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# MOLECULAR DETECTION OF SOME VIRULENCE GENES OF *E. COLI* ISOLATED FROM CALF DIARRHEA IN SIWA OASIS, EGYPT

By

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## ABSTRACT

Pathogenic *Escherichia coli* (*E. coli*) is the bacteria that causes diarrhea in calves most often, especially in young calves So, this study showed the prevalence and antibiogram pattern of *E. coli* found in calves with diarrhea in different areas in SIWA oasis ,Egypt. In addition, the polymerase chain reaction (PCR) was used to look at six genes that code for virulence (*IutA, iss, fimH, papC, adrA* and *TSH*). One hundred seventy calves were checked to see if they had *E. coli*. From samples of 170 calves with diarrhea, 86 (50, 5%) were found to be *E. coli* infected isoletes were studied were very sensitive to amoxicillin/clavulanic acid, polymixin, enrofloxacin, and ciprofloxacin. On the other hand, they were very resistant to streptomycin, gentamycin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol and neomycin. The most common virulence genes found in all of the tested strains were *iss*, *fimH* and *iutA*. This was followed by (*papC*) gene (76%) (*AdrA*) gene (35%) while TSH (9%). Our research showed that colibacillosis in calves is caused by a strain that is resistant to multiple antibiotics and has virulence genes. This is important for coming up with new ways to control colibacillosis in calves.

**Keywords:**

Bovine, Escherichia coli, Virulence Gene, Antibiogram, Siwa Oasis.

## INTRODUCTION

*E. coli* is the primary member of the large bacterial family Enterobacteriaceae, or enteric bacteria. These facultative anaerobic Gram-negative rods are found in both healthy and ill animals' intestinal systems **(Melha *et al.*, 2002).** Even common "nonpathogenic" strains of *E. coli* can cause infection in weak or immunocompromised hosts, or when gastrointestinal barriers are breached. *E. coli* often remains harmlessly contained in the intestinal lumen **(Aman *et al.*, 2021).**

Escherichia coli is commonly considered a commensal bacterium **(Lugsomya *et al*, 2018).** It is a major cause of diarrhea in calves and causes neonatal colibacillosis. It causes high death rates and slows growth and it hurts the global livestock industry economically **(Lorenz *et al*., 2011).** Diarrhea-causing *E. coli* strains are broken up into six pathotypes based on the bacterial virulence and the clinical signs they cause in the host. Enterotoxigenic *E.coli* (ETEC), enteropathogenic *E. coli* (EPEC), shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (DAEC) are examples **(Gomes *et al*., 2016).** Each of these pathotypes is a group of clones that share certain traits that make them dangerous. Still, it's important to note that, the flexibility of the *E. coli* genome has made it hard to identify certain *E. coli* isolates as pathotypes. This is because some isolates combine the main virulence traits of different pathotypes and are therefore thought to be hybrid pathogenic strains that could be more dangerous **(Croxen *et al*, 2013).** ETEC has been found to be the main cause of diarrhea in calves. The other causes are often found in both diarrheal and normal feces **(Kolenda *et al*., 2015).** The main things that make ETEC dangerous and cause diarrhea are fimbrial adhesins and enterotoxins Fimbrial adhesins (F5, F17, and F41) help bacteria attach to epithelial surfaces in the small intestines. This makes it easier for bacteria to colonize with both heat-labile (LT) and heat-stable (STa and STb) enterotoxins. The toxins cause the intestinal epithelial cells to release fluids and electrolytes, which leads to diarrhea **(Acres 1985; Kaper *et al*., 2004; VuKhac *et al*., 2008).** It's not clear yet if the STEC is a cause of calf diarrhea, but there have been reports of STEC infections in cattle that killed them **(Kolenda *et al*., 2015).** Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) are made by STEC. It also makes several virulence factors, such as intimin (Encoded by the eae gene), and the STEC auto-agglutinating adhesin (saa), the most important sources of STEC are known to be domestic ruminants, especially cattle **(Paton *et al*., 2001). Berge *et al*. (2009**) showed that, the use of antimicrobial agents is usually a good way to avoid and treat diarrhea in newborn calves. **Hammerum and Heuer (2009**) reported that this method could lead to the development of bacterial types that can't be killed by several antimicrobial drugs. Also, in the past few years, a lot of foreign surveillance has found animal or human *E. coli* strains that were resistant to multiple drugs **(Woodford *et al*., 2011).** It is important to understand the prevalence, antimicrobial pattern, and distribution of the (*IutA, iss, fimH, papC, adrA, and TSH*) virulence genes between *E. coli* isolates recovered from

diarrheal calves because diarrhea causes significant economic losses in Egypt's farms and smallholder farmers, especially in the desert.

