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The Burden of *Escherichia coli* Pathotypes among Diarrheic Farm Animals: A Possible Zoonotic Relevance

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Abstract

Pathogenic strains of *Escherichia coli* possess virulence factors that contribute to both intestinal and extraintestinal infections in both humans and animals. Farm animals can serve as a potential source for these types of *E. coli* strains. This study aimed to determine the virulence genes and related pathotypes of *E. coli* isolated from diarrheic farm animals and their public health importance. Rectal swabs were collected from 175 diarrheic farm animals (49 cattle, 69 sheep, and 57 goats). Samples were prepared for isolation of *E. coli* through enrichment in tryptic soya broth and then plating on Eosin methylene blue agar, whereas the identification of *E. coli* was performed based on colony morphology, biochemical tests, and molecular confirmation by PCR. Furthermore, the determination of the virulence factors associated with *E. coli* pathotypes was done by molecular technique to amplify the virulence genes including adhesins (*sfa*, *papC*, *sepA*, *etrA*, *aer*, *feaG*, *fsaA*, and *eaeA*), capsule synthesis (*rfc*), and toxins (*cnf1*, *hlyA*, *eltA*, *estA*, *exhA*, *stx1*, and *stx2*). Moreover, phylogenetic analysis was done via sequencing of the 16s rRNA genes from the strains that carry virulence genes, as well as the statistical analysis was done through the production of the hierarchically clustered heat map. Pathogenic *E. coli* was found in 39.4% of the examined animals. Fifteen out of sixteen virulence genes were detected among *E. coli* isolates from different farm animals, including cattle, sheep, and goats. ExPEC pathotype was predominated among cattle and sheep isolates whereas, ETEC pathotype is more frequent among goat isolates. The sequence analysis of 16s rRNA sequences revealed similarity between farm animal isolates and those from humans that were retrieved from GenBank. In conclusion, this study highlights the potential role of diarrheic farm animals in the epidemiology of pathogenic *E. coli* pathotypes which may have public health implications.

KEYWORDS
Extraintestinal pathogenic *E. coli*, *E. coli* pathotypes, Pathogenic *Escherichia coli*, Virulence factors.

INTRODUCTION

Escherichia coli is a bacterium that can cause various diseases in humans and animals. Some *E. coli* strains are harmless and live in the intestinal tract, but others may be harmful (Kaper *et al*., 2004). Pathogenic *E. coli* are classified according to their pathogenicity into two main categories: intestinal pathogenic *E. coli* (IPEC), which causes a wide array of diarrheal illnesses, and extraintestinal pathogenic *E. coli* (ExPEC), which causes infections outside the gastrointestinal tract (Sarowska *et al*., 2019).

IPEC comprises enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enteroaggregative (EAEC), and diffusely adherent (DAEC) *E. coli* whereas; ExPEC includes neonatal meningitis (NMEC), sepsis-associated (SEPEC), uropathogenic (UPEC), (Croxen *et al*., 2013). ExPEC may induce metastatic infections to affect various organs causing serious systemic illnesses such as urinary tract infections, septicemia, pneumonia, and meningitis specifically in newborn children and animals (Croxen *et al*., 2013, Santos *et al*., 2020).

The Pathogenic *E. coli* strains can produce various virulence factors such as toxins, fimbriae, and intimin that damage or kill host cells, facilitate bacterial attachment, and mediate intimate adherence, respectively, which are encoded by mobile genetic elements (Sarowska *et al*., 2019). The virulence genes that are associated with different types of virulence factors include adhesins (*sfa*, *papC*, *sepA*, *etrA*, *aer*, *feaG*, *fsaA*, and *eaeA*), capsule synthesis (*rfc*), and toxins (*cnf1*, *hlyA*, *eltA*, *estA*, *exhA*, *stx1*, and *stx2*) (Alabsi *et al*., 2014). These virulence factors varied depending on the type and site of infection, and *E. coli* strains that have more virulence factors are usually more pathogenic and cause more severe infections. (Gebisa *et al*., 2019).

The investigation of these virulence factors and their corresponding encoding genes provides valuable insights into how they interact with pathotypes, enabling the classification of *E. coli* strains into different pathotypes (Zhao *et al*., 2021).

