PREVALENCE, ANTIMICROBIAL RESISTANCE AND SUBSTANTIAL VIRULENCE-ASSOCIATED GENES OF ESCHERICHIA COLI ISOLATED FROM COLIBACILLOSIS IN NEONATAL CALVES IN EGYPT

Walid S. Mousa and Usama H. Abo Shama

Address(es):
1 Department of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City, Egypt.
2 Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Sohag University, Sohag 82524, Egypt.

*Corresponding author: Usama.shama@gmail.com


ARTICLE INFO

Received 13. 11. 2019
Revised 4. 2. 2020
Accepted 5. 2. 2020
Published 1. 6. 2020

ABSTRACT

Pathogenic Escherichia coli (E. coli) consider the most common bacterial agent causes calf diarrhea, particularly in newborn calves. Therefore, this study highlights prevalence, serotyping, antigen detection, polymrase chain reaction (PCR) and virulence pattern of E. coli recovered from diarrheic calves. In addition to the investigation of six virulence encoding genes (tsb, stx1, stx2, eae, ipaH, cnf1, cnf2, cdhl and f7c) were isolated and predominated as virulence properties of E. coli strains particular of calves origin (Shahrani et al., 2014). The use of antimicrobial agents is usually an effective tool applied for the prevention and treatment of neonatal calf diarrhea. However, this method may lead to the evolution of resistant bacterial strains to several antimicrobial drugs. Moreover, in recent years, much international surveillance recorded multidrug-resistant of E. coli strains of animals or human origin. The significant economic losses due to diarrhea in the dairy farm as well as in small-holder in Egypt encourage this work to investigate the prevalence, antimicrobial pattern, and distribution of the iss, fimbH, tsh, ipaH, stx2 and eaeA virulence genes between E. coli isolates recovered from diarrheic calves.

KEYWORDS: Escherichia coli, calf diarrhea, serotyping, antimicrobial profile, virulence genes

INTRODUCTION

Calf diarrhea represents a prevalent financial dilemma in the animal industry worldwide (Shahrani et al., 2014). Diarrhea is a common cause for high morbidity and case fatality rates, particularly in newborn calves. Various microorganisms, such as protozoa (Cryptosporidium parvum), viruses (coronavirus and rotavirus) and bacteria can cause diarrhea (Izzo et al., 2011). Although various bacterial types have been recovered from calves with diarrhea, E. coli remains an important noteworthy pathogenic bacterium in diarrheic calves and its prevalence varies according to geographic area (Garcia et al., 2000). In general, this bacterium lives naturally in both human and animal intestines. However, certain strains can become pathogenic by producing virulence factors and can, therefore, cause enteric infections (Pico et al., 2015). Many researchers recorded that among ruminant, cattle may implemented the use of antimicrobial agents is usually an effective tool applied for the prevention and treatment of neonatal calf diarrhea (Berge et al., 2009). However, this method may lead to the evolution of resistant bacterial strains to several antimicrobial drugs (Hammerum and Heuer, 2009). Furthermore, in recent years, much international surveillance recorded multidrug-resistant of E. coli strains of animals or human origin (Woodford et al., 2011). In animals, rapid evolution of antimicrobial resistance has been recently reported within E. coli isolates recovered from neonatal calves, especially those received receipt of antimicrobial therapy. The significant economic losses due to diarrhea in the dairy farm as well as in small-holder in Egypt encourage this work to investigate the prevalence, antimicrobial pattern, and distribution of the iss, fimbH, tsh, ipaH, stx2 and eaeA virulence genes between E. coli isolates recovered from diarrheic calves.

MATERIALS AND METHODS

Sample collection and Study area

The current study was performed from October 2018 to April 2019, through which cattle parturition rate is high. One-hundred and twenty calves under one-month-old were monitored clinically for the incidence of diarrhea cases from small-holder in Menoufiya Province, Egypt. The clinical examination was done as described by Jackson and Cockcroft (2002), including clinical signs, systemic reaction, dehydration, pulse and respiratory rates. Forty fecal samples were aseptically collected from diarrheic calves using sterilized bacteriological swabs and directly transferred in the icebox to the lab for bacteriological examination.

