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Prevalence of pathogenic E. coli in diarrhoeic cattle calves and antibiotic resistance genes

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Abstract

A total of 190 fecal samples were collected from diarrheic cattle calves aged from 1-6 months from different private farms in Kalubia and Sharkia Governorates, Egypt during the period from November 2019 to September 2020. Samples were carried out to elucidate the prevalence of *Escherichia coli (E. coli*). Serological identification of *E. coli* isolates was done to detect the incriminated serogroups. Detection of Congo red binding activities hemolysis production and enterotoxin assay by infant mouse test as virulence factors. Also, antimicrobial sensitivity test by disc diffusion. Detection of *bla*TEM and *tet*A (A) antibiotic resistance genes of 10 isolated *E.coli* isolates represented by one isolate from each serogroup and the untypable strain was done by PCR. The bacteriological examination revealed that the prevalence of *E. coli* in diarrheic calves was 37.4%. The serotyping of the isolated *E. coli* revealed serogroups; O55, O25, O111, O119, O126, O78, O157, O186, and O128, and other untypable 8 strains. Furthermore, the Congo red binding activity revealed that 43 *E.coli* isolates showed binding activity with an incidence of 60.6%. Out of 71 isolates, 56 showed hemolytic activity with a percentage of (78.9 %) but enterotoxin production was found only in 26 isolates with a percentage of 36.6. The antibiogram of the isolated strains was investigated; the majority of *E. coli* were resistant to amoxicillin, ampicillin, and tetracycline. On other hand, all serogroups were sensitive to ciprofloxacin and cefotaxime. Polymerase chain reaction (PCR) was used to detect the antibiotic-resistant genes *bla* TEM and *tet*A (A) genes and the obtained results showed that all tested isolates contained these genes except the first serogroup O_{55}.

Keywords: E. coli, diarrhea, calves, antibiotic resistance genes

1. Introduction

Escherichia coli is one of the serious pathogens that can cause a tremendous therapeutic problem by developing resistance to antibiotics (Gupta *et al*. 2014). Diarrhea continues to be one of the most common causes of morbidity and mortality in developing countries (Allam *et al.* 2019). *Escherichia coli* is an emerging agent among pathogens that cause diarrhea (Nguyen *et al*. 2005). *E. coli* is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are facultative anaerobic Gram-negative rods that live in the intestinal tracts of healthy and diseased animals (Melha *et al*. 2002). *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal “nonpathogenic” strains of *E. coli* can cause infection (Aman *et al.* 2021). Moreover, even the most robust members of our species may be susceptible to infection by one of several highly adapted *E. coli* clones which together have evolved the ability to cause a broad spectrum of human diseases. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body (Islam *et al*. 2015). The species *E. coli* is serologically divided into serogroups and serotypes on the basis of its antigenic composition (somatic O antigens for serogroups and flagellar or H antigens for serotypes). Many strains express the third class of antigens (capsular, K antigens) which is important in pathogenesis (Campos *et al*. 2004; Gharieb *et al.* 2015)

The virulence factors of *E.coli* are usually complex and the contribution of individual determinants cannot be considered in isolation, the relevant determinants may vary with the locus and nature of the infection. Virulence in microorganisms is associated with the capacity of *E. coli* to attach and colonize at the site of the infection with subsequent damage to the host and is promoted by aggressions that interfere with host defense (Gillhuber *et al.* 2014). The expression of these virulence factors disrupts the normal host physiology and elicits disease. Certain pathogenic strains cause enteric disease ranging in symptoms from cholera-like diarrhea to

severe dysentery, other *E. coli* may colonize the urinary tract, resulting in cystitis, pyelonephritis, and other strains that cause septicemia and meningitis. These virulence factors included toxins, adhesion, invasiveness, and the ability to resist serum complement. Bacterial virulence factors are required to fight the host selection pressure and for the bacteria to colonize, multiply and survive (Kaipainen *et al.* 2002).

Virulence represents a balance among the multiplicity of factors, some related to the microorganisms are associated with the capacity to at- tack and colonize at the site of infection with damage to the host (Herrera-Luna 2009). The gastrointestinal disease involves the oral ingestion of pathogenic *E.coli* strains and their- subsequent colonization and infection of the small intestine or colon, or both, with disease manifestations caused by the expression of one or more virulence factors. In almost every instance, it is the coordinate expression of multiple virulence determinants by pathogenic bacteria that culminate in overt disease (diarrhea). In any case, at least two factors are required to produce enteric disease: mechanisms for attachment and colonization of the gastrointestinal mucosa and some mechanisms for induction of diarrhoeal disease (Deverdier *et al*. 2012). Enterotoxigenic *E. coli* (ETEC) infection is the most common type of colibacillosis in young animals (primarily pigs and calves). The main virulence attributes of ETEC are adhesions and enterotoxins production, which are mostly regulated on large plasmids. (Nagy and Fekete. 2005). Enteropathogenic *Escherichia coli* (EPEC) induce watery diarrhea similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins. They produce a non-fimbrial adhesion designated as intimin, an outer membrane protein that mediates the final stages of adherence, moderately invasive, with some reporting Shiga-like toxins (Addy et al. 2004).

