Microbiological and molecular characterization of E. coli and Salmonella isolated from diarrheic calves

Michael Salama1; Waleed Younis1; Hams. M. A. Mohamed1 and Serageldeen Sultan1*

1Department of Microbiology, Faculty of Veterinary Medicine, South Valley University. Qena, Egypt

Abstract

Diarrhea is one of the most common fatal disease of neonatal calves regardless of farm welfare. What makes treatment of these cases challenging is the increase in the incidence of multi-drug resistant bacteria, including E. coli and Salmonella. To isolate, identify, molecular characterize of E. coli and salmonella in diarrheic calves, 50 fecal samples were collected and analyzed aseptically. The preliminary result showed that 62% and 14% samples were positive for E. coli and salmonella respectively. Among the E.coli isolates O86:K59, O128:K-, O55:K59 (2), O86:K61, O119:K58, O08:K61, O126:K71 serotypes was detected in eight isolates while one isolate was untypeable. Three salmonella serotypes, Salmonella Typhimurium, Salmonella Anatum and Salmonella Florida was detected in the isolated samples. All of the E. coli strains had eaeA genes while 16.7 and 11.1% of them harbored stx1 and stx2 genes respectively. About 71.4% of salmonella isolates were positive for all five pathogenicity island genes from SPI-1 to SPI-5, while SPI-1, SPI-2 and SPI-5 were detected in 14.3% and SPI-2, SPI-3, SPI-4 and SPI-5 were positive in 14.3%. All E. coli strains were resistant to Ampicillin, Amoxicillin/Clavulanic, Cefazolin and Aztreonam., and all Salmonella serovars were resistant to Cefazolin, Chloramphenicol, Gentamycin, Kanamycin and Aztreonam. The present study identified multidrug resistant E. coli and salmonella as the common pathogen of calf diarrhea in the study area and the pathogens harbored common virulence markers of human diarrheic strains which might cause a food borne outbreak in the future.

Keywords: calves, diarrhea, E. coli, Salmonella, multi-drug resistant, PCR.

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*Corresponding Author: Serageldeen Sultan E-mail: s.sultan@vet.svu.edu.eg

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Introduction

Calves diarrhea is a global dilemma and it is considered one of the most threatening diseases of a newly born calf below one month in the whole world (Wei et al. 2021), as well as being one of the most common diseases of calves under the age of three months (Svensson et al., 2003).

Its threat is not only because of its drastic economic impact on farmers, as it causes enormous economic and productivity losses (Cho and Yoon, 2014), but also because of their public health and zoonotic importance as food born source of infection. Calf diarrhea is a multifactorial disease characterized by being a seasonal disease. In Egypt, according to Ezzat et al, 2023 most cases take place in the winter.

Several infectious agents are associated with calf diarrhea, such as viruses, bacteria and protozoa (Smith, 2009). Among bacterial pathogens, *E. coli* and Salmonella are the most important causative agents (Achá et al., 2004). However, *Clostridium perfringens*, *Pasteurella* spp., *Klebsiella* spp., *Proteus* spp. and *Pseudomonas aeruginosa* could also cause diarrhea in calves (Diwakar et al. 2014).

The importance of *E. coli* is attributed to causing severe watery diarrhea in the first four days of a newly born calf resulting in death within 24 hours (Cho et al, 2010). According to the virulence properties, *E. coli* is divided into 6 pathotypes; Enterohaemorrhagic (EHEC) or Shiga toxin-producing (STEC), Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enteroaggregative (EAEC) and diffusely adherent *E. coli* (DAEC). Many of these pathotypes represent a risk to public health and are responsible for several deadly outbreaks (Pakbin et al. 2020). Salmonella enterica causes severe symptoms in calves below 3 months because it is characterized by watery and mucoid diarrhea with the presence of blood and fibrin. The severity of the illness determines how long and how frequently calves shed salmonella (Molossi et al. 2021). Moreover, recovered cases become carriers and shed the bacteria to the environment for their lifetime (Radostits et al., 2007). There are about 2500 salmonella serovars (Davies, 2008), and many of them are implicated in causing calf diarrhea. Salmonella Typhimurium and Salmonella Dublin are incriminated of causing enteritis, diarrhea and septicemia (Costa, et al, 2012). Salmonella enterica and *E. coli* are two major zoonotic foodborne pathogens in the entire world (WHO, 2015). Salmonella was the most prevalent foodborne pathogen identified in the European Union in 2019 and caused 926 outbreaks (European Food Safety Authority, 2021). Furthermore, Salmonella Typhimurium and Salmonella Anatum are among the most prevalent serovars found in animal-based food (Ferrari et al, 2019).

