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Phylogenetic genotyping, virulence genes and antimicrobial susceptibility of *Escherichia coli* isolates from cases of bovine mastitis

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Research Article

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Abstract

The study described in this research communication used phylogenetic genotyping to identify virulence genes and antimicrobial susceptibility in *Escherichia coli* recovered from cases of bovine mastitis. From 385 mastitic milk samples, 30 (7.8%) isolates were confirmed as *E. coli*. Most isolates (80%) belonged to phylo-group A. These 30 *E. coli* isolates were also screened for 11 different virulence genes. The majority of isolates (63%) harbored no virulence gene. Only 11 (37%) isolates tested positive for two virulence genes, either the iron uptake gene *iucD* in 3 (10%) isolates or the serum resistance gene *traT* in 2 (7%) isolates or both *traT* and *iucD* in 6 (20%) isolates. The *E. coli* isolates showed highest susceptibility to gentamicin, meropenem, and pipracillin. Most isolates were resistant to ampicillin, cefotaxime and streptomycin. This study suggests that mastitis causing *E. coli* might originate from commensal bacteria and that the presence of these virulence genes, common in extra-intestinal pathogenic *E. coli* (ExPEC) strains could be attributed to high genetic variability of mastitis-causing *E. coli*.

Escherichia coli are important pathogens causing bovine mastitis, inflammation of mammary gland, in certain environmental conditions (Ali *et al*., 2016; Lan *et al*., 2020; Zhang *et al*., 2018). *E. coli* have been categorized into phylo-groups A, B1, B2, and D (Clermont *et al*., 2000). The occurrence and severity of mastitis depends on the immune response and the genetic makeup of the host (cow factors) and the virulence of the causative bacterial strains. Virulence determinants in *E. coli* include afimbrial adhesins, fimbrial proteins, resistance to the serum complement, outer membrane proteins and cytotoxic necrotizing factor (Kaipainen *et al*., 2002; Cruz-Soto *et al*., 2020). No particular strain, genotype or virulence factor in *E. coli* had yet been commonly associated with bovine mastitis infection (Cruz-Soto *et al*., 2020). The emergence of antimicrobial resistance in mastitis-causing *E. coli* has been reported (Ali *et al*., 2016; Zhang *et al*., 2018). Several studies have been carried out across the world for the characterization of *E. coli* isolated from bovine mastitis. However, such studies are lacking in Peshawar region of Khyber Pakhtunkhwa, Pakistan, therefore, the current study was designed. The aims of this study were to determine any phylogenetic groupings, virulence genes and to determine antimicrobial susceptibility profiles of *E. coli* isolated from cases of bovine mastitis.

Material and methods

Milk samples ($n = 385$) from suspected cases of mastitis were brought to laboratory either by local farmers having 1–3 dairy animals or from small dairy farms (20–50 cattle) of Peshawar and surrounding rural areas. The California mastitis test (CMT) was used to confirm mastitis using recommendations of the kit supplier (Techni. Vet., Inc. USA). Milk samples tested positive for mastitis were processed for the isolation of bacteria and biochemical characterization according to standard protocols (Ali *et al*., 2016).

Genomic DNA was extracted from *E. coli* isolates by boiling water. Triplex PCR was conducted to identify the phylo-groups of *E. coli* on the basis of presence or absence of the *chuA and, yjaA* genes and TspE4.C2 DNA fragments (Clermont *et al*., 2000). The virulence genes intimin (*eae*), shiga toxin producing *E. coli* (STEC) agglutinating adhesion (*saa*), S Fimbriae (*sfa*), cytotoxic necrotizing factors (*cnf*), aerobactin biosynthesis (*iucD*), P fimbriae (*papC*), shiga toxin 1 and 2 (*stx1, stx2*), fimbriae 41 (F41), fimbriae 17-A (*f17A*) and serum resistance gene (*traT*) were detected as described by Paton and Paton (2002), Kaipainen *et al*. (2002), Suojala et al. (2011), Zhang *et al*. (2018) and Cruz-Soto *et al*. (2020).

Antibiotic susceptibility profiles of all the *E. coli* isolates were tested on Muller–Hinton agar (Oxoid™, Thermo Scientific Inc. US) by the Kirby–Bauer disk diffusion method. A panel of 11

different antimicrobial agents (Oxoid™, Thermo Scientific Inc.) was used; ampicillin, amoxicillin with clavulanic acid, cefotaxime, erythromycin, enrofloxacin, gentamicin, kanamycin, meropenem, norfloxacin, pipracillin and streptomycin. Interpretation of the results was done according to the Clinical and Laboratory Standard Institute method (Ali *et al*., 2016).