Molecular biology techniques have become integrated into the practice of infectious disease epidemiology (Galane and Le-Roux 2001). Yadegari *et al.* 2019 found that PCR was used on large scale as a recent technique for the detection of virulence factors enterotoxigenicenic *E.coli*. Antibiotic resistance is an increasing Phenomenon due to the astonishing ability of bacteria to adapt to a different and continuously changing environment. Antibiotic abuse in animal husbandry for therapy, prevention, and animal growth promotion is considered to be responsible for the spread of resistant bacteria. Utilized antimicrobial agents act not only against pathogenic bacteria but also against commensal bacteria that are a part of normal microbial communities in animals and humans. These commensal bacteria, especially inhabitants of the intestinal tract, are constantly exposed to antibiotics and develop resistance in order to survive, thus becoming an important reservoir of resistance genes. (Knezevic and Petrovic 2008) Antibiotic resistance genes are ubiquitous in clinical pathogens and environmental bacterial species. This is due to the wide usage of antibiotics (Cantas *et al.* 2013).

Antibiotic resistance is a major factor that drives change in the pattern of antibiotic prescribing and is the most important stimulus to the development of new antibiotics by the pharmaceutical industry (Al Gamal *et al.* 2020). Resistance to β - lactam antimicrobial agents in gram-negative bacilli is primarily mediated by β-lactamases. Although a variety of β-lactamases have been described, the *bla*TEM and *tet*A(A) enzymes are those most frequently observed among members of the family Enterobacteriaceae (Bakhshi *et al.* 2014). The antimicrobial resistance is usually associated with pathogenic *E. coli* that could be attributed to the widespread improper use of antibiotics. Multidrug resistance (MDR) is common in *E. coli* and is primarily associated with several genes like; *bla-TEM*, *blaCTX* (β-lactamase genes), *sul1* (sulfonamide resistance gene), and *aadB* (aminoglycoside resistance gene). (Algammal,et al 2020). Antibiotic resistant bacteria such as *E. coli* from animals can colonies or infect the human population via contact (occupational exposure) or via the food chain. Moreover, resistance genes can be transferred from bacteria of animals to human pathogens in the intestinal flora of humans.

This study aimed to determine the incidence of pathogenic *E. coli* in calves, the virulence markers (Congo red, hemolysin, enterotoxin), and the resistance genes. (*bla* TEM and *tet*A) in examined isolates.

2. Materials and methods

2.1. Sampling

A total of 190 fecal samples of diarrhoeic cattle calves aged from 1-6 months from different private farms in Kalubia and Sharkia Governorates during the period from November 2019 to September 2020 were collected under aseptic conditions and each in separate sterile polyethylene bags, labeled kept in an icebox and sent to the lab.

2.2. Isolation of E. coli

Pepton water was added fecal swab, samples were then incubated at 37°C for 24 h, centrifugated, and the supernatant was cultured. A loopful of supernatant was streaked directly onto plates of 5% sheep blood agar, Sorbitol MacConkey agar, and incubated at 37°C for 24 h. Lactose fermenter colonies were sub-cultured into Eosin methylene blue agar and incubated aerobically at 37°C for 18-24hr (Quinin *et al.,* 2011).

2.3. Identification of E. coli isolates

Suspected colonies were subjected to morphological, cultural, and biochemical identification according to (Murrary *et al.* 2003). Serological identification of *E. coli* isolates was done according to (Edwards and Ewing, 1972).

2.4. Detection of virulence factors

Congo red binding activity was done according to (Berkhoff and Vinal 1986). Haemolysin Production was done as previously described (Koneman *et al.* 1997). Isolates were tested for hemolytic activity on 5-7 % sheep blood agar for 24 hours at 37 °C aerobically. Enterotoxin assay was done by infant mouse test according (Galiero *et al.* 1995).

2.5. Antimicrobial susceptibility test

Bacterial isolates were examined for their susceptibility to the following antimicrobial discs by the disc difference method: amoxicillin (25µg) amoxicillin/clavulanic acid (20µg), ampicillin (5µg), gentamycin (10µg), ciprofloxacine (10µg), cefotaxim (30µg), Trimethoprim-sulfamethoxzle (300µg) tetracycline (30µg), and streptomycin (10µg) by disc diffuse technique (CLSI. 2017).