This study aimed for detecting *E. coli* and salmonella as causative agents for calf diarrhea, exploring their antimicrobial sensitivity and the occurrence of the main virulence genes, from fecal samples, which were taken from calves suffering from diarrhea in farms located in Qena, Egypt.

Materials and methods

Sample preparation

A total of 50 fecal samples were aseptically collected in sterile plastic tubes from diarrheic calves of less than 3 months old age, during the winter and spring seasons of 2020-2022, and subjected directly for laboratory of Microbiology, faculty of Veterinary Medicine ,South Valley University for bacteriological identification.
Isolation and biochemical identification

The fecal samples were inoculated into 10 ml Buffered Peptone Water and incubated aerobically at 37°C for 24 hrs., a loopful from bacterial suspension were subcultured on onto MacConkey agar (Oxoid, England) and incubated at 37°C for 24 hrs. The pink colonies were subcultured on Eosin methylene blue (EMB) agar (Oxoid, England). In the same time a loopful from bacterial suspension were inoculated on Tetra Thionate broth (TTB) (Oxoid, England) at 37°C for 24 hrs, then a loopful from tetrathionate suspension were streaked onto Xylose Lysine Deoxycholate (XLD) media (Oxoid, England). Suspected isolates were morphologically identified by Gram’s stain and biochemical tests; IMViC, TSI and Urease test (Varnam and Evans, 1991) and (ISO 6579, 2002).

Serological identification

Serological testing was performed on 10 E. coli isolates out of 31 E. coli isolates (10/31), which were randomly selected according to farm location and clinical signs, and were carried on all Salmonella isolates (7). They underwent serotyping using standard polyvalent and monovalent E. coli antisera on basis of somatic (O) and capsular (K) for E. coli isolates and somatic (O) and flagellar (H) salmonella antisera for salmonellae (Sifin diagnostics GmbH, Germany).

Antibiotic susceptibility testing

The antimicrobial susceptibility test were carried by disc diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018), using the following selected discs; ampicillin (AMP, 10 µg), amoxicillin/clavulanic (AMC, 30 µg), cefazolin (CZ, 30 µg), chloramphenicol (C, 30 µg), sulfamethoxazole/trimethoprim (SXT, 25 µg), tetracycline (TE, 30 µg), gentamicin (GEN, 10 µg), kanamycin (K, 30 µg), azithromycin (AT, 15µg), aztreonam (AZT, 30 µg), nitrofurantoin (F, 30 µg), ciprofloxacin (CIP, 5 µg). Results of zone of inhibition were interpreted according to (CLSI, 2018).

Polymerase chain reaction (PCR)
DNA extraction

Bacterial DNA were extracted using WizPrep™ gDNA Mini Kit (Wizbiosolutions, Republic of Korea), following the manufacturer’s instructions.

Amplification of E. coli virulence genes

PCR was carried out to detect Shiga toxin genes (stx1, stx2) and intimin gene (eaeA) in (18/31) randomly selected E. coli isolates, based on farm location. Both stx1 and stx2 genes were prepared to be done in duplex PCR, which was performed in volume 50 µL containing: 25 µL Emerald Amp GT PCR master mix (Takara, France) Code No.RR310A, 1 µL of each forward and reverse primer (Metabion Germany) as shown in table (1), 6 µL DNA template and 15 µL PCR grad water. The thermal profile of PCR were performed as following: Initial denaturation at 94 ºC /5 min followed by 35 cycles of denaturation at 94ºC/30 seconds, annealing at 58 ºC /40 seconds and extension at 72 ºC /45 seconds followed by final extension at 72 ºC /10 min. Regarding eaeA gene, it was prepared in uniplex PCR in volume of 25 µL, which contains 12.5 µL Emerald Amp GT PCR master mix (Takara, France) Code No.RR310A, , 1 µL of forward primer and 1 µL of reverse primer (Metabion Germany) as shown in table (1), 5 µL DNA template and 5.5 µL PCR grad water. The temperature and time conditions of PCR were done as following: Primary denaturation at 94 ºC /5 min followed by 35 cycles of denaturation at 94 ºC /30 seconds, annealing at 51 ºC /30 seconds and extension at 72 ºC /30 seconds followed by
final extension at 72 °C /7 min. PCR for *E. coli* genes was performed using Thermal cycler (Biometa, Germany). Documentation was done by Gel documentation system (Alpha Innotech, USA).