Some of the pathotypes of *E. coli* that cause different diseases in humans are EPEC, which expresses intimin (*eaeA*) and enterohemolysin (*exhA*) genes to cause attaching and effacing lesions on intestinal epithelial cells; ETEC, which produces heat-labile (*eltA*) and heat-stable (*estA*) enterotoxins and fimbrial adhesins (*faeG* and *fasA*) to cause diarrhea; EAEC, which forms aggregative adherence fimbriae (AAF) and expresses a component of ETT2 type III secretion system (*etrA*) gene to cause persistent diarrhea; EIEC, which expresses *aer*obactin (*aer*) gene to invade intestinal epithelial cells and cause dysentery; EHEC, which produces Shiga toxins (*stx1* and *stx2*) and expresses intimin (*eaeA*) and enterohe-

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molysin (*exhA*) genes to cause hemorrhagic colitis and hemolytic uremic syndrome (Pakbin *et al*., 2021).

Furthermore, ExPEC expresses P fimbriae (*papC*), S fimbriae (*sfa*), cytotoxic necrotizing factor 1 (*cnf1*), alpha-hemolysin (*hlyA*), lipopolysaccharide synthesis (*rfc*), and secreted serine protease of the auto-transporter family (*sepA*) genes to cause urinary tract infections, septicemia, meningitis, and other infections (Denamur *et al*., 2021).

Certain pathogenic forms of *E. coli* that lead to intestinal illnesses in animals are Shiga toxin-producing *E. coli* (STEC). These types of bacteria, which can be transmitted between animals and humans, are responsible for generating Shiga toxins *Stx1* and *Stx2*, that block protein synthesis and cause cell death. STEC can also cause serious complications in humans, such as hemolytic uremic syndrome, the pathogenicity, and severity of the infection in humans depend on the types of virulence genes related to this pathotype (Kim *et al*., 2020). Additionally, ETEC produces adhesins and enterotoxins that induce fluid secretion and diarrhea in animals (Melton-Celsa, 2014).

Cattle and other ruminants are the main reservoirs of zoonotic STEC, which can be transmitted to humans through contaminated food, water, or direct contact with infected animals or their feces (Dubreuil *et al*., 2016). Different *E. coli* pathotypes, such as ExPEC, ETEC, and EHEC can infect farm animals and some studies have examined the epidemiology and pathogenesis of these infections (Dubreuil *et al*., 2016, Denamur *et al*., 2021).

Therefore, the current study was carried out to shed more light on the burden of *E. coli* pathotypes and their virulence factors among diarrheic farm animals for better control of such pathogens.

MATERIALS AND METHODS

Ethical approval

This study was done according to the guidelines of the ethical committee in the Faculty of Veterinary Medicine, Cairo University, Egypt, and approved by the Institutional Animal Care and use committee, Vet Cu 12/10/2021/357.

Sampling

Rectal swabs were collected from 175 farm animals suffering from diarrhea, cattle (n.= 49), sheep (n.= 69), and goats (n.= 57). The samples were obtained from farms and veterinary clinics in the Giza governorate of Egypt.

Isolation and identification of E. coli

Each sample's rectal swab was placed into a sterile tube containing tryptic soya broth and incubated overnight at 37°C, after enrichment a loopful of the broth was streaked onto Eosin methylene blue agar (EMB) and incubated at 37°C for 24-48 hrs. Colonies with a metallic green color were picked from the plate and subcultured into tryptic soya broth. The suspected colonies were confirmed as *E. coli* by conventional biochemical tests.

DNA extraction

A boiling method was used to extract DNA from the obtained isolates; 2 ml of the enriched tryptic soya broth were centrifuged at 13,000 rpm for 10 min. The supernatant was poured out and 200 ul of nuclease-free water was added to the pellet. The dissolved pellets were heated at 100°C for 10 min, and subsequently centrifuged at 13,000 rpm for 5 min; the supernatants were used as DNA templates in all PCR (Ragheb *et al*., 2020).

Molecular identification of E. coli

For molecular identification of *E. coli*, primers for the 16S rRNA gene of *E. coli* were selected according to Wang *et al*. (2002). The PCR mixtures were performed according to Fahim *et al*. (2019). The amplified fragments from three isolates from cattle, sheep, and goat were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions and sequenced using the forward and reverse primers of 16S rRNA gene of *E. coli*. Sequences in this study were compared with sequences available in the NCBI database using BLAST analysis.

Table 1. Annealing temperatures of primers used, and amplicons size.

