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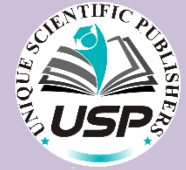

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Phenotypic and Genotypic Characterization of *Escherichia coli* Isolates Recovered from Equines with Respiratory Problems in Egypt

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ABSTRACT

The gradual increase of antibiotic resistance of *E. coli* has become alarming to public health; moreover, isolates have virulence genes affecting the severity of infections. Sequencing virulence genes and studying their phylogenic relations are effective for infection control. The current study aimed to determine the prevalence of *E. coli* isolates from horses suffering from respiratory manifestation and their antimicrobial resistance, virulence determinants, biofilm production, and phylogeny of these strains. Polymerase Chain Reaction (PCR) was used to detect virulent genes such as eaeA, Iss, papC, astA, AdrA, iutA, Stx1, and Stx2. Furthermore, isolates were screened for antibiotic-resistance genes such as *blaTEM and Sul1*. All identified *E. coli* strains were positive for the following virulence genes: *eaeA, Iss, papC*, *astA*, *iutA,* and *AdrA* with 100% incidence. Shiga toxin genes (*Stx1* and *Stx2*) were not revealed in all *E. coli* isolates examined. Furthermore, the examined bacterial isolates harbored the β-lactamase resistance gene (*blaTEM*) and sulphonamide resistance gene (*Sul1*). PCR products were sequenced, and four sets of DNA-sequences for *E. coli* isolates were produced and deposited in GenBank for *ast*, *iss*, *eae,* and *pap*C gene. In the present study, virulent antibiotic-resistant strains of *E. coli* in equines threaten the public's health. Phylogenic analysis of these genes exhibits an effective method for disease control.

Key words: *E. coli,* Virulence, Antibiotic resistance, Equine, Egypt

INTRODUCTION

Equine athletes are greatly affected by respiratory illnesses, which are frequently identified as the second most common reason for low performance and high veterinary expenses costs (Melo and Ferreira 2022; Melo et al. 2024). Horses frequently suffer from lower and upper respiratory system disorders, ranging from minor virus infections to major bacterial infections (Arafa et al. 2021; Melo and Ferreira 2022). Interest in the health of the lungs and heart of equines has increased recently (Stucchi et al. 2023). The most prevalent cause of pneumonia in adult horses is the aspiration of bacteria from the outside world, the nose, or the oropharynx, which overwhelms the body's defense mechanisms in the lower airways, as opposed to the case with neonates, where bacterial pneumonia frequently has a hematogenous etiology (Melo and Ferreira 2022). Among the bacteria that are frequently recovered from horses that have pleuropneumonia are *Streptococcus sp*., *Streptococcus equi subsp zooepidemicus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pasteurella* sp., *Actinobacillus sp.* and *Enterobacter sp.* Anaerobic bacterial isolates include *Clostridium sp*., *Bacteroides sp*., *Peptostreptococcus sp*. and *Fusobacterium sp*. (Reuss and Giguere 2015; Arafa et al. 2021).

Choosing suitable antimicrobials is essential for treating these infections effectively and lowering the chance of multiple antibiotic-resistant *E. coli* strains emerging, which has been identified as the main source of infection globally (Weese et al. 2015; Reshadi et al. 2021). Additionally, according to Ramos et al. (2020), the existence of resistant genes in commensal *E. coli* may propagate resistance to pathogenic strains horizontally as well as to humans through fecal-oral contamination (Salinas et al. 2021).

Virulent strains of *E. coli* have been observed to have an impact on the infection severity. Numerous virulence factors, including those related to adhesion (*iha*, *afaB/C*,

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fimH, *papC*, *papG2,* and *sfaD/E*), iron acquisition (*ironN*, *fyuA*, iucD, and *iutA*), capsule formation (kpsMT2), toxicity (*hlyA*, *sat*, *cnf1*, and *usp*) and miscellaneous genes (*ompT*, *tcpC*, *cvaC*, *ibeA,* and *traT*), have been demonstrated to be expressed by *E. coli* strains. These virulence factors are significant at every stage of the infection process (Flores-Mireles et al. 2015).