**Table (1): Oligonucleotide primers sequences of *E. coli* genes.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Stx1</td>
<td>ACACTGGATGATCTCAGTGG</td>
<td>614 bp</td>
<td>Dipineto <em>et al.</em>, 2006</td>
</tr>
<tr>
<td></td>
<td>CTGAATCCCCTCCATTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> Stx2</td>
<td>CATGACACGCCAGACAGTT</td>
<td>779 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCTGTCAACTGAGAGCAGCTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> eaeA</td>
<td>ATG CTT AGT GCT GTT TTA GG</td>
<td>248 bp</td>
<td>Bisi-Johnson <em>et al.</em>, 2011</td>
</tr>
<tr>
<td></td>
<td>GCC TTC ATC ATT TCG TTC T</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Amplification of Salmonella virulence genes**

PCR assay was performed to detect 5 salmonella pathogenicity island genes; *InvE/A* for SPI-1, *ssaQ* for SPI-2, *mgtC* for SPI-3, *spi4R* for SPI-4 and *sopB* for SPI-5 in all Salmonella isolates (7). PCR primers that used for the amplification of virulence genes are listed in table (3). PCR tests were carried out in volume of 20 µL containing: 10 µL WizPure™ PCR 2X Master (Wizbiosolutions, Republic of Korea). 1 µL of each forward and reverse primer (Metabion Germany) as shown in table (2), 5 µL DNA template and 3 µL nuclease-free H2O. The thermal conditions of PCR were performed as following: Initial denaturation at 95 °C /5 min followed by 30 cycles of denaturation at 95 °C /1 min, annealing at 51 °C /1 min (*invaE/A* and *spidR*), 53 °C /1 min (*sopB*), 54 °C /1 min (*mgtC*) or 58 °C /1 min (*ssaQ*) and extension at 72 °C /1 min before a final extension at 72 °C /5 min. A 200 Gradient Thermal cycler (Japan) was used to perform PCR tests. PCR products were separated by gel electrophoresis in 1.5% agarose in Tris–acetate–EDTA (TAE) buffer at 100 V. And Solis Bio Dyne 100 bp ladder was included in each agarose run, visualized and documented by UV light illumination Gel documentation system (UVP Photo Doc) U.K.

**Table (2): Oligonucleotide primers sequences were used for characterization of Salmonella isolates**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>InvE/A</em> (F)</td>
<td>SPI-1</td>
<td>TGCCCTACAAGCATGAAATGG</td>
<td>450 bp</td>
<td>Sánchez-Jiménez <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAACTGGACCACGGTAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>InvE/A</em> (R)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ssaQ</em> (F)</td>
<td>SPI-2</td>
<td>GAATACGCAATGAAGAGCGTCC</td>
<td>677 bp</td>
<td>Soto <em>et al.</em>, 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATCGGTATATCCCTGTCAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ssaQ</em> (R)</td>
<td>SPI-3</td>
<td>TGACTATCAATGCTCCAGTGAAAT</td>
<td>655 bp</td>
<td>Sánchez-Jiménez <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATTTACTGGCCCGCTATGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mgtC</em> (F)</td>
<td>SPI-4</td>
<td>GATATTTATCGTCTATAACAGC</td>
<td>1269 bp</td>
<td>Sánchez-Jiménez <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATTCTATCCAGAGTGGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mgtC</em> (R)</td>
<td>SPI-5</td>
<td>GATGTTGATATAATGAAGAAATGCC</td>
<td>1170 bp</td>
<td>Soto <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>spi4R</em> (F)</td>
<td></td>
<td>GCAAACATTTAAAATTTACTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>spi4R</em> (R)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sopB</em> (F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sopB</em> (R)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table (3): Antimicrobial resistance profile of *E. coli* strains isolated from diarrheic calves.**