Virulence factors	Primer sequence (5–3)		Annealing Temperature (°C)	Amplicon sizes (bp)
	Forward	Reverse		
sfa	CTCCGGAGAACTGGTCATCTTAC	CGGAGGAGTAATTACAAACCTGGCA	64	410
cnf1	AAGATGGAGTTTCCTATGCAGGAG	CATTCAGAGTCCTGCCCTCATTATT	63	498
papC	GTGGCAGTATGAGTAATGACCGTTA	ATATCCTTTCTGCAGGGATGCAATA	64	200
hylA	AACAAGGATAAGCACTGTTCTGGC	ACCATATAAGCGGTCATTCCCGTCA	63	1177
rfc	ATCCATCAGGAGGGGACTGGA	AACCATACCAATGCGAG	63	788
sepA	TAAAACCCGCCGCCTGAGTA	TGCCGGTGAACAGGAGGTTT	62	611
etrA	CTTCTTCCTAACGAAACTATCATTA	TGACATATCAACTTTCTCTTACGC	55	913
aer	TACCGGATTGTCATATGCAGACCGT	AATATCTTCCTCCAGTCGGAGAAG	60	602
faeG	GAATCTGTCCGAGAATATC	GTTGGTACAGGTCTTAATGG	55	499
fasA	GTAACTCCACCGTTGTATC	AAGTTACTGCCAGTCTATGC	62	409
eltA	GGCGTTACTATCCTCTCTAT	TGGTCTCGGTCAGATATGT	55	272
estA	CAACTGAATCACTTGACTCTT	TTAATAACATCCAGCACAGG	55	158
eaeA	GACCCGGCACAAGCATAAGC	CCACCTGCAGCAACAAGAGG	63	384
exhA	GCATCATCAAGCGTACGTTCG	AATGAGCCAAGCTGGTTAAGCT	63	534
stx1	TGTCGCATAGTGGAACCTCA	TGCGCACTGAGAAGAAGAGA	58	655
stx2	CCATGACAACGGACAGCAGTT	TGTCGCCCGATTATCTGACATTC	58	477

Determination of the virulence genes of E. coli

Uniplex PCR was used to detect 16 virulence genes associated with *E. coli* pathotypes, which included adhesins (*sfa*, *papC*, *sepA*, *etrA*, *aer*, *feaG*, *fsaA*, and *eaeA*), capsule synthesis (*rfc*), and toxins (*cnf1*, *hlyA*, *eltA*, *estA*, *exhA*, *stx1*, and *stx2*). The sequences and annealing temperatures for each primer set are shown in Table 1. The reaction was performed according to Zhao *et al.* (2021). The PCR cycling conditions were 94°C for 5 min and 30 cycles of 95°C for 30s, annealing temperature for 30s, and 72°C for 30s, followed by a final extension at 72°C for 10 min.

Phylogenetic analysis

The 16s rRNA gene sequences in this study were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers OR069350, OR400940, and OR069352 for sheep, goat, and cattle, respectively.

Using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/), the gene sequences were aligned with the publicly available sequences from NCBI GenBank. Multiple sequence alignments and sequence similarities were calculated using the CLUSTALW 1.8® program in BioEdit version (v. 7.0.9.0). The Phylogenetic analysis was conducted with MEGA X software using the Maximum Likelihood method with 1000 bootstrap replicates. The analysis involved genes from animal isolates and human samples retrieved from GenBank, resulting in a phylogenetic tree.

Statistical analyses

The hierarchically clustered heat map was produced by R (Version 3.6.1, R Foundation for Statistical Computing) using "pheatmap" package.

RESULTS

Out of 175 examined diarrheic farm animals, 69 yielded pathogenic *E. coli* with an overall prevalence of 39.4%. The occurrence of pathogenic *E. coli* in different animal species was displayed in Table 2.

Fifteen out of sixteen virulence genes were detected among the obtained isolates, the most frequent virulence gene among different pathotypes was *eaeA* followed by *etrA*, and *papC* (Fig 1) while none of isolates yielded *faeG* gene.

Most of the *E. coli* isolates from cattle and sheep were classified as ExPEC pathotype but ETEC is the frequent one among goat isolates. In addition, EHEC, EIEC, and EAEC pathotypes were also detected (Fig 2). Moreover, most of the *E. coli* isolates carried the combinations of virulence genes, associated with ExPEC and ETEC pathotypes (Table 2, Fig 2). A phylogenetic tree based on 16s rRNA genes obtained from farm animals and those retrieved from GenBank highlighted the public health threat posed by such strains (Fig 3).