Phylogenic research has revealed that *E. coli* is included in four major phylogenetic groups: A, B1, B2, and D. Group B2 and, somewhat less significantly, group D comprises the most virulent extra-intestinal *E. coli* strains, whereas the commensal *E. coli,* considered less virulent, belong to groups A or B1 (Clermont et al. 2000; Čurová et al. 2020; Riaz et al. 2023). Therefore, the current work aimed to determine the prevalence of *E. coli* to detect their resistance to antimicrobial agents, virulence determinants, biofilm production, and phylogeny of *E. coli* isolates from horses exhibiting respiratory manifestation. Therefore, this study aimed to evaluate the potential virulence of equine strains of *E. coli* in Egypt.

MATERIALS AND METHODS

Ethical approval

The Medical Research Ethics Committee-NRC approved the project (approval number 19153).

Study period and location

The study was carried out from 2021 to 2023. The research was done at the National Research Centre, and the samples were processed at the Microbiology and Immunology Department, Veterinary Research Institute, National Research Centre, Egypt.

Samples

A total of 108 nasal swabs were obtained from Giza Governorate, Egypt, and divided into three groups: native breed (68), foreign breed (36), and Arabic breed (4). Using sterile cotton swabs soaked with normal saline, nasal swabs were taken from the nasopharynx, which connects the nasal cavity to the oropharynx. All collected samples were immediately placed in an icebox in the laboratory after being thoroughly packaged, numbered, and dispatched.

Bacterial isolation and identification

Nasal swabs were immersed in modified tryptic soy broth (TSB, Oxoid, UK). For 24 hours, the specimens are incubated at 37°C. MacConkey agar plates (Oxoid, UK) are used to cultivate every sample (Feng et al. 2011). Following an 18-24 hour incubation period at 37°C, distinct colonies exhibiting characteristic *E. coli* morphology were identified and moved to EMB agar (Oxoid, UK) for additional purification. Subsequently, the potentially pathogenic *E. coli* isolates were cultivated on nutrient agar and subjected to biochemical analyses, including methyl red, oxidase, citrate, and triple sugar iron tests, according to Liu et al. (2017).

Serotyping of *E. coli* isolates

The examined isolates were serogroup in Animal Health Research Institute, Giza, Egypt, via Sifin antisera "Berlin, Germany" Polyvalent and monovalent diagnostic *E. coli* antisera. The serological typing based on agglutination reactions of their antigens was detected as described by Edwards and Ewing, 1972.

DNA extraction

According to the manufacturer's recommendations, the GF-1 Bacterial DNA Extraction Kit (Cat No. GF-BA-100, Vivantis Technologies, Malaysia) was used to extract DNA from bacterial cultures.

Molecular identification of *E. coli* Spp. virulence genes and antibiotic resistance genes using conventional Polymerase Chain Reaction (PCR)

All isolated *E. coli* strains were examined for the existence of the following virulent genes: *eaeA*, *Iss, papC*, *astA*, *AdrA, iutA, Stx1,* and *Stx2* genes; furthermore, all isolates were examined for the presence of the antibiotic resistance genes as *blaTEM and Sul1* genes. The used primers with the expected products are listed in Table 1.

PCR reaction was performed using SimpliAmp™ Thermal Cycler (Cat. No. A24811, Applied Biosystems, USA) in a total volume of 25µL reaction including 12.5µL of 2x MyTaq™ Red Mix Master Mix (Cat. BIO-25043, Meridian Bioscience, UK), 1µL (10µM) of each primer and 1µL of target DNA and 9.5µL of DDW. Electrophoresis on a 1.5% agarose gel was used to separate the PCR products, then photographed and analyzed by the InGenius3 gel documentation system (Syngene, UK). Table 2 lists the cycling conditions.