<table>
<thead>
<tr>
<th>NO</th>
<th><em>E. coli</em> strains</th>
<th>Antimicrobial resistance profile</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O86:K59</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AZT, CIP.</td>
<td>0.833</td>
</tr>
<tr>
<td>2</td>
<td>O128:K-</td>
<td>AMP, AMC, CZ, TE, GEN, K, AZT.</td>
<td>0.583</td>
</tr>
<tr>
<td>3</td>
<td>O55:K59</td>
<td>AMP, AMC, CZ, C, SXT, TE, K, AT, AZT.</td>
<td>0.750</td>
</tr>
<tr>
<td>4</td>
<td>O55:K59</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AT, AZT, CIP.</td>
<td>0.917</td>
</tr>
<tr>
<td>5</td>
<td>O86:K61</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AT, AZT, CIP.</td>
<td>0.917</td>
</tr>
<tr>
<td>6</td>
<td>O119:K58</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AZT.</td>
<td>0.750</td>
</tr>
<tr>
<td>7</td>
<td>O08:K61</td>
<td>AMP, AMC, CZ, C, SXT, TE, K, AT, AZT, CIP.</td>
<td>0.750</td>
</tr>
<tr>
<td>8</td>
<td>O126:K71</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AZT.</td>
<td>0.750</td>
</tr>
</tbody>
</table>

Ampicillin (AMP), Amoxicillin/clavulanic (AMC), Cefazolin (CZ), Chloramphenicol (C), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (TE), Gentamycin (GEN), Kanamycin (K), Azithromycin (AT), Aztreonam (AZT), Nitrofurantoin (F), Ciprofloxacin (CIP).

MAR index: Multi antibiotic resistant index

**Table (4): Antimicrobial resistance profile of Salmonella strains isolated from diarrheic calves.**

<table>
<thead>
<tr>
<th>NO</th>
<th><em>Salmonella</em> strains</th>
<th>Antimicrobial resistance profile</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. Typhimurium</td>
<td>CZ, C, GEN, K, AT, AZT</td>
<td>0.500</td>
</tr>
<tr>
<td>2</td>
<td>S. Typhimurium</td>
<td>AMP, AMC, CZ, C, TE, GEN, K, AT, AZT, CIP.</td>
<td>0.833</td>
</tr>
<tr>
<td>3</td>
<td>S. Anatum</td>
<td>AMP, AMC, CZ, C, TE, GEN, K, AZT.</td>
<td>0.667</td>
</tr>
<tr>
<td>4</td>
<td>S. Anatum</td>
<td>AMP, AMC, CZ, C, TE, GEN, K, AT, AZT, CIP.</td>
<td>0.833</td>
</tr>
<tr>
<td>5</td>
<td>S. Anatum</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AZT, CIP.</td>
<td>0.833</td>
</tr>
<tr>
<td>6</td>
<td>S. Anatum</td>
<td>AMP, AMC, CZ, C, TE, GEN, K, AZT, CIP.</td>
<td>0.750</td>
</tr>
<tr>
<td>7</td>
<td>S. Florida</td>
<td>AMP, AMC, CZ, C, SXT, TE, GEN, K, AT, AZT, F, CIP.</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Ampicillin (AMP), Amoxicillin/clavulanic (AMC), Cefazolin (CZ), Chloramphenicol (C), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (TE), Gentamycin (GEN), Kanamycin (K), Azithromycin (AT), Aztreonam (AZT), Nitrofurantoin (F), Ciprofloxacin (CIP).

MAR index: Multi antibiotic resistant index

**Results**

**Bacterial isolation and biochemical testing**

Bacterial isolation and biochemical tests revealed that out of 50 samples, there are 62% (31/50) *E. coli* isolates and 14% (7/50) salmonella isolates. salmonella isolates were isolated from samples that also *E. coli* were isolated from it, while *E. coli* colonies were detected in 48% of samples without detection of salmonella on it.