**Material and methods:**

**Samples:**

A total of 170 fecal samples of diarrheic cattle calves aged from 1-8 months from , Siwa oasis, Egypt during the period from September 2022 to May 2023 were collected under aseptic conditions and each in separate sterile polyethylene bags, labeled sent on ice to the Desert Research Center. This study was approved by the Bedouin calves breeder who gave us permission to take diarrhea samples, and all international and governmental rules on how to use and care for the animals were followed.

**Isolation of *E. coli:***

Fecal swabs received Peptone water addition, samples were incubated at 37°C for 24 hours, centrifuged, and the supernatant was cultured. A loopful of supernatant was streaked immediately onto 5% sheep blood agar and Sorbitol MacConkey agar plates and incubated for 24 hours at 37°C. Colonies of lactose fermenters were sub-cultured onto Eosin methylene blue agar and incubated aerobically for 18-24 hours at 37°C **(Quin *et al.*, 1994).**

**Identification of *E. coli* isolates:**

Suspected colonies were subjected to morphological, cultural, and biochemical identification according to **(Murrary *et al.* 2003).** Each fecal sample was inoculated on MacConkey agar (MBcell, Seoul, and ROK) and blood agar (Asan Pharmaceutical Co., Ltd., ROK) and put in an incubator at 37 °C for 12-18 hours.

Pure colonies that looked like *E. coli* were chosen at random, and their identities were confirmed using standard biochemical tests (API 20E System, BioMérieux, France).

**Antibiogram profile:**

According to (CLSI, 2002), the antimicrobial susceptibility test of *E. coli* strains was carried out using a Kirby-Bauer disk diffusion assay. The preparation of the bacterial solution followed **(Tenover, 2009).** The susceptibility of 11 Oxoid antimicrobial discs (Tetracycline, chloramphenicol, amoxicillin, clavulanic acid, streptomycin, enrofloxacin, sulfamethoxazole /trimethoprim, gentamicin, neomycin, ciprofloxacin, and polymixin) was then tested on *E. coli* isolates in vitro. According to the interpretation criteria provided by the CLSI standard, the inhibition zone was measured to evaluate the susceptibility or resistance.

**DNA extraction, and PCR Detection of virulence Genes and antibiotic resistance gene**

**DNA extraction:**

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to get DNA from samples, but the instructions from the company were slightly modifications. In short, 10 minutes were spent at 56OC mixing 200 µl of the sample solution with 10µl of proteinase K and 200 µl of lysis buffer. After the lysate had been incubated, 200µl of 100% ethanol was added to it. The sample was then washed and put through a centrifuge, just as the maker said to do. 100µl of purification buffer was used to get rid of the nucleic acid.

**Oligonucleotide Primers:**

Primers used were supplied from Metabion (Germany) are listed in (Table 1).

**PCR amplification:**

Primers were used in a 25µl reaction that had 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan),1 µl of each primer at a concentration of 20 pmol, 4.5 µl of water, and 6 µl of DNA template. A machine called an applied biosyst em 2720 thermal cycler was used to do the process.

**Analysis of the PCR Products:**

Electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature with 5V/cm gradients was used to separate the PCR results. For gel analysis, each gel slot was filled with 20µl of the uniplex PCR products and 40 µl of the duplex PCR products. Gelpilot 100 bp and 100 bp plus ladders (Qiagen, gmbh,Germany) and the Generuler 100 bp ladder (Fermentas, Germany) were used to figure out the sizes of the fragments. A gel documentation device (Alpha Innotech or Biometra) took pictures of the gel, and computer software was used to look at the data.

**Oligonucleotide Primers:**

The primers (Metabion, Steinkirchen, Germany) used for detecting the virulence genes of *E. coli* are listed in (Table1). (IutA) **(Yaguchi *et al.*, 2007),** adrA **(Bhowmick *et al.*, 2011),** iss **(Yaguchi *et al.*, 2007),** TSH **(Delicato *et al.*, 2003)** and for papC **(Wen-jie *et al.*, 2008).** Positive and or negative controls were represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference laboratory for veterinary quality control on poultry production, Animal health research institute.