DNA sequencing and phylogenetic tree building

The Gene JET Gel Extraction Kit (K0691, Thermo Fisher, USA) was used to purify two of the positive PCR products targeting *ast*, *eae*, *Iss* and *papC* virulence genes of *E. coli* isolated from nasal swabs of horses. The sequences were then run by Macrogen Company (Korea). Two-way sequencing using the specific primers used in PCR served as a confirmation of the data's accuracy. BioEdit 7.0.4.1 and MUSCLE were used to examine the nucleotide sequences acquired in this work. Using a neighbor-joining technique of the aligned sequences implemented in the application CLC 6, the obtained sequences were aligned with reference sequences of virulence genes of *E. coli.* Four sets of DNA sequences for *E. coli* isolates were deposited in GenBank under the accession numbers PP236041-PP236046 for *ast* gene, PP196348- PP196353 for *iss* gene, PP196354-PP196359 for *eae* gene and PP196360-PP196365 for *papC* gene.

RESULTS

Isolation and identification of *E. coli*

Colonies of suspected *E. coli* on MacConkey agar appeared pink to dark pink, and colonies examined by Gram's stain showed Gram-negative bacilli when examined under a light microscope. All the biochemical tests were interpreted as methyl red positive, Voges-Proskauer negative, oxidase negative, citrate negative, urease negative, and acid produced with gas and without hydrogen sulfide formation on TSI were identified as *E. coli*. Out of 108, 68 isolates of *E. coli* were recovered with an incidence of 62.9%, which isolated from foreign and

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Table 1: Primers used for detection of virulence and antibiotic resistance genes

Gene	Sequence (5´-3´)	Amplicon size (bp)	References
eaeA	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	Guion et al. (2008)
Iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266	Tawfik et al. (2016)
papC	GTGGCAGTATGAGTAATGACCGTTA ATATCCTTTCTGCAGGGATGCAATA	200	López-Banda et al. (2014)
astA	CCATCAACACAG TATATCCGA GGTCGCGAGTGACGGCTTTGT	111	Yamamoto and Echeverria (1996)
AdrA	ATGTTCCCAAAAATAATGAA TCATGCCGCCACTTCGGTGC	1113	Bhowmick et al. (2011)
iutA	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	Yaguchi et al. (2007)
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Dipineto et al. (2006)
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Dipineto et al. (2006)
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	Colom et al. (2003)
Sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	Ibekwe et al. (2011)

Table 2: Cycling condition for screened genes

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
AdrA	94°C/5min	94°C/30s	50°C/1min	72°C/1min	72°C/10min	35
astA	94°C/5min	94°C/30s	55°C/30s	72°C/45s	72°C/10min	35
eaeA	94°C/2min	94°C/20s	53°C/30s	72°C/45s	72°C/10min	35
Iss	94°C/5min	94°C/30s	54°C/30s	72°C/45s	72°C/10min	35
iutA	94°C/5min	94°C/30s	63°C/30s	72°C/30s	72°C/7min	35
papC	94°C/5min	94°C/30s	58°C/30s	72°C/45s	72°C/10min	35
Stx1	94°C/5min	94°C/30s	63°C/30s	72°C/45s	72°C/10min	35
Stx2	94°C/5min	94°C/30s	63°C/30s	72°C/45s	72°C/10min	35
Sul1	94°C/5min	94°C/30s	60°C/40s	72°C/45s	72°C/10min	35
blaTEM	94°C/5min	94°C/30s	54°C/40s	72°C/45s	72°C/10min	35

native breeds with an incidence of 14.8 and 48.1%, respectively.

E. coli serotyping

Six *E. coli* isolates were investigated for the serotyping of biochemically identified *E. coli,* which revealed that the most predominant serotypes were O27 (16.6%), O18 (16.6%), O158 (33.3%), and O157 (33.3%).

Molecular identification of *E. coli* Spp. virulence genes

All the serotyped identified *E. coli* isolates were positive in PCR for the following virulence genes with an expected product of 248, 266, 200, 111,300 and 1113bp for *eaeA, Iss, papC*, *astA*, *iutA,* and *AdrA* with 100% incidence. Shiga toxin genes (*Stx1 and Stx2*) were not noticed in all screened *E. coli* isolates.

Molecular identification of *E. coli* Spp. antibiotic resistance genes

All the serotyped identified *E. coli* isolates were positive in PCR for sulphonamide resistance gene (*Sul1*) and beta-lactamase gene (*blaTEM*) with the expected product of 433 and 516bp, respectively.