**Serotyping**

The results of *E. coli* serotyping revealed that 7 different serotypes of *E. coli* and the results of serotyping of salmonella revealed only 3 serotypes. *E. coli* serotypes were as (1\(\text{\textregistered}\)10) 10% O86:K59, (1\(\text{\textregistered}\)10) 10% O128:K-, (2\(\text{\textregistered}\)10) 20% O55:K59, (1\(\text{\textregistered}\)10) 10% O86:K61, (1\(\text{\textregistered}\)10) 10% O119:K58, (1\(\text{\textregistered}\)10) 10% O08:K61, (1\(\text{\textregistered}\)10) 10% O126:K71 and (1\(\text{\textregistered}\)10) 10% untypeable with the available antisera and 1 sample was not identified as *E. coli*. While, Salmonella isolates serotypes were (2\(\text{\textregistered}\)7) 28.6% Salmonella Typhimurium 1,4,[5],12:i:1,2., and (4\(\text{\textregistered}\)7) 57.1% Salmonella Anatum.
Antimicrobial susceptibility Testing

Antibiotic susceptibility test which was done to *E. coli* and Salmonella isolates showed multiple multidrug resistance patterns and the most significant pattern of all isolates is Salmonella Florida which was resistant to all the tested antibiotics. Figure (1) highlights the percentage of the resistance, intermediate sensitivity and sensitivity *E. coli* isolates. All tested *E. coli* isolates were resistant to ampicillin, amoxicillin/clavulanic, aefazolin and aztreonam. Whereas Figure (2) shows the sensitivity of salmonella isolates of 12 antimicrobials, depicting that all Salmonella serovars are resistant to cefazolin, chloramphenicol, gentamycin, kanamycin and aztreonam. The antimicrobial resistance profiles for each serotype are shown in tables (3 and 4).

![Figure 1](image1.png)

**Figure (1).** Results of antimicrobial sensitivity of *E. coli* isolates.
Ampicillin (AMP), Amoxicillin/clavulanic (AMC), Cefazolin (CZ), Chloramphenicol (C), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (TE), Gentamycin (GEN), Kanamycin (K), Azithromycin (AT), Aztreonam (AZT), Nitrofurantoin (F), Ciprofloxacin (CIP).

![Figure 2](image2.png)

**Figure (2):** Results of antimicrobial sensitivity of Salmonella isolates.
Ampicillin (AMP), Amoxicillin/clavulanic (AMC), Cefazolin (CZ), Chloramphenicol (C), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (TE), Gentamycin (GEN), Kanamycin (K), Azithromycin (AT), Aztreonam (AZT), Nitrofurantoin (F), Ciprofloxacin (CIP).
Molecular characterization and identification.

Firstly, all *E. coli* isolates which were tested for *eaeA* gene were positive as shown in figure (3). Secondly, *stx1* and *stx2* genes were detected in 16.7 and 11.1% respectively, as shown in figure (4). On the other hand, all 5 Salmonella pathogenicity islands genes were detected in 71.4% of isolates, 14.3% possess only SPI-1, SPI-2 and SPI-5 and 14.3% possess SPI-2, SPI-3, SPI-4 and SPI-5 as shown in figure (5, 6 and 7).

Figure (3): Result of PCR to detect *eaeA* gene of *E. coli* obtained from diarrheic calves by 1.5% agarose gel electrophoresis. Lanes 1-18: Positive *E. coli* strains for *eaeA* gene 248 bp, Lane L: GeneRuler 100 bp DNA ladder (Lane P: Positive control. Lane N: Negative control.