2.6. Detection of blaTEM and tetA (A) genes by PCR

DNA extraction from 10 *E. coli* isolates represented by one isolate from each serogroup and untypable was done using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit. Primers were

listed in Table (1). Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. A 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Table 1. Primers sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondar denaturation	Annealing	Extension		
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 45 sec.	54˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	Colom *et al.* 2003
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94˚C 5 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	35	Randall *et al.* 2004

3. Results and Discussion

The role played by *E. coli* in producing diarrhea in claves has received great attention from many authors (Gupta *et al.* 2014). Some workers claimed that the cold, dampness, increased humidity, overcrowdness, and rains would lower the resistance of the animals, particularly the newly born calves, and could be the cause of mortality especially (Sayed *et al.* 2002).

The present study revealed that 71 isolates out of 190 samples of newborn calves showing signs of diarrhea harbored *E. coli* with an incidence of 37.4 % table (2), which are similar to previous studies (Abdelazeem *et al*. 2020) (52%), Solmaz *et al.* (2000) (26.5%). This difference may be due to different factors such as predomination of other microorganism breeding systems, environmental, immunity status methods, age group, factors of isolation, or antibiotics therapy. Regarding the age of the examined calves, the prevalence of E. coli was 45.5%, 38.5%, and 30.6 % in the first 2 months old, 2–4 months old, and 4–6 months old aged calves, respectively. Previous studies reported that the prevalence of *E. coli* was high in the young calves, and then decreased as the age increased Osman *et al.* (2012).

The serological identification of the retrieved isolates revealed that a total of 63 (88.7%) strains were typable, while 8 isolates (11.3%) were untypable. The most common serogroup was O55 (14) followed by O25 (11) and O111 (10), O119 (8), O126 (8), O78 (5), O157 (3), O186 (2) and O128 (2). *E. coli* serogroups were illustrated in Table (3). These results support the finding of (Campos *et al.* 2004). In the present study, *E.coli* isolates were tested for their virulence activity (the ability to produce hemolysis to bind with Congo red and the production of enterotoxin in suckling mice). The Cong red is a simple dye that can be incorporated into agar media and uptake of dye has been proved to be a virulence marker to distinguish between invasive and noninvasive isolates (Quinn *et al.* 1994). In this study, 60.6% of *E.coli* strain isolated from diarrhoeic calves showed CR positive with a different degree in red colour. This significant result agreed with the results of (Berkhoff and Vinal 1986) who found that more than half of the examined *E.coli* isolates were CR positive. Haemolysin was used as a phenotypic marker for the virulence factor of *E.coli* because it suggested that haemolyin causes damage to the cell membrane. The contribution of hemolysin production was attempted in the present study using 5% sheep blood agar plates. Results showed that 78.9 % of *E.coli* isolates were beta-hemolytic. These are in agreement with that reported by (Abd El-Wahed 2005) who stated that 66.07% of tested *E.coli* isolates were haemolytic. Moreover, *E.coli* strains produced both alpha and beta haemolysins. The detection of enterotoxins in isolates was considered an indicator of enteropathogenicity. The infant mouse assay test was used in the detection of enterotoxigenic *E.coli* strains isolated from diarrhoeic calves. The incidence of enterotoxigenic strains was 36.6% (Table 3). These results are nearly similar to that mentioned by (Alexa *et al.* 1997) who isolated enterotoxigenic *E.coli* stains in the incidence of 47%, 31.2% and 41% respectively. On the other hand, (Galiero *et al.* 1995) recorded a lower incidence of production than our results shown in Table (3).

Table 2. Prevalence of pathogenic *E. coli* in diarrheic calves at different ages

Ages (Months)	Number of Samples	No. of *E. coli*	Prevalence (%)
1-2	37	17	45.9
2-4	91	35	38.5
4-6	62	19	30.6
Total	190	71	37.4

Table 3. Detection of the virulent isolates using Congo red blinding activity, hemolytic activity, and Enterotoxigenic production of *E.coli* serovars

E.coli serovars	No. of isolates	CR^{++}	Haemolytic activity	Toxin production
		NO.	NO.	NO.
O 55	14	10	13	5
O 25	11	7	8	4
O 111	10	5	7	4
O119	8	6	6	3
O126	8	4	6	2
O78	5	3	4	2
O157	3	2	3	2
O186	2	1	2	1
O128	2	1	2	1
Untypable	8	4	4	2
Total	71	43 (60.6%)	56 (78.9%)	26(38.6%)

*Percentages were calculated according to the number of the same serogroup. CR: Congo red .