Phylogenetic tree building

Based on its sequence alignment, the present sequences were firmly embedded within *E. coli* virulence genes. Concerning *ast* gene, the accession numbers (PP236041-PP236046) showed 100% similarity to that from poultry with cellulitis in Brazil (KJ149567) and only 40% similarity with that from calves with diarrhea in Egypt (HM099899) as shown in Fig. 1.

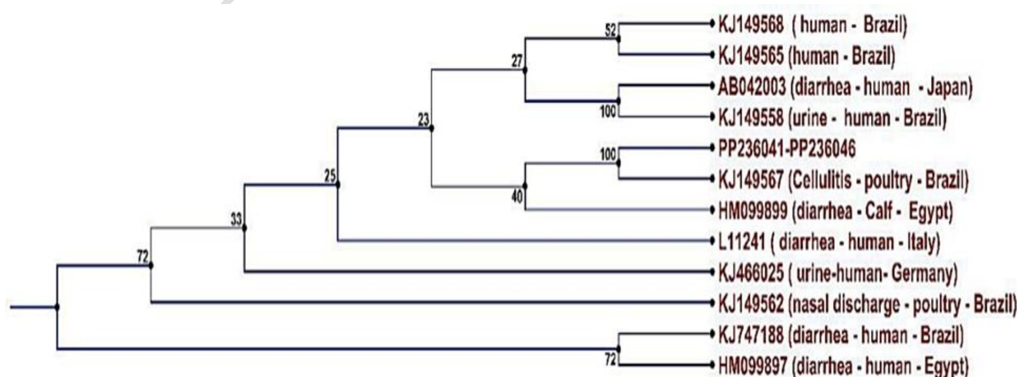

Fig. 1: Phylogenetic tree constructed based on the *ast* gene sequences of *E. coli* (PP236041- PP236046) and its closest sequences using the neighbor-joining method.

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Concerning *iss* gene, the accession numbers (PP196348 - PP196353) showed 100% similarity to that from poultry from Brazil (FJ824853), Iran (FJ416147), and USA (AF042279) and China (JX466844) and 97 % similarity with that from poultry in Egypt (KU904254) as shown in Fig. 2. Concerning th*e eae* gene, the accession numbers (PP196354-PP196359) showed 100% similarity to that from human stools in India (MF326955), as shown in Fig. 3.

Regarding the *papC* gene, the accession numbers (PP196360- PP196365) showed 100 % similarity to those of patients with urinary tract infections in India (HQ165752 and ON911574) and water in Brazil (OK001333), as shown in Fig. 4.

DISCUSSION

Horses, as companion animals, have been regarded as a possible source of microbial pathogens that trigger infections and problems in a variety of hosts, including humans. regarding these microbes, several strains of *E. coli* exhibit virulence attributes and antibiotic resistance (AMR), which can be spread by direct or indirect contact (Lyimo et al. 2016). One of the commensal microorganisms in horses' gut microbiome is *E. coli*, but it is correspondingly a major opportunistic extra-intestinal bacterium in both adult and young horses (Van Duijkeren et al. 2000; Albihn et al. 2003; Clark et al. 2008). It is the leading cause of neonatal horse septicemia, and while it isn't usually the reason for gastrointestinal disorders, certain strains might be involved with foal enteric disorders (Magdesian 2005; Olivo et al. 2016).

The Native and Arabian breed of horses showed strangle signs clinically as respiratory distress and nasal discharge and recorded *E. coli* with an incidence of 100% for each (Omair et al. 2018). Kohnen et al. (2023) revealed that of 721 samples cultured for *E. coli* isolated from horses at the hospital, 85% (613/721) were positive. This high incidence matches our study as 68 isolates of *E. coli* were recovered with an incidence of 62.9% (68/108), which isolated from foreign and native breeds with an incidence of 14.8% (16/108) and 48.1% (52/108), respectively. According to several studies, the frequency of resistant *E. coli* in hospitalized horses is expected to be much greater than in non-hospitalized horses (47-73% versus 13–21%) (Bryan et al. 2010; Maddox et al. 2011; Schoster et al. 2012). The O antigen is present in the side chain of the lipopolysaccharide found in cell walls, a crucial *E. coli* component. The current gold standard for epidemiological judgment is the O antigen serotype classification (Liu et al. 2020). We found that *E. coli* isolates showed that the most prevalent serotypes were O27 (16.6%), O18 (16.6%), O158 (33.3%) and O157 (33.3%).