Figure (4): Result of PCR to detect *stx1* and *stx2* genes of *E. coli* obtained from diarrheic calves by 1.5% agarose gel electrophoresis. Lanes 1, 2, 3, 4, 5, 6, 8, 9, 11, 14, 16, 17 and 18: Negative *E. coli* strains for *stx1* 614 bp and *stx2* 779 bp., Lanes 7, 12 and 13: Positive *E. coli* strains for *stx1* 614 bp., Lanes 10 and 15: Positive *E. coli* strains for *stx2* 779 bp., Lane L: GeneRuler 100 bp DNA ladder, Lane P: positive control., Lane N: Negative control.
Figure (5): Results of PCR products of five pathogenicity island genes (SP-1 (450 bp), SP-2 (677 bp), SP-3 (655 bp), SP-4 (1269 bp) and SP-5 (1170 bp)) of salmonella serotypes isolated from diarrheic calves, by 1.5 % agarose gel electrophoresis. Lane L: 100 bp DNA ladder, Lanes (1-5): Pathogenicity islands of Salmonella Florida (10), Lanes (6-10): Pathogenicity islands of Salmonella Anatum (25) Lanes (11-15): Pathogenicity islands of Salmonella Anatum (39).

Figure (6): PCR results of 3 genes (SP-1 (450 bp), SP-2 (677 bp), SP-3 (655 bp) of Salmonella Anatum and Salmonella Typhimurium serotypes isolated from diarrheic calves, by 1.5% agarose gel electrophoresis. Lane L: 100 bp DNA ladder (Solis BioDyne, Estonia), Lanes 1-3: Positive results for SP-1 (450 bp) of Salmonella Anatum (41), Salmonella Anatum (42) and Salmonella Typhimurium (43). Lane 4: Negative result for SP-1 (450 bp) of Salmonella Typhimurium (50). Lanes 5-8: Positive results for SP-2 (677 bp) of Salmonella Anatum (41), Salmonella Anatum (42), Salmonella Typhimurium (43) and Salmonella Typhimurium (50). Lanes 9-12: Positive results for SP-3 (655 bp) of Salmonella Anatum (41), Salmonella Anatum (42), Salmonella Typhimurium (43) and Salmonella Typhimurium (50).
Figure (7): PCR result of 2 genes SP-4 (1269 bp) and SP-5 (1170 bp) of Salmonella Anatum and Salmonella Typhimurium serotypes isolated from diarrheic calves by 1.5% agarose gel electrophoresis. Lane L: 100 bp DNA ladder (Solis BioDyne, Estonia), Lanes 1-4: Positive results for SP-4 (1269 bp) Salmonella Anatum (41), Salmonella Anatum (42), Salmonella Typhimurium (43) and Salmonella Typhimurium (50). Lanes 5-8: Positive results for SP-5 (1170 bp) of Salmonella Anatum (41), Salmonella Anatum (42), Salmonella Typhimurium (43) and Salmonella Typhimurium (50).

Discussion

Diarrhea continues to be a pressing economic problem for cattle producers not only in Egypt but also in the world (Farid et al., 2001 and Ibrahim, 2007). E. coli and Salmonella as significant bacterial agents, with Rotavirus and Coronavirus are the most predominant causes of calf diarrhea (Foster and Smith, 2009). This study reveals that the percentage of E. coli and Salmonella are 62 and 14% respectively. The occurrence of E. coli is in agreement with the results of Osman et al, (2013) which was detected E. coli by 63.6%, higher than (El-Tawab et al, 2017) who reported E. coli by 47% and lower than (El-Seedy et al, 2016) who isolated E. coli by 75.6%. The incidence of Salmonella is within the same range of other studies in Egypt, such as; (Gharieb et al, 2015):16.25%, lower than (Mousa et al, 2010) 45,53% and higher than (Younis et al, 2009):4.09%. The variation in the results may be due to geographical location, weather, and management protocols.

Serological tests results unveil eight serovars for E. coli; O86:K59, O128: K-, O55:K59(2), O86:K61, O119:K58, O08:K61, O126:K71. O-Serogroups O8, O55, O86, O119, O126 and O128 have shown frequency in most previous studies that concerned calf diarrhea. For example, O8, O55 and O126 were among results of (Maher et al 2017), whereas O119 and O126 were the most prevalent serotypes with (El-Seedy et al, 2016). According to (Nataro & Kaper, 1998), O55, O86, O119, O126 and O128 were recognized as EPEC. On the other hand, O08 and O128 serogroups belong to ETEC, in addition to that, some EPEC serogroups identified as EHEC, such as O55, O119, O126 and O128 (Stenutz et al, 2006). Several publications linked possession of eaeA and stx to the pathotype of E. coli. According to (Kolenda, et al, 2015), serovars that are eaeA- positive and stx- positive are EHEC, while those which possess eaeA solely are EPEC and serovars which are positive to either stxl or stx2 and negative to eaeA are
STEC, and this presumption largely correlates with our results.

