamase, quinolones, rifampicin, macrolide, aminoglycoside and phenicols resistance. We detected 39 virulence genes in IVRI KOL CP4 and 52 in CM IVRI KOL-1 which include toxins, adhesions, invasins, secretion machineries or iron acquisition system. High prevalence of mastitis strains belongs to phylogroups A, although few isolates were also assigned to phylogenetic groups B1 and B2. In conclusion, the present study reported the presence of genes involved in Adherence, Iron acquisition, secretion system and toxins which shown to be crucial in MPEC pathogenicity. This is the first whole genome analysis of MPEC strains to be carried out in Indian isolate to highlights the spread of resistance and virulence genes in food animals.

Keywords: *Escherichia coli*, Mastitis, Pangenome, Virulence genes

In terms of milk production from all mammalian species, India ranks first in the world and second number in cows' milk production. Indeed, India actually accounts for 9.5% of all milk consumed within the world. Mastitis continues to be the most economically important disease of dairy cattle; the distribution of mastitis incidence varies from country to country. The national economic loss caused by mastitis is approx Rs 71.655 billion every year. Out of which approx Rs 41.511 billion is caused by subclinical mastitis while rest Rs 30.144 billion is caused by clinical mastitis (Bhat *et al.* 2017, Bansal *et al.* 2009). Cost of treatment, culling of animals, death, and decreased milk production are the main factor of losses caused by clinical mastitis. Apart from financial losses, the importance of mastitis with regard to public health should not be overlooked. The extensive use of antibiotics in treatment and control of mastitis have probable implications on human health (White *et al.* 2001).

Mastitis is inflammation of the mammary gland tissue caused by bacterial infection and about 70% of all losses being due to the infection (Zadoks *et al.* 2011). *E. coli* is a major cause of clinical mastitis (CM) in well managed dairies with low milk somatic cell counts (SCC) (Dogan *et al.* 2012), which affects the health and welfare of cows and in extreme cases may be fatal. The most common antimicrobials for the treatment of mastitis are β-lactam antimicrobials, particularly penicillin, fluoroquinolones, and cefquinome. Mastitis causing virulence factors is yet unknown and pathotypes are still uncharacterized (Blum and Leitner 2013). Different structural component (e.g. capsules, fimbriae or other cell wall components) or active secretion of substances are associated with bacterial pathogenicity that protects the bacteria against host defences. Hence, the main factor of bacteria by which they cause disease is adhesins, toxins, and haemolysins (Kalita *et al.* 2014).

E. coli has been classified into different groups based on human and animal diseases. The majority of *E. coli* isolates belong to the four major phylogenetic groups

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(A, B1, B2 or D), most of the virulent strain lie under phylogroups B2 and D and while group A belongs to commensal isolates (Carlos *et al.* 2010). The previous study also found that most of the mastitis causing isolates belonged to phylogroup A. Cow, goat, and sheep samples were more commonly found to be cluster with group B1 (Carlos *et al.* 2010).

With the help of next-generation sequencing (NGS), the genomes sequencing have become much easier and accessible which provides a huge amount of data for in depth analysis of intra-species diversity. Large number of genome should be included to better identify the genome diversity and pangenomes study may help to define species (Medini *et al.* 2005, Dagan and Martin 2006). The pangenome has been defined as the entire set of genes for all strains within a clade (Medini *et al.* 2005). *E. coli* species have been classified into different pathotypes, including enterohemorrhagic *E. coli* (EAEC), enterohemorrageic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and extra-intestinal pathogenic *E. coli* (ExPEC). Mammary pathogenic *E. coli* (MPEC) has been considered as a new pathotype (Blum *et al.* 2015). Many studies (Richards *et al.* 2015, Kempf *et al.* 2016) have been conducted to examine MPEC cohorts at the genomic level. Till now, there is no published phylogenetic study that includes Indian MPEC isolates. The objective of this study is to conduct a comparative study of newly sequenced isolate with other *E. coli* pathovars collected from the NCBI database and to identify the potential virulence properties of newly sequenced *E. coli* isolates. The outcome of our study provides new insights into the diversity and phylogeny of *E. coli*, particularly in regards to the hybrid genome that carry both resistance and virulence determinants.