**Table (1):** Primers sequences, target genes and amplicons sizes.

| pcr | Target gene | Primers sequences | Amplified segment (bp) | Reference |
|---|---|---|---|---|
| ***E. coli*** | ***iutA*** | **GGCTGGACATGGGAACTGG** | **300** | **Yaguchi *et al*., 2007** |
| | | **CGTCGGGAACGGGTAGAATCG** | | |
| | ***adrA*** | **ATGTTCCCAAAAATAATGAA** | **1113** | **Bhowmick *et al*., 2011** |
| | | **TCATGCCGCCACTTCGGTGC** | | |
| | ***iss*** | **ATGTTATTTTCTGCCGCTCTG** | **266** | **Yaguchi *et al*., 2007** |
| | | **CTATTGTGAGCAATATACCC** | | |
| | ***fimH*** | **TGCAGAACGGATAAGCCGTGG** | **508** | **Ghanbarpour and Salehi , 2010** |
| | | **GCAGTCACCTGCCCTCCGGTA** | | |
| | ***papC*** | **TGATATCACGCAGTCAGTAGC** | **501** | **Wen-jie *et al*., 2008** |
| | | **CCGGCCATATTCACATAA** | | |
| | ***tsh*** | **GGT GGT GCA CTG GAG TGG** | **620** | **Delicato *et al*., 2003** |
| | | **AGT CCA GCG TGA TAG TGG** | | |

## RESULTS AND DISCUSSION

**Gupta *et al.* (2014**) paid a lot of attention to the role that *E. coli* plays in causing diarrhea in claves. Some workers said that, the animals' resistance would be lowered by the cold, dampness, high humidity, overcrowding, and rain, especially in the young calves, and that, this could be the main cause of death **(Sayed *et al.*, 2002).** The present study revealed that samples were taken from calf that were clinically examined for the frequency watery and mucoid diarrheal cases from small-holder in SIWA oasis, Egypt, Of 170 examined calves samples, 86 (50, 5%) of the examined diarrheic calves were positive for *E. coli.* Our result, nearly similar to previous studies (52%) **Solmaz *et al.* (2000)** in Türkiye**.,** in india **Majueeb *et al.*, 2014** (50%) ,On contrary, this result was higher than other studies in Egypt reported by **Mousa, and Shama, (2021)** who isolated *E. coli* from diarrheic calves samples with a prevalence rate of (40%) and **(Galal *et al.*, 2013 and Abdelazeem 2020)** 28.7%,**(El-Seedy *et al.*, 2016)** 18.1%, from India **(Malik *et al.*, 2013 and Shekhar *et al.*, 2017)** 37.61% and 41.6%, respectively from Sweden **(Duse *et al.*, 2015)** 14.6%. This result was lower than that reported by **(Osman *et al.*, 2012, Egypt and Anwarullah *et al.*, 2014 Pakistan)** with a prevalence rate of 63.6% and 72.8% respectively. The variations in the outcomes caused by various factors, such as other

microorganism breeding systems predominating, the environment, immunity status methods, differences in the number of samples collected, geographical regions, stress factors, age groups, isolation factors, antibiotic therapy, or hygienic and management systems.