Concerning Salmonella serotyping, the test resulted in 3 serovars; Salmonella Typhimurium, Salmonella Anatum and Salmonella Florida with a percentage of 57.1, 28.6 and 14.3% respectively. These outcomes may not coincide with most studies in Egypt, however, Salmonella Typhimurium is always one of the most common serovars in most previous studies, such as (Youssef and El-Haig 2012) and (El-Tawab et al, 2017). In respect of Salmonella Anatum which is the most prevalent serovar in the current study. It came after Salmonella Typhimurium according to (Zahran et al, 2014). Salmonella Anatum and Salmonella Typhimurium are the most noteworthy serovars because they are frequently isolated from cattle lymphatic system (Gragg et al, 2013), which implies that they may contaminate meat during the slaughter or inspection process then to consumers, causing serious health problems.

In this current study, despite its powerful molecular and antimicrobial resistance profile, Salmonella Florida is the least prevalent serovar. Nevertheless, Salmonella Florida has not been reported or isolated from diarrheic calves in previous studies in Egypt or around the world.

Molecular detection of virulence genes shows some significant findings. Starting with *E. coli*, all tested isolates are positive for *eaeA* gene, 16.7% *stx1* and 11.1% *stx2*. *eaeA* gene codes for intimin, which plays an important role in the adherence of bacteria to intestinal epithelial cells. therefore, it results in severe damage to the adjacent microvilli (Ateba et al, 2014). According to the results of PCR of *E. coli* positive only for *eaeA* gene, there is around 13 *E.coli* are EPEC and there is 5 *E.coli* isolates were positive for *eaeA* with *stx1*and/or *stx2* so it is classified as EHEC. The results of *eaeA* gene is higher than other recent studies in Egypt such as; (Mousa et al, 2021) and (Ezzat et al, 2023), which are relatively higher than most studies in Egypt. Salmonella molecular identification of Salmonella pathogenicity islands (1-5) virulence genes shows variable results; the majority of isolates possess all of the 5 genes with a percentage of 71.4%, while SPI-1, SPI-2 and SPI-5 detected in 14.3% and SPI-2, SPI-3, SPI-4 and SPI-5 were positive in 14.3%. Notably, from the molecular profile of these serovars, they presumably are implicated in causing calf diarrhea. It is known that SPI-1 and SPI-5 are involved in the type III secretion system, and are primarily responsible for calf diarrhea caused by Salmonella (Tsolis et al.1999), while SP-2 is concerned with the second type III secretion system and is responsible for the survival of the organism intracellularly (Ochman et al., 1996).

Antimicrobial susceptibility testing revealed several multi-drug resistant profiles. Tables (3) and (4) show that all serovars are multi-drug resistant and the markedly significant serovars are *E. coli* O55:K59, O86:K61, which are resistant to 11 drugs out of 12 and Salmonella Florida which is resistant to all of the 12 selected antibiotics. Antibiotic resistance is multifactorial and complex and constitutes a major problem in treatment of calf diarrhea. Perhaps, one of the most critical causes of antibiotic resistance in Egypt is the administration of antibiotics without doing the appropriate laboratory tests. Another reason for this is some farms adopt prophylactic antibiotic administration from the first day of birth. Here comes the role of veterinarians to ensure responsible antibiotic use on-farm, even though they do
not directly administrate the medicine and considering the ideal way of the choice of antibiotics is running culture and sensitivity testing, which ensure the best practice (BVA, 2019). In addition, with increasing the rate of multi-drug resistant bacteria, applying the preventive measures and vaccination are the best way to reduce the disease (Pereira et al, 2017)

**Conclusion**

*E. coli* and Salmonella keep the predominance of being the major bacterial causes of calves diarrhea and it is worthy of note that developing of multi-drug resistant strains makes treatment of these cases is a real struggle in the meantime. In addition, these pathogens are infectious to human and might transfer the antibiotic resistance genes to human via food chain.

**References**


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