MATERIALS AND METHODS

Sample collection and bacterial confirmation: Samples were collected from milk sample (25 ml) of infected animal from Nadia, West Bengal, India (22.9747° N, 88.4337° E). For molecular confirmation a penta-plex PCR covering five genes (*lacY, lacZ, cydA, uidA* and *phoA*) and a duplex PCR for three genes (*rpoB, gyrA* and *pehX*) were conducted for confirmation of *E. coli* and *K. pneumonia*, respectively.

Phenotypic characterization of the isolates: The isolates were checked for their sensitivity to the 13 antibiotics (ampicillin, cefpodoxime, aztreonam, ceftazidime, cefotaxime, ceftriaxone, amikacin, nalidixic acid, enrofloxacin, imipenem, meropenem, chloramphenicol, tetracycline) belonging to eight separate classes using commercially available antibiotic discs (BD, USA). Minimum inhibitory concentration (MIC) of IMP and colistin (MICIMP, MICCIP, and MICCOL) were determined only for the isolates having reduced susceptibility to IMP/MEM. Both the isolates (IVRI KOL CP4 and CM IVRI KOL-1) were selected based on their resistance towards β-lactam, carbapenem (meropenem and imipenem), fluoroquinolone (enrofloxacin and ciprofloxacin), tetracycline and amikacin.

Whole genome sequencing and assembly: Bacterial culture (*E. coli*) grown overnight was centrifuged and the pellet was taken for total DNA isolation. Initially, the cell was lysed using SDS and the proteins digested by proteinase K. DNA was isolated through phenol chloroform method (Fourth, 2012). PacBio SMRTbell template preparation kit v1.0 was used to prepare genomic DNA library. Whole-genome sequencing (WGS) was performed using a PacBio RS II system (Song *et al.* 2019) using P5 polymerase-C3 sequencing chemistry. This data is processed to remove low quality reads. These subreads were de novo assembled with HGAP3 (Hierarchical Genome Assembly Process) within the SMRT Analysis version 2.2.0 (Pacific Biosciences, USA). Blast2GO, Glimmer algorithm (Conesa *et al.* 2005) and RAST server (https://rast.nmpdr.org/) was used from Genome Annotation. Complete genome sequences were submitted to NCBI BioProject (PRJNA496565 and PRJNA496607), (Genome Accession Num: CP034253 and CP033158).

Resistance genes/Virulence factors analysis: Res-finder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari *et al.* 2012) was used for in-silico identification of antibiotic resistance genes in the genomes with default parameters. For the prediction of virulence genes we used two approaches, In the first approach we used Virulence finder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) tool and VFanalyzer from the Virulence Factor Database (VFDB) to identify the virulence gene. In the Second approach local TBLASTX search was performed with both of the genome using a list of 302 virulence genes as database (Kempf *et al.* 2016) to find the putative virulence genes. Only the hits with 100 coverage and sequence identity ≥99% were retained.

*E. coli pangenome analysis:*Bacterial Pan Genome analysis Pipeline (https://iicb.res.in/bpga/index.html) was used for pangenome analysis (Chaudhari *et al.* 2016). Genes were clustered using USEARCH algorithm (using 80% identity cut off) and binary matrix of the gene was used for pan genome based phylogeny. Sequence alignment and Phylogeny trees based on core gene sequences was generated using MUSCLE. It is provided with the BPGA package. *E. coli* strains included in the Pangenome analyses are listed in (Supplementary File 1), including 25 mastitis related strains (MPEC) (including IVRI KOL CP4 and CM IVRI KOL-1), 7 commensal, 4 ExPEC, 1 avian pathogenic *E. coli* (APEC), 1 neonatal meningitis *E. coli* (NMEC), 2 enteropathogenic *E. coli* (EPEC), 4 enterohemorrhagic *E. coli* (EHEC), 2 adherent-invasive *E. coli* (AIEC), 2 enteroaggregative *E. coli* (EAEC), 2 enterotoxigenic *E. coli* (ETEC) and 1 Asymptomatic Bacteria. In total a set of 50 *E. coli* isolates were used for Pangenome analysis. This involved data for 48 *E. coli* representing all the major phylotypes taken from GenBank and 2 of our newly sequenced genome.