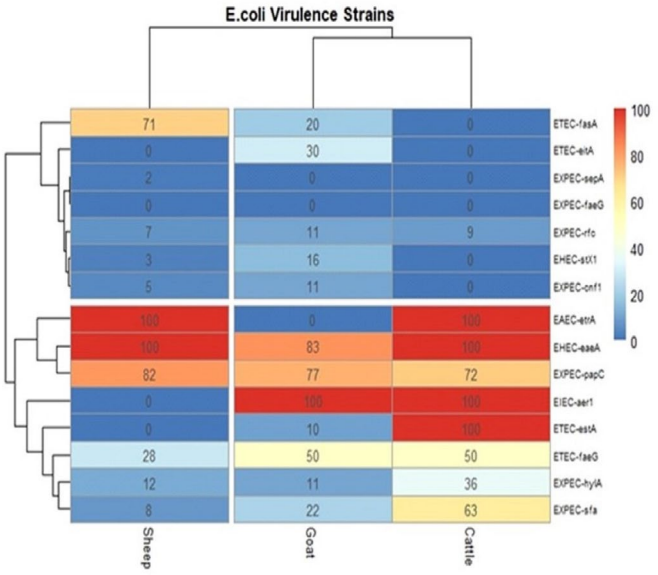

Fig 1. The hierarchical heat map displayed the distribution of virulence genes among different pathotypes in different farm animal species (expressed as a percentage).

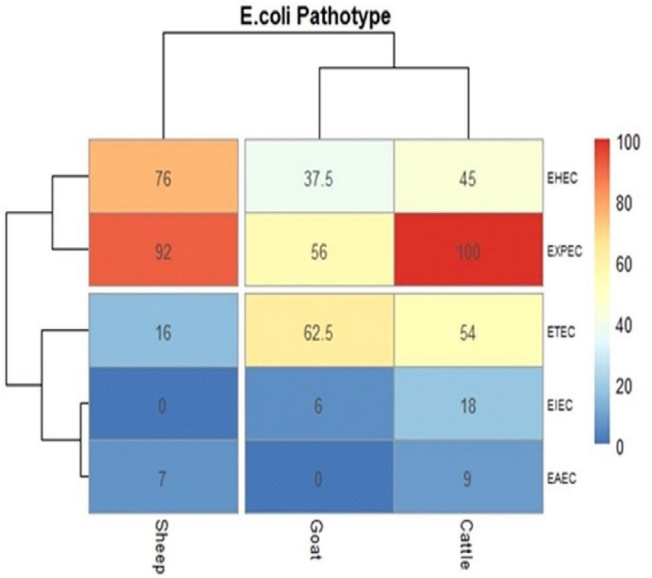

Fig 2. The hierarchical heat map demonstrated the distribution of different pathotypes among different animal species. data presented in the heat map are percentages of different pathotypes.

DISCUSSION

Pathogenic *E. coli* isolates from animal sources, animal products, and human infections have been increasing globally (Dorado-García *et al*., 2018; Wang *et al*., 2020, Seleem *et al*., 2021).

In this study, a total of 69 (39.4%) pathogenic *E. coli* strains were isolated from 175 diarrheic farm animals, of which, 11 (22.4%), 42 (60.9%), and 16 (28%) were isolated from cattle, sheep, and goat, respectively. However, there are limited data

Table 2. Occurrence of pathogenic *E. coli* and mixed pathotypes among different farm animals.

Host	No. of examined animals	No. of pathogenic *E. coli* (%)	No. of isolates contain mixed pathotype (%)
Cattle	49	11 (22.4%)	11 (22)
Sheep	69	42 (60.9%)	33 (47)
Goat	57	16 (28%)	10 (17)
Total	175	69 (39.4%)	54 (30)

about the prevalence of different pathogenic E coli pathotypes among farm animals, some studies have found different rates of *E. coli* O157 in fecal samples. For instance, Bosilevac *et al.* (2015) detected *E. coli* O157:H7 in 10.7% of cattle feces, while Al-Ajmi *et al.* (2020) found *E. coli* O157 in 2% of goat feces and 16% of cattle feces, but not in sheep feces. These variations may be influenced by the methods of sampling and detection, as well as the geographic regions of these studies.

