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Genotypic detection of virulence factors from different types of *E. coli* isolated from diarrheic Camel Calves in Sudan

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Abstract

The study conducted to isolate and characterized *Escherichia coli (E.coli)* associated with diarrhea in camel calves in Sudan. Conventional bacteriological methods, Api 20 E. strips were used for primary identification of *E. coli* and polymerase chain reaction technique was used for molecular characterization. Two hundred fecal samples taken from calve camels suffering diarrhea were collected from Gezira, West Kordofan and North Kordofan States. The collected samples subjected to bacteriological examination for isolation and identification of *E. coli*. Sixty E. *coli* were isolated and characterized (30%). The characterized *E. coli* were tested for the presence of virulence genes using multiplex PCR for fimbriae F41, F5, *eae*, heat-labile (LT) and heat-stable (STa), enterotoxins Stx1, saa and hly.

72 virulent genes were detected from 60 isolates carried genes for at least one of the virulence factors tested. The gene encoding for eae was the most prevalent 20(27.78%) followed by those encoding for stx 15 (20.83%), f5 14(19.44%), 41 7(9.74%), LT 6(8.34%), sta 5(6.94%), saa 4(5.55%) and hly 1(1.38%). Different pathogenic *E.coli* from camel calves were detected containing various pathogroup this indicated the important role of *E.coli* in contamination of environment. The study concluded that, camel calves considered as a reservoir for many pathogens carrying resistance genes as *E. coli*.

Keywords: *E.coli*, diarrhea, PCR, resistance genes, camel calves

Introduction

Escherichia coli (*E.coli*) is a bacterial strain lives as commensal in the digestive tract of humans and warm blooded animals. Nevertheless some strains have evolved the capability to cause both intestinal and extra intestinal illnesses [1, 2]. *E.coli* strains are genetically diverse species that includes many pathotypes, both intestinal and extra intestinal, most of which own specialized mechanisms characterized by their high efficiency in both colonization and pathogenicity. The appearance of different bacterial pathotypes is mainly due to horizontal transfer and exchange of genes responsible for virulence [3].

The different pathogenic *E. coli* strains are characterized by particular subsets of genes associated with the virulence, identifying distinct groups or pathogroups pathogenic forms of *E. coli* that can cause a variety of diarrheal diseases in hosts due to the presence of specific colonization factors, virulence factors and pathogenicity associated genes, which are generally not present in other *E. coli*. Of the strains that cause diarrheal diseases, six pathotypes are now recognized. These pathotypes are: verocytotoxigenic *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) [1]. All humans and animals carry *E. coli* in their intestines; they are Gram-negative, rod-shaped, flagellated, nonsporulating and facultative anaerobic bacteria which belonging to the family *Enterobacteriaceae* and are usually harmless [4]. Pathogenic *E. coli* strains are common agents responsible for a variety of intestinal disorders, diarrhea remains an important cause of morbidity and mortality in livestock and is one of the most common diseases of camel calf worldwide [5]. Pathogenic *E. coli* is associated with many disease conditions including the enteric involvements in both animals and humans [6].

Materials and Methods

Collection of samples

Two hundred rectal swabs from clinical cases associated with diarrhea were collected from camel calves with ages up to one year. The samples collected from Gezira State (*n*=119), West Kordofan (*n*=48) and North Kordofan State (*n*=33). They were collected using sterile cotton swabs that placed into tubes containing Stuart medium and immediately placed into ice- box containing ice and transported to the Central Veterinary Research Laboratory, Department of Bacteriology for bacteriological analysis.

Isolation and identification of isolates

Nutrient agar, blood agar, MacConkey's agar and Eyothene methylene blue (EMB) agar were used for *E. coli* isolation and differentiation. All samples were cultured and incubated at 37˚C for 24 hours. Primary identification of the isolates were identified as *E. coli* by standard conventional primary and secondary biochemical tests (Gram stain, oxidase, Indole, methyl red, Voges-Proskauer and citrate utilization

tests). For more confirmation, API 20 E kits were used for identification of *E. coli* according to manufacturer instructions.

Genotypic characterization of virulent factors of *E. coli* strains using multiplex PCR

Multiplex PCR was used for characterization of virulent factors of isolated *E. coli* according to **--**

Extraction of DNA

Each isolate grown heavily onto nutrient agar, incubated at 37°. The growth was harvested using 3ml normal saline C for 24 hours. Bacterial DNA was obtained by centrifugation of samples at 13000 RPM for 15 min, and the supernatant was discarded, the pelleted cells boiled at 100ºC for 10 min after addition of 1.5 ml of deionized water and then cooled immediately.