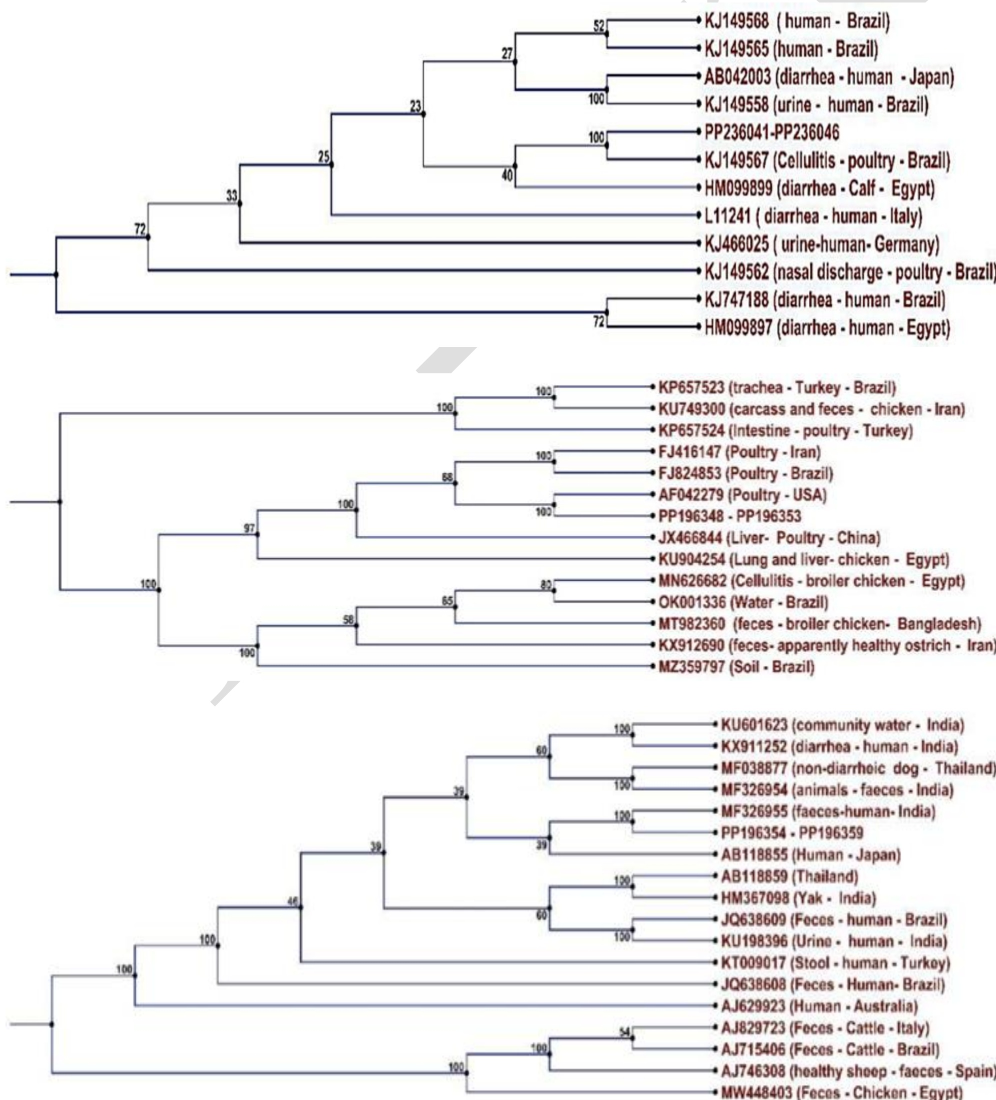

Fig. 1: Using the neighbor-joining method, a phylogenetic tree is constructed based on the *ast* gene sequences of *E. coli* (PP236041-PP236046) and its closest sequences.