Bacteriological examination of E. coli strains

Based on their virulence factors, pathogenic E. coli strains can be divided into six types: (i) enteropathogenic E. coli (EPEC); (ii) enteroaggregative E. coli (ETEC); (iii) enterohemorrhagic E. coli (EHEC), also known as shiga toxin-producing E. coli (STEC); (iv) enteroaggregative E. coli (EAEC); (v) diffusely adherent E. coli (DAEC); and (vi) enteroinvasive E. coli (EIEC) (Kaper et al., 2004). All these pathotypes are mainly associated with diarrheagenic strains. A number of probable virulence of E. coli strains are potential based on the presence of substantial factors, such as adhesins, fimbriae, invasins, toxins, capsules, siderophores and hemolysins which play a critical role to avoid or suppress defenses and inflammatory response of the host (Bekal et al., 2003 and Croxen and Finlay, 2010). Among the ETEC and EPEC pathotypes, molecular analysis has revealed a considerable divergence in virulence genotypes (Behin et al., 2005). The pathogenicity of E. coli based mainly on adhesion and binding to the receptor of the mucosa of the small intestine using of fimbrial factors and exotoxins (Kaper et al., 2004) including F5 (K99), F41 and heat-stable enterotoxins (Acres, 1985).
The collected fecal swab samples were inoculated into tryptose soy broth (mTSB- Difco La Jolla, CA, USA) and incubated at 37°C for 12 hours and then sub-cultured on a selective culture medium (MacConkey agar, MAC, Difco) at 37°C for one day. Pink color colonies were then picked up and cultured in eosin methylene blue (EMB; Difco) medium. Metallic green colonies were considered E. coli. Confirmation of all isolates were done through morphological characters. Gram staining and standard biochemical tests, including indole, citrate utilization, Voges-Proskauer, methyl red, Triple Sugar Iron (TSI), and urease tests as described by (Cowan and Steel, 1985) and biofilm activity on Congo red medium according to (Nivedith et al., 2012).

E. coli isolates Serotyping

Serotyping using the slide agglutination test for E. coli isolates was done as described by (Edwards and Ewing, 1972).

Antibiogram profile

The antimicrobial susceptibility test of E. coli strains was performed using a Kirby-Bauer disk diffusion assay according to (CLSI, 2002). Bacterial suspension was prepared according to (Tenover, 2009). E. coli isolates were then examined in vitro for their susceptibility to 10 antimicrobial discs (Oxoid): tetracycline: TET, amoxicillin/clavulanic acid: AMC, chloramphenicol: C, amikacin: AK, streptomycin: S, enrofloxacin: ENR, sulfamethoxazole/trimethoprim: SXT, gentamicin: CN, neomycin: N, ciprofloxacin: CIP, and polymyxin: PB. The inhibition zone was measured to survey the resistance or the susceptibility according to the interpretation criteria established by CLSI standard.

Molecular characterization of E. coli serovars virulence genes

Freshly grown typical E. coli-like colonies were collected and the DNA extraction was done according to the manufacturer's guidelines using QiAamp DNA Mini Kit DNA extraction kit (Quagen, GmbH, Germany). All E. coli strains were screened for six virulence genes (iss, fimH, tsh, iutA, stx2 and eaeA) by PCR. Several PCR protocols were used to detect the target genes of E. coli isolates. Lists of primers used, the PCR conditions, and the amplified products are listed in Tables 1. Amplification of DNA was carried out a total reaction of 25 μl including 12.5 μl of PCR Master Mix, 1 μl of each primer of 20 pmol concentration, 6 μl of DNA template and 4.5 μl of purified water. The primers (Metabon, Steinkirchen, Germany) used for detecting the virulence genes of E. coli (fimH and tsh) (Colom et al., 2003), eaeA (Archambault et al., 2006), iss and iutA (Ghanbarpour and Salehi, 2010) and for stx2 gene (Bisi-Johnson et al., 2011).

The reactions were performed in an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster, CA). Five microliter aliquots of all PCR products and a 100 bp DNA ladder were loaded in the wells, electrophoresed through 1.5% agarose gels, stained with ethidium bromide and photographed under UV transilluminator.