Multiplex PCR: **Polymerase chain reaction**

Specific primers from Mytacg Bioscience Enterprise (Malaysia) were used to amplify the STX1, STA, LT, EAE, F5, F41, SAA and HLY, genes as shown in Table 1. The multiplex PCR assay was carried out according to Franck SM [8], for work1(STX1, STA, EAE, F5, F41) in a total volume of 20 µL of mixture containing 2 µL Maxime PCR containing 1X PCR buffer, 2 µM MgCl, 0.5 µM dNTP, 0.25 µM Taq DNA polymerase, 2.5 µL of each of the virulence gene-specific primers, forward and reverse primers for each gene (a total of 12 .5 µL for the 5 target genes), 2 µL of template DNA and 12.8 µL of deionized water. The amplification conditions included three steps: heating at 94°C for 5 min; 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 45 s, extension at 70°C for 1min; and final extension at 70°C for 10 min. in work 2(saa, sta and hly), the same protocol was applied with increasing of the number of cycle to 35 and annealing at 62°C instead of 50°C

Table (1) shows primers used for multiplex PCR, target gene primer sequences, size of product (bp). All isolates were examined for the presence of the stx1, stx2, sta, f5, f41 and eae genes by multiplex PCR (work1) as described by Franck *et al* [8]. Then multiplex PCR (work 2) for the presence of lt, saa and hly genes.

Table 1: primers used for multiplex

Target gene	Primer sequences	bp
stx1	STx1-F TTCGCTCTGCAATAGGTA Tx1-R TTCCCCAGTTCAATGTAAGAT	55
sta	STa-F GCTAATGTTGGCAATTTTTATTTCTGTA STa-R AGGATTACAACAAAGTTCACAGCAGTAA	190
it	LT-F ATTTACGGCGTTACTATCCTC LT-R TTTTGGTCTCGGTCAGATATG	281
eae	EAE-F ATATCCGTTTTAATGGCTATCT EAE-R AATCTTCTGCGTACTGTGTTCA	425
f5	F5-F TATTATCTTAGGTGGTATGG F5-R GGTATCCTTTAGCAGCAGTATTTC	314
f41	F41-F GCATCAGCGGCAGTATCT F41-R GTCCCTAGCTCAGTATTATCACCT	380
Saa	SAA-F CGTGATGAACAGGCTATTGC SAA-R ATGGACATGCCTGTGGCAAC	119
ehxA	Hly-F GGTGCAGCAGAAAAAGTTGTAG Hly-R TCTCGCCTGATAGTGTTTGGTA	1,551

Results

72 genes were detected from 60 isolates of *E. coil* as shown in Table 2

Table 2: shows the number of positive samples and each percentage from total.

Pathogenic genes	Positive samples	percentage
NZ	20	2738%
six1	15	20.83%
F5	14	19.44%
F41	7	9.74%
It	6	8.34%
a	5	634%
Wig	4	5.55%
biy,	1	138%
Total		100%

12 isolates of *E. coli* contain more than one genes (8 contain 3 genes +4 contain 2 genes) and S contain no genes.

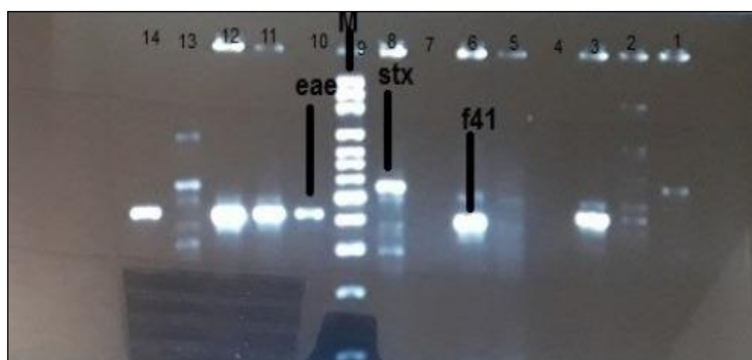

Fig 1: Agrose gel electrophoresis of multiplex PCR product: 4= negative sample 6= positive f41 gene, 8= positive stx gene, 9= 100 bp ladder, 10= positive eae gene.

Fig 2: Agrose gel electrophoresis of multiplex pcr prodect: 1=100 bp ladder, 2, 3 = negative samples, 6= positive eae gene, 8= positive f5 gene, 9= positive stx gene, 14= positive sta gene.

Fig 3: Agrose gel elctrophorsis of multiplex pcr product M= 100 bp ladder, 4= positive hly gene

Fig 4: Agrose gel electrophoresis of multiplex pcr 1: 100 pb ladder, 5, 6= positive it gene 7, 8= positive saa gene.