RESULTS AND DISCUSSION

Bacterial characterization: Two isolates (IVRI KOL CP4 and CM IVRI KOL-1) exhibited resistance to the entire drug tested except chloramphenicol, including β-lactams (Am, CPD, CAZ, CTX, CTR, ATM) and carbapenems (IMP and MEM). MIC testing reconfirmed the imipenem and meropenem resistance of the isolates (≥ 4 µg/mL). The isolates were sensitive to colistin as revealed by broth microdilution assay. Further, both the isolates were found carbapenemase producer using the assays like Carba NP test, modified carbapenem inactivation test, IMP-IMP/EDTA disk synergy test. However, they were negative in double disk approximation method, AmpC disk test, and CCDDS assay. Although, CM IVRI KOL-1 was found negative in combination disk method, IVRI KOL CP4 exhibited an indication of ESBL production in CD assay.

Genome annotation: The sequencing run of strain (*IVRI KOL CP4*) yielded 334,101,910 bases within 38,171 reads (N50 size 14,361 and mean subread length 8,752) and strain (*CM IVRI KOL-1*) generated 767,722,447 bases within 76,133 reads (N50 size 17,389 and mean subread length 10,083). Complete circular genome sequence of both the strains was generated without any gaps with genome length of 4,690,918 bp (*IVRI KOL CP4*) and 4,945,239 bp (*CM IVRI KOL-1*) and Genome Coverage of 59.24x and 129.75x respectively. IVRI KOL CP4 strain is having 4232 and *CM IVRI KOL-1* having 4686 coding genes.

Resistance gene identification: In total, 10 (*AmpE, AmpH, ParE, gyrB, rpoB, rpoC, mdfA, aadA1,oxa-1*,and *catA1*) resistance genes were located on the CM IVRI KOL-1 (CP033158) strain and 7 resistance genes (*AmpH, AmpE, ParE, gyrB, rpoB, rpoC and mdfA*) on IVRI KOL CP4 (CP034253) strain, responsible for resistance to β-lactamase (*AmpE, AmpH,Oxa-1*), quinolones (*ParE, gyrB*), rifampicin (*rpoB*), macrolide (*mdfA*), aminoglycoside (*aad1/2*) and phenicols (*catA1*). Ampicillin (AMP), a β-lactam antibiotics, is widely used to treat of human and livestock *E. coli* infection, and β-lactamase inhibitor susceptibility is affected by the hyperproduction of AmpC/E β-lactamase and some type of OXA β-lactamase (Briñas *et al.* 2002, Juan *et al.* 2005). *Oxa-1* gene hydrolyzes broad-spectrum cephalosporins resulting in reduced susceptibility to penicillin/inhibitor combinations (Ragupathi *et al.* 2020, Sugumar *et al.* 2014). Previous studies (Taylor *et al.* 2018, Poirel *et al. 2018*) reported, that cooccurance of aminoglycoside, and β-lactamase genes, further increase the carbapenem resistance. Another gene, *parE* homologous to the QRDR of the *gyrB* gene, plays a role in the development of quinolone resistance (Gómez *et al.* 2004).