Table 4. Antimicrobial sensitivity of *E.coli* isolated from diarrheic calves.

Type of antibiotics	O55 (14)	O25 (11)	O111 (10)	O119 (8)	O126 (8)	O78 (5)	O157 (3)	O186 (2)	O128 (2)	Untypable (8)
Amoxicillin	6	0	0	0	0	0	0	0	0	0
Amoxicillin and clavalonic acid	7	6	4	3	3	2	0	2	1	2
Ampicillin	0	0	0	0	0	0	0	0	0	0
Gentamycin	6	5	4	3	3	0	0	0	0	1
Ciprofloxacin	12	9	8	6	5	4	2	2	2	0
Cefotaxime	14	10	9	7	5	4	3	2	2	7
Trimethaprine sulphamethozle	5	7	4	2	4	2	1	0	0	0
Tetracycline	8	3	3	3	2	1	0	0	0	0
Streptomycin	3	2	2	2	1	2	0	0	0	0

The percent calculated for the sensitivity to antibiotics in relation to total No. of the same serogroup.

It had been shown that enterotoxin Concerning the result of antimicrobial susceptibility in this study, Cefotaxime, and ciprofloxacin was the most effective antimicrobial drug agent with high susceptibility percentage as shown in table (4), similar to (Liu *et al.* 2007) reported that *E.coli* isolates were more sensitive to Cefotaxime and ciprofloxacin. Most *E.coli* isolates were resistant to amoxicillin, ampicillin, and tetracycline similarly (De Verdier *et al* 2012) isolated *E.coli* with high resistance to amoxicillin, however, Giurov, (1985) recorded highly sensitivity to amoxicillin was. In this study multidrug-resistant recorded in all isolated strains. Also (Shahoni *et al.* 2014) found that *E.coli* isolated from diarrheic

calves resist penicillin followed by tetracyclin as shown in table (4). (Bakhshi *et al.* 2014) found that *E.coli* from diarrhoeic calves resist to ampicillin and tetracycline. Regarding the result of PCR for the detection of β –lactamases encoding genes among 10 *E.coli* isolated strains, all isolates contain β – lactamase encoding genes except one isolate.

These results indicate that *tet*A (A) and *bla*TEM genes play an important role in increasing antibiotic resistance (Ahmed *et al.* 2013). Also, they found antimicrobial resistance genes that encode resistance to ampicillin (Shahrani *et al.* 2014). Bradford 2001 proved that *bla*TEM is mostly encountered in β-lactamase in gram-negative bacteria. Up to 90% ampicillin resistance in *E. coli* is due to the production of *bla*TEM. Most isolates probably shared resistance to amoxicillin, ampicillin, and tetracycline. Similar results were obtained by (Chaibi *et al.* 1999) who stated that *bla*TEM variants were resistant to inhibition by clavulanic acid and sulbactam, thereby showing clinical resistance to the β –lactam - β –lactamases inhibitor combinations of amoxicillin /clavulanate, tricarcillin/clavulanate, and ampicillin/ sulbactam, they remained susceptible to inhibition by tazobactam and subsequently, the combination of piperacillin / tazobactam. (Henquell *et al.* 1995) stated that concerning *E.coli* isolates, the most recently discovered mechanism of resistance to amoxicillin/clavulanic is the production of inhibitor-resistant tem β –lactamases. Colom *et al.* (2003) found that overproduction of *E.coli* chromosomal β –lactamase is one cause of resistance to β –lactomase inhibitor combinations such as amoxacillin calvulanate and also results in results in reduced susceptibility to all β –lactams except carbapenems.

Table 5. The antimicrobial resistance genes of *E. coli* isolated from diarrheic calves.

E .coli serogroup	*blaTEM*	*tet*
O 55	-	-
O 25	+	+
O 111	+	+
O119	+	+
O126	+	+
O78	+	+
O157	+	+
O186	+	+
O128	+	+
Untypable	+	+