Discussion

E. coli is part of the natural microflora of the digestive tract of human and animals but certain strains have evolved the capability to cause a wide range of diseases [1, 2]. Pathogenic *E. coli* strains are common agents responsible for a variety of intestinal disorders like diarrhea that remains an important cause of morbidity and mortality in livestock [5].

The bacterium can effectively survive either in the host or in the environment where it released with human sewage and animal excreta. Pathogenic *E. coli* with a zoonotic origin, such as Shiga toxin producing *E. coli* (STEC), can also be released in the environment with the discharges from slaughterhouses [2] The previous paragraphs boost the importance of this study.

STEC are characterised by the production of cytotoxins that disrupt protein synthesis within host cells [1]. In this study, 40 (55.55%) of virulent factor producing Shiga toxins (STEC) identified from various serogroups of *E. coli* and this is very serious as reported by European Centre for Disease Prevention and Control [9] that STEC may cause great damage from uncomplicated diarrhea to hemorrhagic colitis, which can progress into hemolytic uremic syndrome (HUS), composed of micro-angiopathic hemolytic anemia, thrombocytopenia and severe acute renal failure. Also, of STEC strains considered to be highly virulent for humans that has the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded on a pathogenicity island termed the locus for enterocyte effacement (LEE). LEE encodes a type III secretion system and *E. coli* secreted proteins, which deliver effector molecules to the host cell and disrupt the host cytoskeleton (4, 5, 20). LEE also carries eae, which encodes an outer membrane protein (intimin) required for intimate attachment to epithelial cells (22); eae has been used as a convenient diagnostic marker for LEE positive

STEC strains [10].

In this study, stx gene represent high percentage 15(20.83%), this finding was in line with Hassan who showed that highest percentage was reported for Stx virulence genes in *E.coli* subtypes isolated from ruminant meat samples [11].

The present study also showed that Enterohaemorrhagic *E. coli* (EHEC) has hly gene which is a subset of STEC that detected in one isolate. Although it was considered to be human pathogens, this may encourage new studies to search the incidence of this gene in different type of animals [1]

Enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) are the main categories of diarrhoeagenic *E. coli* which was detected in this study and appeared as similar to enteric infections in pigs [12]. ETEC is defined as a pathogen containing *E. coli* isolates that elaborate at least one member of two defined groups of enterotoxins, namely heat-labile (LT) and heatstable enterotoxins (STs) [12, 14]. Detection of ETEC in this study was in agreement with Salih [13] who detected sta, lt and f41, this confirmed that the main virulence factors associated with ETEC in diarrhoea are enterotoxins and fimbrial adhesins. Fimbrial adhesins mediate the attachment of bacteria to the surface of the intestinal epithelium cells, thus allowing bacterial colonization. Both heat-labile (LT) and heat-stable (sta and stb) enterotoxins cause an imbalance in intestinal homeostasis, causing hypersecretion of fluids that results in diarrhea [5].

Most ETEC isolated from diarrheic camel calves in this study produce fimbriae genes (f5, f41), this similar in pigs in which ETEC can produce one or more of the following fimbriae: F4 (K88), F5 (K99), F6 (987P), F17, F18 and F41 [15, 16, 17, 18, 19]. Fimbriae are surface proteins that are responsible for adhesion of pathogenic bacteria to intestinal epithelial cells [19]. In addition to ETEC and STEC, strains inducing attaching and effacing lesions similar to those produced by enteropathogenic *E. coli* (EPEC) in humans, have also been associated with diarrhea in pigs [20]. Also, eae gene represents the highest percentage, this gene belong to attaching and effacing *E. coli* (AEEC) which possess an outer membrane protein termed intimin, that involved in attachment of the bacteria to enterocytes [21, 22].

Conclusions

The high number of STEC strains isolated from diarrheic dromedary camel calves in this study implied that these animals are an important reservoir of STEC strains that are potentially pathogenic for other farm animals and humans.

The findings of this study provide further evidence that pathogenic *E. coli* of zoonotic origin can contaminate the environment as a result of the discharge of untreated abattoir. Additionally, the lack of enforcement of good hygiene practices may ease the release and persistence of multiple pathogenic *E. coli* strains in the abattoir environment, including those belonging to human-borne pathogroups, making such a setting a unique favorable environment for bacteria to bacteria interaction and exchange of genetic material possibly leading to the emergence of new pathogenic strains with new virulence features. Enterohaemorrhagic *E. coli* (EHEC) was isolated in only one sample, this may indicated that it could be a human pathogens.

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