Virulence gene prediction: We detected total 39 virulence genes in IVRI KOL CP4 and 52 in CM IVRI KOL-1 which included the *E. coli* common pilus (ECP), *E. coli* laminin-binding fimbriae (*ELF*), fimbriae (*ELF*), Hemorrhagic pilus (HCP), Type I fimbriae (FimF/G), Hemolysin (hlyE), TTSS effectors (*ESp*), secretion system (T6SS), iron acquisition (*Fec*), glutamate decarboxylase (*gad*), serum resistance (*iss*) and long polar fimbriae (*lpfA*) (Tables 1, 2). Adherence; Autotransporter, Invasion, Secretion system, non-LEE encoded TTSS effectors, hemolysin, and iron uptake were the most putative functional categories for the virulence factors in both strains. As shown in Fig. 1, high prevalence of virulence genes belongs to Adherence, Iron acquisition and secretion system. The first step in bacterial infection is binding to the host cell, which is initiated by adhesin such as fimbriae or pilus (Liu *et al.* 2014). *ecp, elf, cfa, hcp* and *fim* gene were found in both the isolates which could be involved in adherence to mammary epithelial cells. Another important gene *FimH*, is the gene encoding type 1 fimbriae and is important in the establishment of infections. As per the latest research (Ragupathi *et al.* 2020), it was discovered that *FimH* is the major cause for producing mannose-sensitive bacterial adhesion. Further, mannose-

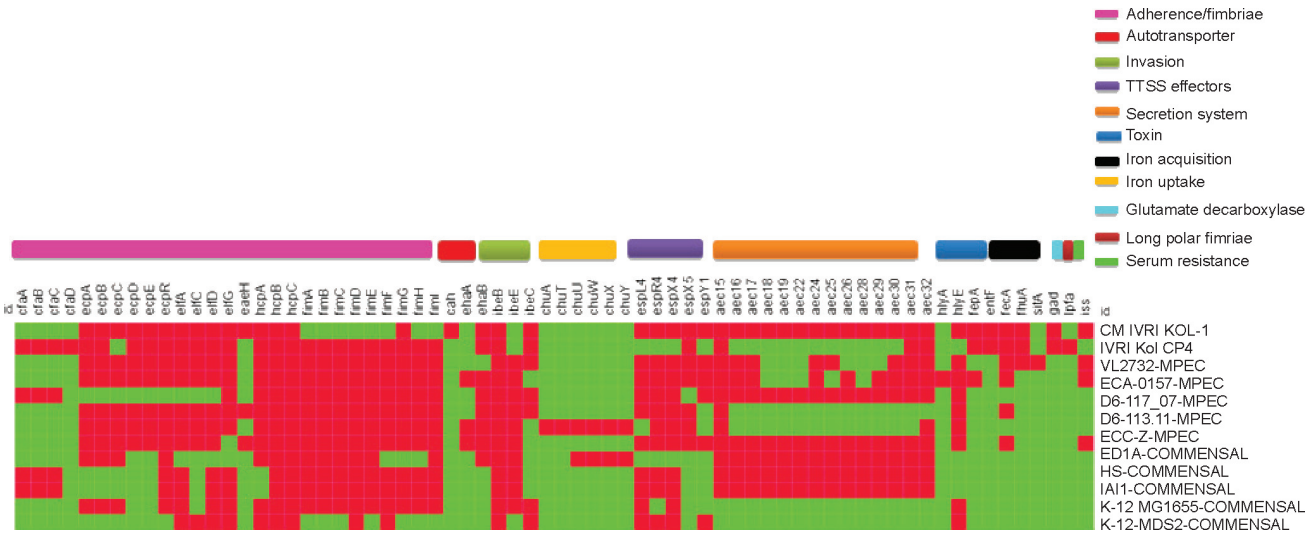

Fig. 1. Schematic diagram of presence/absence of virulence genes in MPEC and other commensal isolates. Pink color circle represents the presence and blue color represents the absence of the virulence gene in all isolates.