Fig. 2: A phylogenetic tree is constructed based on the *iss* gene sequences of *E. coli* (PP196348-PP196353) and its closest sequences using the neighbor-joining method.

Fig. 3: Using the neighbor-joining method, the phylogenetic tree was constructed based on the *eae* gene sequences of *E. coli* (PP196354-PP196359) and its closest sequences.

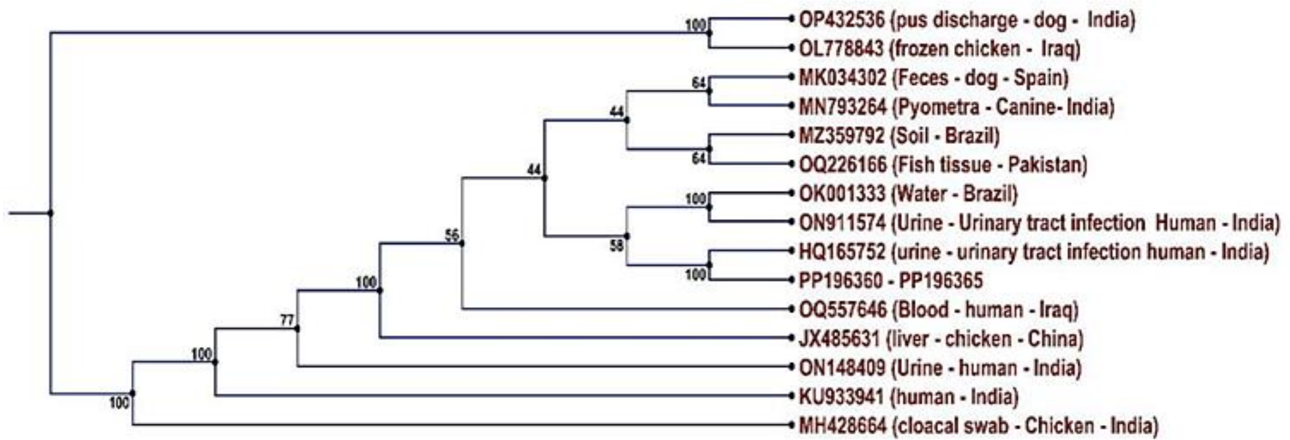

Fig. 4: Phylogenetic tree constructed based on the *papC* gene sequences of *E. coli* (PP196360-PP196365) and its closest sequences using the neighbor-joining method.

Virulence elements are unique compounds, primarily proteins, generated and released by protozoa, fungi, bacteria, and viruses. Certain genes found on chromosomes or mobile genetic components (such transposons or plasmids) in pathogenic bacteria encode these components (Wu et al. 2008; Alegbeleye et al. 2018). Pathogenicity-related genes can encode functions including toxin activity, attachment, adhesion, invasion, motility and iron acquisition, among others (Pakbin et al. 2021).

Two *E. coli* isolates were recovered from the tracheal wash of mule foals. Out of the six studied virulence genes, five were present in the samples. The following genes *fim*H, *kps*, *pap*, *hly*A, and *cnf* were present in two isolates from a sick animal, whereas the genes *afa*, *fim*H, *kps*, *pap*, and *cnf* were present in one isolate from a healthy animal (Carneiro et al. 2017). In our study, all the serotyped identified *E. coli* isolates were positive in PCR for the following virulent genes with the expected product of 248, 266, 200, 111, 300, and 1113bp for *each, Iss, papC*, *astA*, *iutA,* and *AdrA* with 100% incidence. Shiga toxin genes (*Stx1* and *Stx2*) were not noticed in all examined *E. coli* strains. According to DebRoy et al. (2008), three genes were identified in an *E. coli* strain recovered from a mare that had died of bronchopneumonia: *cnf*, *fim*H, and *pap*.