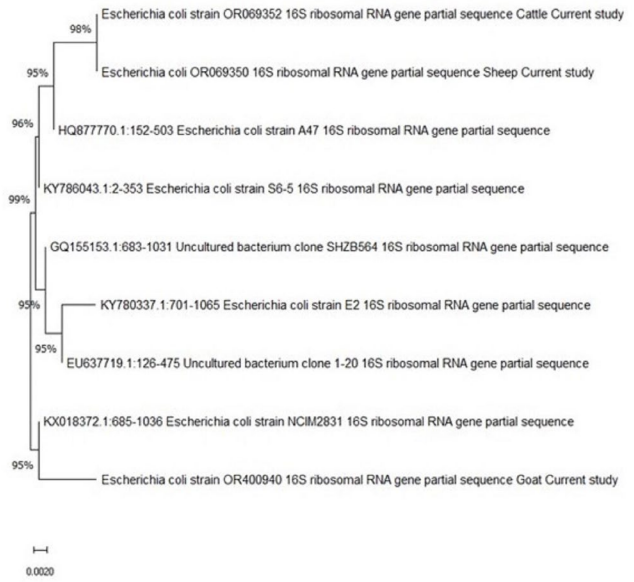

Fig 3: Phylogenetic relationships based on the 16s rRNA region sequences of selected isolates The trees were constructed and analyzed by the Maximum Likelihood with 1000 bootstrapping.

E. coli strains can carry genes that encode toxins or virulence factors that can cause intestinal or extra-intestinal infections. The genetic diversity of *E. coli* strains can show their evolutionary origins, phylogenetic relationships, and potential risks of transmission to humans (Denamur *et al*., 2020, Muloi *et al*., 2022).

The virulence genes of *E. coli* are the key factor for causing disease, a matter which has attracted a lot of research interest. In this study, fifteen out of sixteen virulence genes were identified in *E. coli* isolates, indicating that diarrheic farm animals may harbor pathogenic *E. coli* strains with abundant and diverse virulence factors, which are a significant public health threat since such strains could be transmitted to humans via direct contact or the food chain (Madec *et al*., 2015).

The most common genes among different pathotypes in this study were *eaeA*, *etrA*, and *papC*, while none of the animal isolates had the *faeG* gene. Such observations differ from those described in previous reports of *E. coli* isolates of animal origin (Lupindu 2017, Zhao *et al*., 2021). Noteworthy, the *eaeA* and *etrA*, and *papC* genes may play a major role in adherence/colonization, causing attaching/effacing lesions and cause intestinal and/or extra-intestinal diseases among humans (Rehman *et al*., 2017). The emergence of virulence gene patterns observed in the current study might be attributed to horizontal gene transfer between related or unrelated bacterial species, mediating the transfer of virulence factors (Sarowska *et al*., 2019).

The present study showed that a significant proportion of *E. coli* from cattle (100%), sheep (92%), and goats (56%) were ExPEC pathotypes, which are known to cause extraintestinal infections among humans. However, among goats, ETEC (62.5%) was the most common pathotype, which is associated with enterotoxigenic diarrhea.

Moreover, this study also found that some *E. coli* isolates were related to EHEC, EIEC, and EAEC pathotypes, which can cause hemorrhagic colitis, dysentery, and persistent diarrhea, respectively. This is consistent with previous studies identifying EAEC and EPEC pathotypes to have emerged as an increasingly important cause of diarrhea worldwide (Bolick *et al*., 2013; Hernandes *et al*., 2020; Ranganathan *et al*., 2020). These findings significantly enhance our understanding of *E. coli* pathotypes' dynamics and their potential to cause infections in both humans and animals.

Seriously, most of the obtained isolates belonged to more than one pathotype. This can be explained by the frequent gains and losses of genes resulting in the diversification of new strains, and sometimes "hybrid" pathotypes. Thus, the characterization of strains with combinations of virulence traits enables defining their pathogenic potential (Tozzoli *et al*., 2014; Stanford *et al*., 2017; Lindstedt *et al*., 2018).

Pathogenic *E. coli* with its virulence genes in diarrheic farm animals pose a serious threat to public health. They can spread from animals to humans through fecal contamination of food, water, and soil, or direct contact. This can lead to human infections and transmission within the community (Zhao *et al*., 2021). The construction and analysis of a phylogenetic tree using 16s rRNA genes from both farm animal isolates and human isolates retrieved from GenBank is a crucial step in assessing the zoonotic potential of specific strains. The analysis of the sequences revealed similarity between farm animal isolates and those from humans and this provides valuable insights into the genetic relatedness and evolutionary relationships between different isolates, shedding light on the potential for *E. coli* transmission between animals and humans.

CONCLUSION

This study underlined the occurrence of pathogenic *E. coli* strains carrying several virulence factors among diarrheic farm animals with a potential zoonotic risk. Special preventive measures must be taken for monitoring diarrheic farm animals to tackle such pathogens.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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