**Antimicrobial susceptibility pattern of *E. coli* from diarrheic calves:**

In this study, (Table 2) shows that 86%, 76%, 76%, and 64.7% of the *E. coli* strains that were tested for their ability to respond to antibiotics were sensitive to ciprofloxacin, enrofloxacin, polymixin, and amoxicillin/clavulanic acid, respectively. At the same time, 100 % of the bacteria were resistant to streptomycin, 82.3 percent were resistant to chloramphenicol and Gentamycin (CN), 81% were resistant to tetracycline and sulfamethoxazole/trimethoprim, and 53% were resistant to neomycin. **Khachatryan *et al.* (2006)** also came to the same conclusion. **Pereira *et al.* (2011) and Shahrani *et al.* (2014)** showed that all samples of *E. coli* were very sensitive to ciprofloxacin and cefepime and resistant to tetracycline, streptomycin, and sulfamethoxazole-trimethoprim. **Srivani *et al.* (2017)** found that STEC strains from calves with diarrhea had high antimicrobial resistance to tetracycline (63.21%) but were responsive to chloramphenicol, gentamycin (96.33%), and imipenem (99.06%). Also, 69.81% of these types were resistant to more than one drug. Also, **Hang *et al.* (2019)** found that tetracycline resistance was highest in *E. coli* types. The next medicines with the most resistant bugs were sulfamethoxazole, ampicillin, trimethoprim, and ciprofloxacin. Otherwise, Trimethoprim/sulfa and enrofloxacin have been shown to be useful at treating diarrhea in young calves **(Ortman and Svensson, 2004). Mous *et al.* (2021)** did a study in Egypt that compared different things. Showed that *E. coli* isolates were very sensitive to ciprofloxacin, enrofloxacin, polymixin,and amoxicillin / clavulanic acid, with rates of 81.25, 75%, 75%, and 62.5%, respectively. On the other hand, streptomycin had 100% resistance, followed by gentamycin, tetracycline, sulfamethoxazole /trimethoprim, chloramphenicol, and neomycin, which all had 81.25% resistance. **El-Seedy *et al*. (2016)** said that marbofloxacin, spectinomycin, and neomycin were all very effective against *E. coli.*

**Table (2):** Antibiogram of different *E. coli* isolates.

| Antimicrobial class | Antimicrobial agents | No of *E. coli* isolates (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | R | % | I | % | S | % |
| Chloramphenicol | Chloramphenicol (C) | 70/85 | 82.3 | 13/85 | 15 | 2/85 | 2 |
| Tetracycline | Tetracycline (TET) | 69/85 | 81 | 16/85 | 18.8 | 0/85 | 0 |
| Sulfonamides | Sulfamethoxazole/trimethoprim (SXT) | 69/85 | 81 | 10/85 | 11.7 | 6/85 | 7 |
| Aminoglycosides | Gentamycin (CN) | 70/85 | 82.3 | 15/85 | 17 | 0/85 | 0 |
| | Streptomycin (S) | 85/85 | 100 | 0/85 | 0 | 0/85 | 0 |
| | Neomycin (N) | 45/85 | 53 | 22/85 | 26 | 18/85 | 21 |
| Fluor quinolones | Ciprofloxacin (CIP) | 0/85 | 0 | 12/85 | 14 | 73/85 | 86 |
| | Enrofloxacin (ENR | 0/85 | 0 | 20/85 | 23.5 | 65/85 | 76 |
| Polymixins | Polymixin B (PB) | 4/85 | 4.7 | 16/85 | 18.8 | 65/85 | 76 |
| Beta - lactams | Amoxicillin/Clavulanic acid (AMC) | 10/85 | 11.7 | 20/85 | 23.5 | 55/85 | 64.7 |

**Molecular detection of *E. coli* virulence genes:**

When it comes to *E. coli* strains, their ability to cause disease is mostly due to the presence of strong toxins and certain virulence determinants. AdrA controls how cilia are made. Also, it is thought that many fimbrial genes play important parts in how *E. coli* sticks to surfaces, attaches to surfaces, and spreads to new areas **(Mainil, 2013). (Kaper *et al.*, 2004)** found that Shiga toxins link to the glycolipids on (Gb3) sites on the cell surface. This stops protein synthesis and kills the cell.

In our study, we used specific primer sets in a PCR test to look for six virulence genes in *E. coli* isolates (*Iss, fimH, iutA, papC, adrA,* and *TSH*). The results demonstrated that *iss*, *fimH* and *iutA* were the genes that were found most often in all of the isolates that were tested Fig. (1, 2, 3) followed by the gene encoding fimbria papC 76% Fig. (4), iron acquisition gene 30\85 (*adrA*) gene in 35% Fig. (5) Followed by8\85 (*TSH*) gene in 9% Fig. (6).