Table 1. Virulence gene in IVRI KOL CP4 isolate

VF Class	Virulence factor	Related genes
Adherence	CFA/I fimbriae	cfaA/B/C/D/E
	E. coli common pilus (ECP)	ecpA/B/D/E/R
	E. coli laminin-binding fimbriae	elfA/C/D/G
	Hemorrhagic *E.coli* (ELF) pilus (HCP)	HcpA/B/C
	Type I fimbriae	fimA/B/C/D/E/F/G/H/I
Autotransporter	EhaB	ehaB
	UpaG adhesin	upaG/ehaG
Invasion	Invasion of brain endothelial cells (Ibes)	IbeB/C
Non-LEE encoded TTSS	EspX5	espX5
Secretion system	ACE T6SS	aec15/31/32
Iron acquisition	Fep	fepA
	Ent	entF
	Fec	fecA
	Fhu	fhuA
Glutamate decarboxylase	GAD	gad
Long polar fimbriae	LPF	lpfa

Table 2. Virulence gene in CM IVRI KOL-1 isolate

VF Class	Virulence factor	Related genes
Adherence	*E. coli* common pilus (ECP)	ecpA/B/C/D/E/R
	E. coli laminin-binding fimbriae (ELF)	elfA/C/D/G
	EaeH	eaeH
	Hemorrhagic *E. coli* pilus (HCP)	hcpA/B/C
	Type I fimbriae	fimF/G
Autotransporter	Cah	cah
	EhaB	ehaB
Invasion	Invasion of brain endothelial cells (Ibes)	IbeB/C
Non-LEE encoded TTSS effectors	EspL4	espL4/R4/X1/X5/Y1
Secretion system	ACE T6SS	aec15/16/17/18/ 19/22 24/25/26/27/ 28/29/30/31/32
Toxin	Hemolysin/ cytolysin A	hlyE/clay, fepA,entF
Iron acquisition	FEC	fecA
	FHU	fhuA
Glutamate decarboxylase	GAD	gad
Serum resistance	ISS	ISS

sensitive bacterial adhesion is one of the top candidate gene in the development of vaccine against pathogenic *E. coli* (Tchesnokova *et al.* 2011).

We also found another set of genes (*fec/fhu*) are those involved in iron acquisition system that play important role in providing resistance to host immunological defences. The presence of the *Fec* system impacts the fitness and ability of *E. coli* to grow in milk. *A recent study* (Blum *et al.* 2018) shown that *Fec* system is a prerequisite for pathogenicity of *E. coli* in the mammary gland, without *Fec*, *E. coli* cannot multiply in the mammary gland sufficiently to allow establishment of an actual infection. They observed *fec* locus (*fecIRABCDE*) for ferric dicitrate uptake was present in the MPEC isolates and that it was absent in other dairy farm *E. coli*. Similar type of pattern is found in presence/absence matrix of virulence genes. Fig. 1 represents the presence of fec/fhu genes in all MPEC isolates while absent in other commensal isolates.

T6SS clusters also plays a major role in conveying pathogenicity to the host as evident from the study of Shrivastava and Mande in 2008; which demonstrate very high frequency of T6SS clusters in pathogenic species while absence in non-pathogenic species. T6SS injects effector proteins into both eukaryotic and prokaryotic target cells using a phage-tail-spike-like injectiosome which is required for virulence in several pathogens (Pukatzki *et al.* 2007, Bingle *et al.* 2008, Filloux *et al.* 2008, Chen *et al.* 2015).