Results

A total of 30 *E. coli* isolates (7.8%) were recovered from 385 milk samples collected from mastitic cows. The majority of isolated *E. coli* strains belonged to group A ($n = 24$; 80%), followed by two isolates from group B1 (6.7%), group B2 (6.7%) and group D (6.7%). Nineteen isolates (63.3%) did not carry any virulence gene. Only 11 (37%) isolates were positive for either the *traT* gene ($n = 2$; 6.7%) or the *iucD* gene ($n = 3$; 10%) or both ($n = 6$, 20%). None of the isolates contained *eae*, *saa*, *sfa*, *cnf*, *pap*C, *stx*1, *stx*2, F41 or *f*17A.

The lowest antimicrobial susceptibility was against ampicillin (10%), followed by cefotaxime (16.7%), streptomycin (23.3%), amoxicillin plus clavulanic acid (30%), erythromycin (33.4%) and kanamycin (50%). The highest susceptibility of *E. coli* isolates was against meropenem (80%), gentamicin (73.4%), piperacillin (73.3%), norfloxacin (56.7%) and enrofloxacin (53.4%).

Discussion

E. coli associated with bovine mastitis are belonging mainly to the phylogenetic groups A and B1 (Suojala *et al*., 2011; Zhang *et al*., 2018; Cruz-Soto *et al*., 2020). The present study also found that 80% of *E. coli* isolates belonged to group A. Previous studies have assigned the commensal and diarrheagenic strains mainly to group A and B1, whereas extra-intestinal *E. coli* strains were assigned to group B2 and D (Clermont *et al*., 2000). Our results are in agreement with the previous studies in which the majority of the isolates belonged to group A, which belongs to commensal *E. coli* strains.

No particular virulence-associated genes in *E. coli* have so far been linked to mastitis pathogenesis (Cruz-Soto *et al*., 2020). Our results showed no virulence gene in 63% isolates despite testing for 11 genes. Suojala *et al*. (2011) also reported a lack of virulence genes in *E. coli* isolates. The lack of virulence genes in the majority of isolates may indicate that mastitis associated *E. coli* may originate from commensal flora already fin the host's environment (Marashifard *et al*., 2019).

In the present study, only two virulence genes, *traT* and *iucD* were detected and only in 37% of isolates. The genes *iucABCD* produce siderophores that can uptake iron from extra-intestinal niches and concentrate it in bacterial cytoplasm, this ability is fundamental to survive in iron poor sites of the host and iron uptake systems are common in extra-intestinal strains. Avian pathogenic *E. coli* (APEC) and uropathogenic *E. coli* (UPEC) are the two main subsets of extraintestinal pathogenic *E. coli* (ExPEC) that harbor various genes for iron acquisition. The gene *traT* is known for serum resistance, the ability to resist the bactericidal activity of host serum. The *traT* is an outer membrane protein that provides resistance to bacteria against killing action of host serum. This characteristic enables bacteria to survive in body fluids such as urine. The *traT* is also one of the important virulence genes of UPEC isolates. Although the *E. coli* tested probably originated from commensal flora, they contained iron uptake and serum resistance genes that are commonly found in UPEC isolates (subset of ExPEC). This could be due to the heterogeneous genome of mastitis *E. coli* isolates. This was described by Cruz-Soto *et al*. (2020), who suggested bovine mastitis *E. coli* might form a new putative pathotype using the same pathogenic mechanisms as ExPEC. Marashifard *et al*. (2019) questioned if bovine mastitis *E. coli* are commensal in origin or if mastitis *E. coli* resemble ExPEC because of the presence of virulence genes. The grouping could be called mammary pathogenic *E. coli* (MPEC) isolates or, better, mammary associated *E. coli* (MAEC) as the isolates are facultative and opportunistic pathogens from the greatly varying bovine gastrointestinal microbiota.

Antibiotics are frequently used to treat mastitis and their extensive use has given rise to the emergence of antimicrobial resistance in *E. coli* (Ali *et al*., 2016; Lan *et al*., 2020; Zhang *et al*., 2018). This despite the fact that routine use of antimicrobial treatment is not recommended for coliform mastitis (Suojala *et al*., 2011). Our study identified the highest resistance in *E. coli* isolates against ampicillin, followed by cefotaxime and streptomycin. This is consistent with the high resistance to ampicillin reported by Zhang *et al*. (2018) and to both ampicillin and streptomycin by Suojala *et al*. (2011).

In conclusion, this study suggests that the *E. coli* isolated from mastitis cases may have originated from commensal flora. Only *traT* and *iucD* genes were detected in *E. coli* isolates, which are reported to be commonly harbored by ExPEC strains. The virulence genes detected in *E. coli* in this study could be an attribute of high genetic variability of *E. coli*.

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