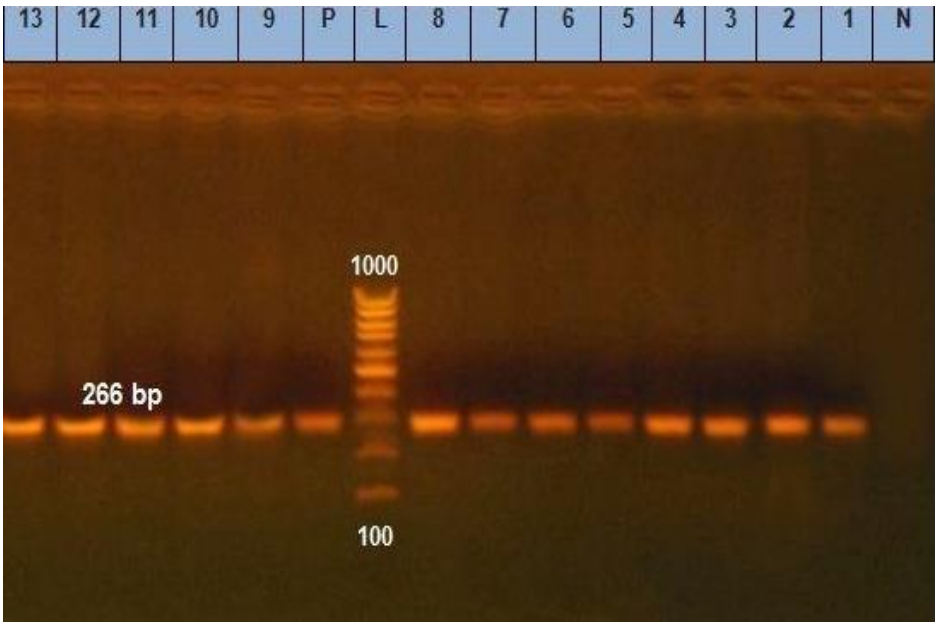


**Fig. (1):** All *E. coli* isolates (1-13) were positive for the *iss* gene (266 bp).

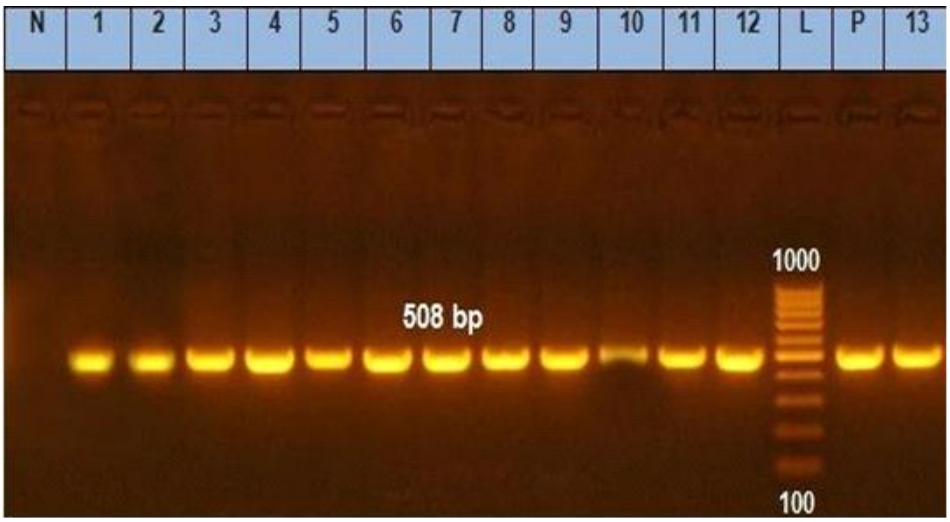


**Fig. (2):** All *E. coli* isolates (1-13) were positive for the *fimH* gene (508 bp).

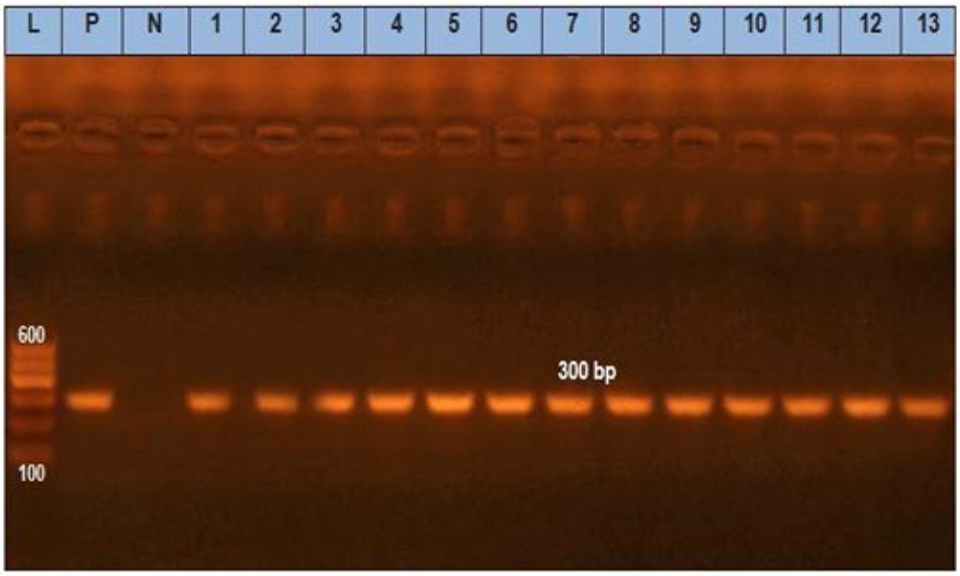


**Fig. (3):** All *E. coli* isolates (1-13) were positive for the *iutA*, gene (300bp).

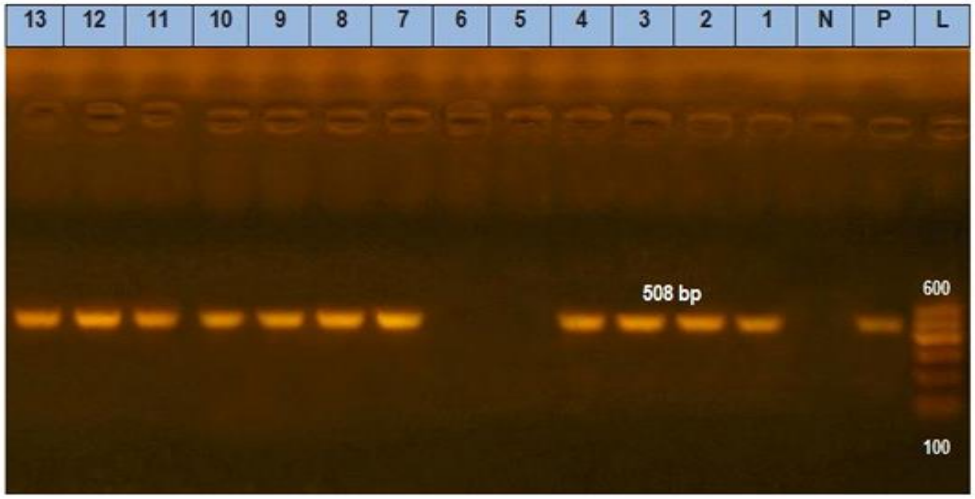


**Fig (4):** All *E. coli* isolates (5 and 12) were positive for the papC, gene (508bp).

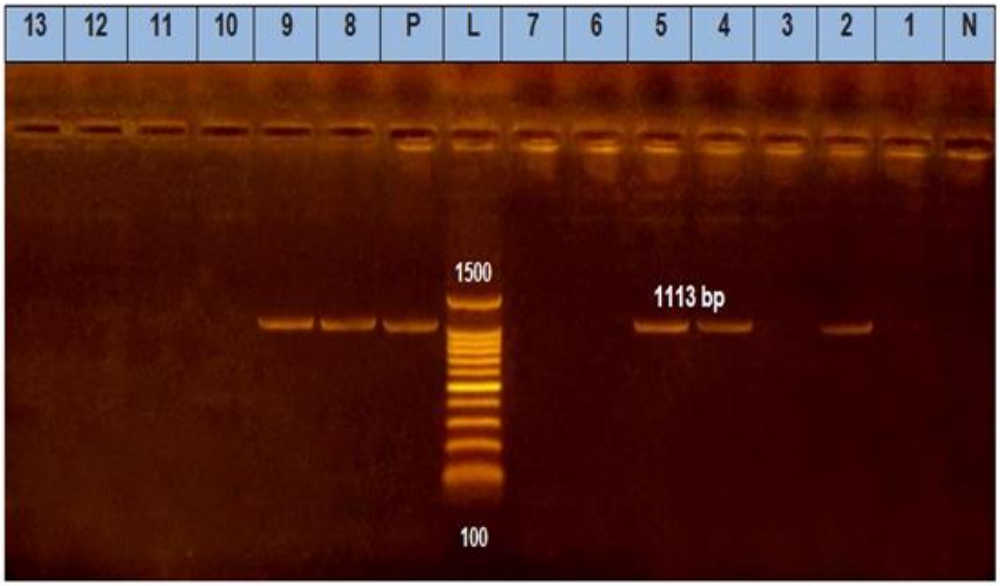


**Fig. (5):** *E. coli* isolates (2, 4, 5, 8 and 9) were positive for the *adrA*, gene (1113bp).

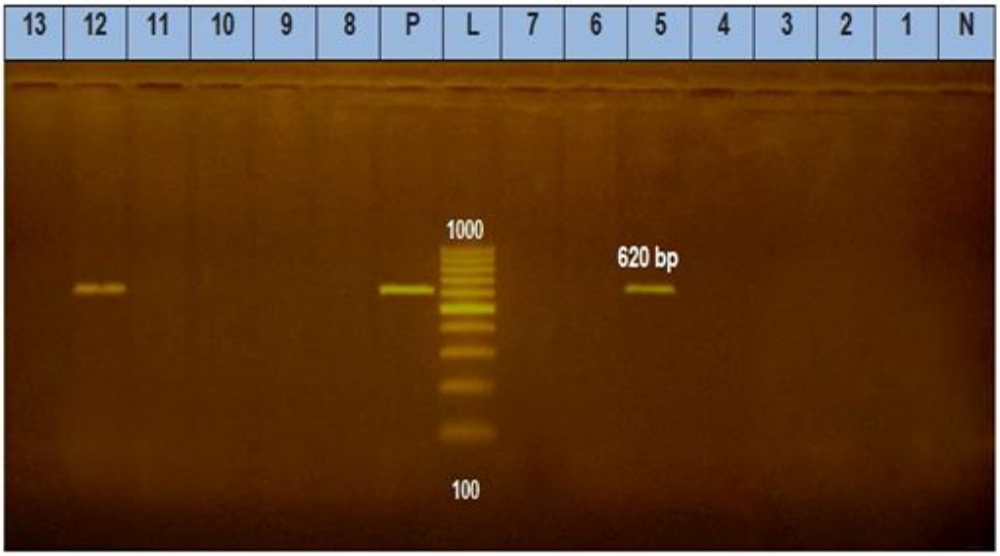


**Fig. (6):** All *E. coli* isolates (5 and 12) were positive for the *TSH*, gene (620bp).

Our results were similar to what **Kwon *et al.* (2002**) found in their study. They talked about how important fimbrial genes like F5 (K99) are for *E. coli* colonization in the mucosa of the small intestine of calves , *iutA* gene encodes the aerobactin siderophore ferric receptor protein, facilitates iron acquisition by mediating the uptake of siderophores They also talked about how