Glutamate decarboxylase (*gadA* and *gadB*), participate in the glutamate-dependent acid-resistance system 2, which increases the survival rate of bacteria in acidic regions of mammalian gastrointestinal tracts and is responsible for bacterial infection (Castanie-Cornet and Foster 2001). We also found *lpfA* gene in IVRI KOL CP4 isolate, as mentioned earlier, *lpfA* gene in mastitis isolates might play an important role in improving virulence in the mammary gland by mediating epithelial adhesion. *E. coli* isolates that encode *lpfA* have an increased ability to invade bovine mammary epithelial cells *in vitro* than strains which do not have this gene (Dogan *et al.* 2012). Serum resistance is a common finding in *E. coli* mastitis isolates and a wide range of mastitis strain (16.7% up to 99.5%) being serum-resistant (Sanchez-Carlo *et al.* 1984, Nemeth *et al.* 1994, Kaipainen *et al.* 2002). The part of outer membrane protein encoded by the *iss* gene involved in the anti-complement effect of bacteria, enhancing the serum resistance of *E. coli* and enabling the strain to rapidly proliferate in the host.

Another important secreted virulence factor is a lipoprotein toxin called α-haemolysin (*hlyA*) which is encoded by *hlyA* gene. *hlyA* belongs to a family of proteins called repeat in toxins (RTX) and plays a major role in pathogenic *E. coli* strains (Goebel *et al.* 1988). hly/A/E gene majorly found in pathogenic isolates while absent in others (Fig. 1). Hemolysin E (*HlyE* also known as *ClyA* or *SheA*) is a, pore-forming toxin synthesized by *E. coli* (Reingold *et al.* 1999).