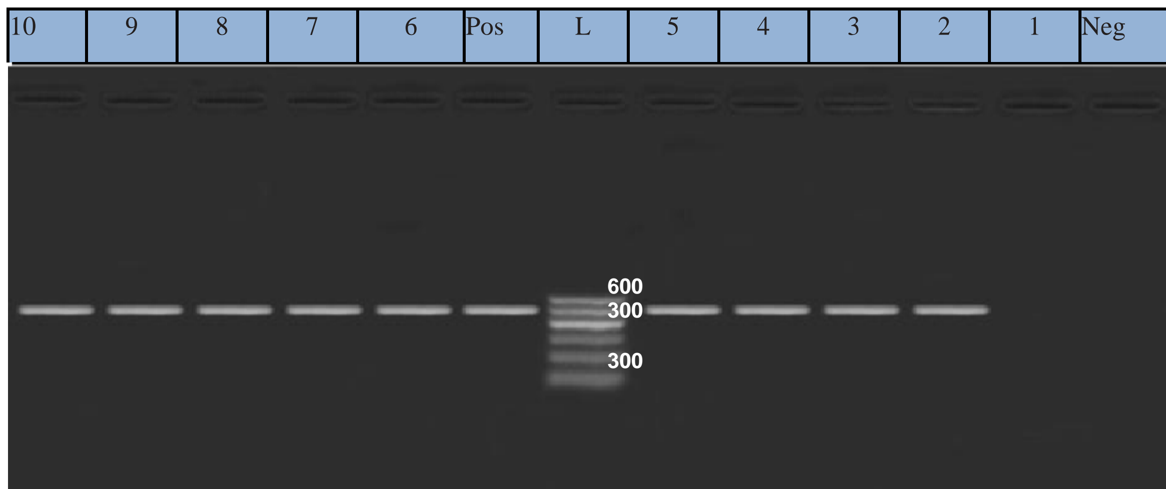

Fig.1. Amplicon of *blaTEM* gene. L, 300- 600bp DNA ladder, Neg, negative control, pos, positive control lanes 2:10 *E. coli* isolates positive for *blaTEM* 516bp, lane (1) negative isolate.

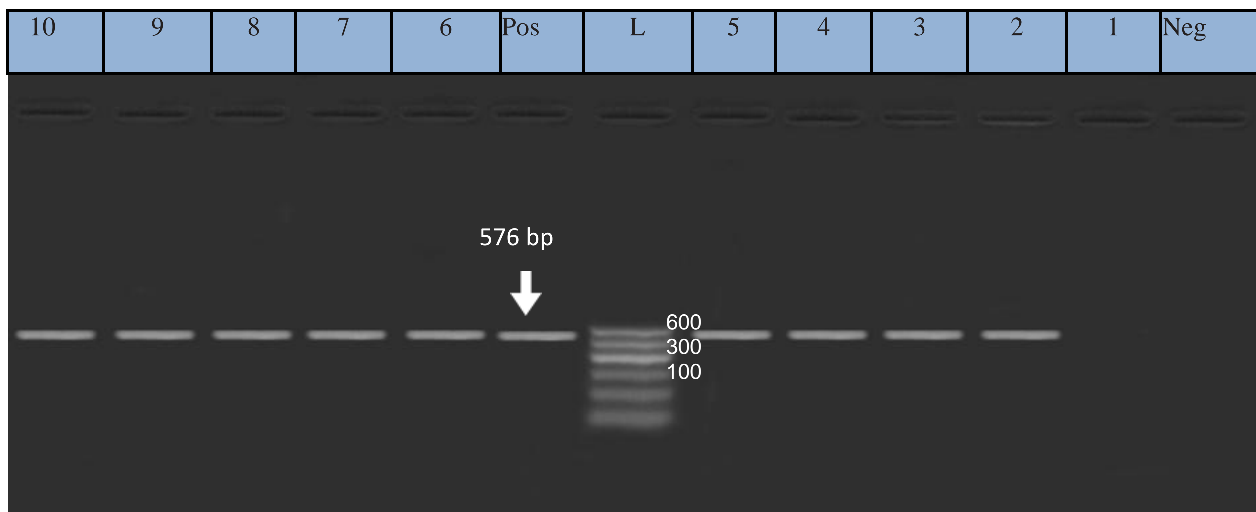

Fig 2. Amplicon of *tet A* gene. Lane 300-600 Pb DNA ladder, lane Neg, negative control, lane pos, positive control lanes 2,10 *E. coli* isolates positive for *tet A* at 576 bp, and lane 1 negative isolate.

From these results, we concluded that β – lactams are broad-spectrum antibacterial agents widely used for the treatment of *E.coli* which cause major economic loss in animals (calves) due to morbidity and mortality, due to intensive use of β –lactam drugs as therapeutic agents so β –lactams resistant is developed therefore the affective of this study was to characterize β-lactamase genes in *E.coli* isolated from diseased calves.

Conclusion

In the current study diarrhegenic, *E. coli* may constitute a prospective human threat as a source of certain zoonotic serotypes infection through milk and meat consumption. The problem is more exacerbating with the marked incidence of multi-antimicrobial resistance isolates, so the attention should be directed to hygienic precautions, as well as careful use of antibiotics on the field.

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