the frequency of the iss gene went from 80% to 100%. **Mellata *et al*. (2003)** found other fimbrial genes (F41 and F17) in ETEC in young calves. **Mousa *et al.* (2021)** found that *iss, fimH* present by 100% but *iutA* gene was not detected in any tested samples. Another study **(Lynne *et al.*, 2007)** explained how the *iss* and *bor* genes affect the pathogenicity of *E. coli* strains. These genes are thought to stop the effect of inhibitory mediators made by the host complement and resist the process of phagocytosis, which makes *E. coli* strains more dangerous to the target host, **(Binns *et al.*,1982)** suggested that, the *Iss* protein may have a role in formation of functional complexes in the junctions between the inner and outer membranes.

In this study, the *TSH* gene was found in the samples by 8%. This led to different results from the researchers. **(Saidenberg *et al.*, 2013)** showed that 85.3% of *E. coli* poultry strains had the TSH gene. **Mousa *et al.* (2021)** cannot detect any positive sample. In another study done in Egypt **(Abdulgayeid *et al.*, 2015),** the TSH gene was found in 100% of the *E. coli* from calves with diarrhea. Because of this difference, more research needs to be done on the role and frequency of this gene in different animal species, as well as the chance of transmission between species and the environmental risk factor.

**Conflict of Interest:**

The authors declare that they have no conflict of interest.

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## CONCLUSION

Based on the facts concluded through the research, it can be said that *Escherichia coli* diarrhea is very important because it can cause diarrhea in newborn calves and is also a source of infection that spreads among animals and causes high economic losses, especially in Siwa Oasis. The problem is compounded by the fact that there are strains that are becoming resistant to more than one type of antimicrobials and that *E. coli* contains virulence genes that can make calves sick. Also, it is important to identify more virulence genes and keep track of antibiotic resistance.

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