Evolutionary analysis: Pangenome is the total number

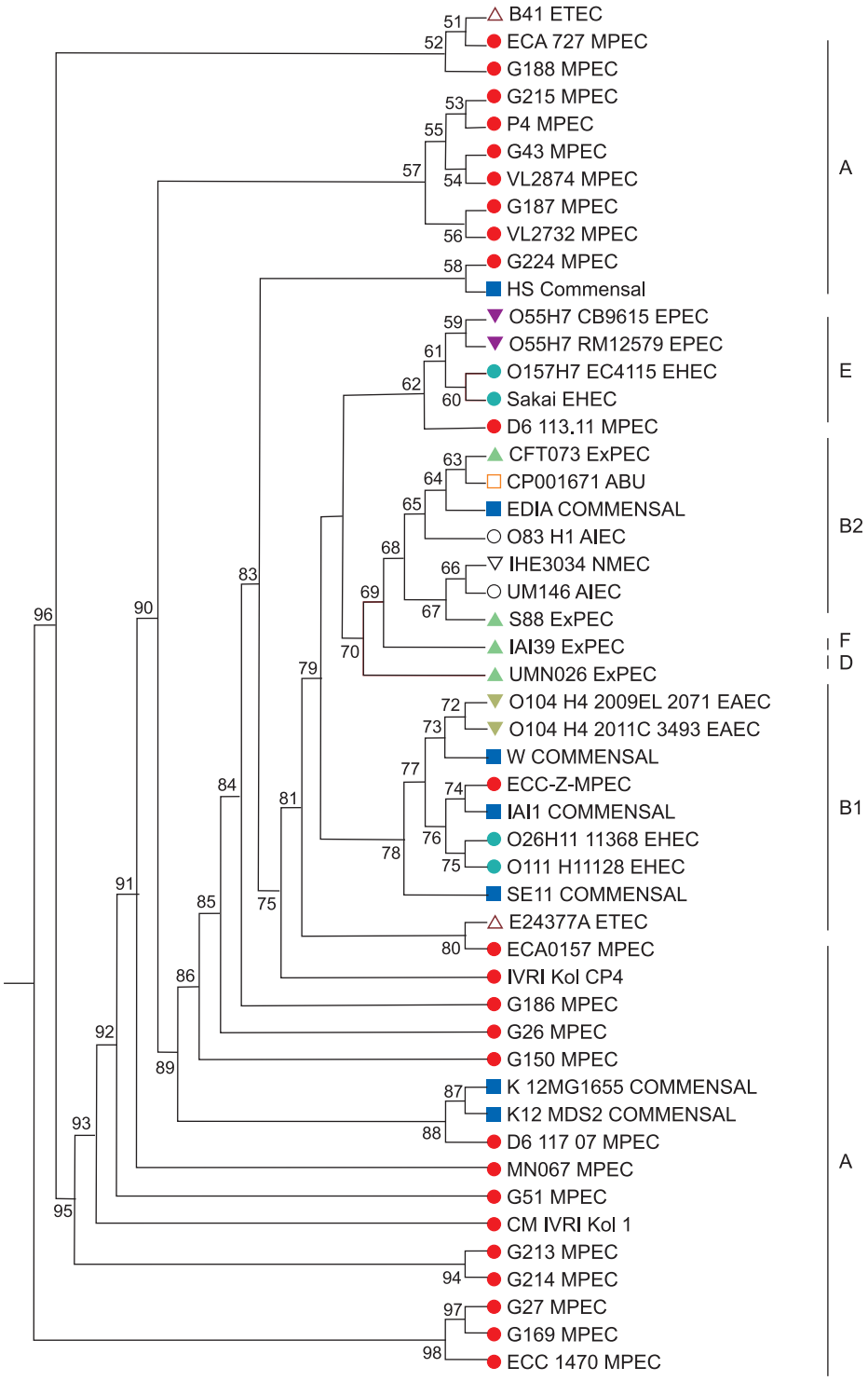

Fig. 2. Phylogenetic tree constructed from the concatenated sequence of 2224 core genes, different pathotypes are represented by different colors and symbols.

of different genes found in all strains within the species (Snipen *et al.* 2009). A pangenome is usually separated into three sets (Rouli *et al.* 2015): the core genome, which includes common genes present in all strains; accessory genome (Medini *et al.* 2005) which include genes present in two or more strains (n-1); and the unique genes, which are specific to a particular strain. As a result, we found a list of core genes shared by 50 *E. coli* strains. A previous study found that *E. coli* pangenome contains 45,000 gene families (Kaas *et al.* 2012). In our analysis, we found a total of 15468 gene families as a pangenome and 2224 core genes common to all 50 strains. The concatenation of the 2224 core genes yielded a 25029 amino acid sequence for each strain. These sequences were further used for phylogenetic analyses. The majority of MPEC isolates fall within phylogroup A (Fig. 2). We reconstructed the phylogenetic

tree using concatenated sequence of the core genes. As shown in the previous study (Richards *et al.* 2015), *E. coli* isolates (n = 50) were clustered into Six major clades, IVRI KOL CP4 and CM IVRI KOL-1 were found to be clustered with other MPEC isolate of phylogroup A. Both of the isolates have suggested to have their origins from commensal isolates. CM-IVRI-KOL-1 isolate is closely related to MPEC strain from Germany (G51) and India (MN067). The Second isolate *IVRI-kol-CP4* is sister group to an isolate of MPEC (ECA0157) and ETEC (E24377A), falling within the *E. coli* phylogroup A. We found that 50% of the isolates fall into phylogroup A. Most of the commensal isolate grouped into phylogroup B1 and B2. All EPEC strains belongs to phylogroup E. Extraintestinal pathogenic *E. coli* (ExPEC) majorly belongs to phylogroup B2, and few belongs to phylogroup D and F.

This is the first whole genome analysis of MPEC strains to be carried out in Indian isolates. We found that high prevalence of mastitis strains belonging to phylogroups A although few isolates were also assigned to phylogenetic groups B1 and B2. Therefore, study of phylo-group A isolates, should be carefully evaluated. Occurrence of β-lactum and antibiotic resistance genes from mastitis causing bacteria which showed resistance to third-generation cephalosporins is also a serious clinical problem. Thus the potential risk of pathogens carrying these resistance genes being transmitted from animals to humans through the food chain cannot be undermined. In conclusion, the present study draws attention to virulence genes belonging to adherence, iron aquision, secretion system, and toxins had an increased risk of being detected in mastitis causing strains and responsible for the pathogenicity of the strains. This type of combination of genetic materials i.e, the Multi Drug resistance and virulence encoding regions in a single Strain is of clinical importance.

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