Antibiotic resistance is seen as a crucial multifactorial and dynamic global concern due to the quick appearance and dissemination of genes linked to antibiotic resistance in the environment, animals, and humans (Rousham et al. 2018). The American Association of Equine Practitioners has released guidelines for antimicrobial stewardship for equine practitioners, acknowledging the importance of using antibiotics responsibly (AVMA 2021). To further assist veterinarians in the practice of equine medicine, insightful papers regarding the use of antibiotics and resistance have been published (Traub-Dargatz and Dargatz, 2009; Weese 2015; Raidal 2019; Knych and Magdesian 2021). Antimicrobial resistance in many *E. coli* strains, whether inherent or acquired, should be taken seriously since it poses a serious risk to public health (Reshadi et al. 2021).

One of three *sul* genes: *ul*1, *sul*2, or *sul*3 mediates sulfonamide resistance in *E. coli* from food producers and companion animals. Due to its membership in the 3′-conserved region of class 1 integrons, the *sul*1 gene is highly prevalent (Recchia and Hall 1995). High levels of MDR strains were found in a retrospective data analysis on the evolution of antimicrobial susceptibility in equine diseases, conducted between 2006 and 2016 (Duchesne et al. 2019). Nocera et al. 2023 reported that 41.7% of the *E. coli* strains collected from horses had multiple antibiotic resistance. The most often detected resistance gene (98.4%) was *bla*TEM, while 26.15% of *E. coli* strains had multidrug resistance (Reshadi et al. 2021). This is consistent with our study in which all the serotyped identified *E. coli* isolates were positive in PCR for sulphonamide resistance gene (*Sul1*) and beta-lactamase gene (*blaTEM*) with the expected product of 433 and 516 bp respectively.

Understanding the phylogenetic background of *E. coli* is crucial to comprehend the connection between illness, antibiotic resistance, and strains (Yılmaz and Aslantaş, 2020; Mwafy et al. 2023). The virulence of *E. coli* varies depending on its serotype, chronological, regional, and source variables which affect the most common serotype. *E. coli* is categorized into groups A, B1, B2, and D based on its phylogenetic categorization; group A is not pathogenic, group B1 is less pathogenic, and groups B2 and D are extremely pathogenic (Coura et al. 2015).

Based on its sequence alignments, the present sequences were firmly embedded within *E. coli* virulence genes. Concerning the *ast* gene, it showed 100% similarity to that from poultry with cellulitis in Brazil and only 40% similarity with that from calves with diarrhea in Egypt. Concerning *iss* genes, they showed 100% similarity to that of poultry from Brazil, Iran, the USA, and China, and 97% similarity with that of poultry in Egypt. In relation to *eae* gene showed 100 % similarity to that from human stools in India. The *papC* gene showed 100% similarity to that of patients with urinary tract infections in India and water in Brazil. This proves the easiness of movement of this pathogen between different species, including poultry and humans, with adding virulence capacity and the zoonotic importance of this pathogen.

Conclusion

In conclusion, our study revealed a significant frequency of *E. coli* in equines and the most predominant serotypes were O27, O18, O158, and O157. Moreover, sulphonamide resistance gene, beta-lactamase gene and

virulence genes: *eaeA, Iss, papC*, *astA*, *iutA* and *AdrA* were present in the identified *E. coli isolates* while Shiga toxins (*Stx1 and Stx2*) were not detected Therefore, designing treatment methods depending on the diverse local and cyclical factors associated with the incidence and resistant virulent *E. coli* strains is particularly crucial. Sequencing of *ast, iss, eae,* and *papC* genes showed different similarities to those from poultry, calves, and humans in different countries such as Brazil, Iran, the USA, China, India, and Egypt, which proves the high virulence of this pathogen, which helps it to move from one host to another including human. Our research will help safeguard public and animal health by efficiently treating and controlling *E. coli* infection.

Declaration of competing interest: The authors declare that any known competing financial interests or personal relationships have impacted none of the work described in this study.

Authors' Contribution: AAA, KAA, MMK and AMA shared in the design of this research, AAA conducting the molecular detection of antibiotic resistance genes, supervision, Funding and Project administration. KAA conducting the PCR for sequencing and phylogenetic analysis. MMK was responsible for serological identification and conducting the PCR genetic markers of virulence. AMA was responsible for bacterial isolation, DNA extraction and sharing in conducting the PCR.

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