### Table 1 Target genes, primer sequences, amplicon sizes and cycling conditions of virulence genes of E. coli isolates

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences Virulence genes</th>
<th>Amplicon amplicon (bp)</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iss</td>
<td>ATGTATATTGTCGCCGCTGTCG</td>
<td>266</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>54˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>72˚C 7 min.</td>
</tr>
<tr>
<td></td>
<td>CTATTGTCGACATATACCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2</td>
<td>CCATGACAAAGGACAGCATGTT</td>
<td>779</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>58˚C 45 sec.</td>
<td>72˚C 45 sec.</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td></td>
<td>CCGTCACTGACAGCAGCCTTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fimH</td>
<td>TGCAAGACGGATAAGCCTGTT</td>
<td>508</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>50˚C 45 sec.</td>
<td>72˚C 45 sec.</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td></td>
<td>GCAGTCACCTGCCCCCTCGTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsh</td>
<td>GGT GGT GCA CTG GAG TGG</td>
<td>620</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>54˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td></td>
<td>AGT CCA GGC TGA TAG TGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iutA</td>
<td>GGCTGACATGGGAAACCTG</td>
<td>300</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>63˚C 45 sec.</td>
<td>72˚C 45 sec.</td>
<td>72˚C 7 min.</td>
</tr>
<tr>
<td></td>
<td>CGTCGAAACGGTAGAATCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>ATG CTT AGT GCT GGT TTA GG</td>
<td>248</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>51˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>72˚C 7 min.</td>
</tr>
<tr>
<td></td>
<td>GCC TTC ATC ATT TCG CTTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All genes were amplified for 35 cycles.

RESULTS AND DISCUSSION

Prevalence and clinical examination of diarrheic calves

Calf diarrhea is considered a major acute disease in neonatal calves causing high mortality and significant economic losses. This syndrome caused by multiple etiology (e.g. bacteria, viruses, protozoa) led to severe mucoid diarrhea, dehydration and rapid weight loss and may end with death in complicated non-treated calves (Galal et al., 2013 and Cho and Yoon, 2014). E. coli is the primary bacterial pathogenic agent responsible for calf diarrhea (Osman et al., 2012).

In our present work, a total of 120 samples were collected from under one month old calves that monitored clinically for the incidence of diarrhea cases from small-holder in Menoufiya Province, Egypt and examined for the incidence of E. coli. The clinical observation and examination of diseased calves revealed profuse watery and mucoid diarrhea with a variable degree of dehydration that was detected by a decrease in skin elasticity, sunken eyes, cold extremities and inability to stand. All affected calves were depressed and had a rapid pulse and respiratory rates and rough coats. Systemic reactions were observed in some of the affected animals.

Of 120 examined calves samples, forty (33.3%) were diarrheic, sixteen (40%) of the examined diarrheic calves were positive for E. coli. Our result, nearly similar to those of other studies in India reported by (Malik et al., 2013 and Shekhar et al., 2017) who isolated E. coli from diarrheic calves with a prevalence rate of 37.61% and 41.6%, respectively. In Egypt (Galal et al., 2013) reported an E. coli prevalence rate of 28.57%. On contrary, this result was lower than that reported by (Osman et al., 2013; Anwarullah et al., 2014 and Majueeb et al., 2014) with a prevalence rate of 63.6%, 72.8%, and 50%, respectively. While, (Duse et al., 2015 and El-Seedy et al., 2016) reported a lower prevalence rate with 14.6% and 18.1%, respectively. The variations in the results might attribute to the difference in the number of collected samples, geographical regions, stress factors, hygienic and management systems.

Serogrouping of E. coli isolates

Serogrouping of sixteen E. coli isolates in our study revealed that O26 and O157 were the most common identified E. coli serogroups with prevalence rate 25% of each type. Other E. coli serogroups (O178, O125, and O146) were identified with a prevalence rate of 12.5% for each and finally E. coli serogroups (O44 and O18) with a prevalence rate of 6.25% for each. Our results are in agreement with (Shahrami et al., 2014) who conducted that E. coli O26 and O157 were the most E. coli serogroups isolated from diarrheic calves. Other studies reported by (El-Seedy et al., 2016) revealed that O26 and O103 were the most prevalent (17.7% of each) E. coli serogroups isolated from neonatal diarrheic calves. However,
(Badouel et al., 2010) recorded that O26 is the most prevalent E. coli serotypes with (18.4%) as well as O157:H7, O111, and O26 were also serotyped from diarrheic and non-diarrheic calves. While, (Sardiakas et al., 1997) reported that O26, O114 and O119 were the most identified E. coli strains obtained from diarrheic calves. In addition to (Mora et al., 2011) who typing 52% of bovine E. coli strains serovars as belonged to O4, O20, O22, O26, O77, O105, O113, O157, and O171 serovars. The variation in the dominate serogroups of E. coli may explain the difference in geographic areas and numbers of the collected samples.

Antimicrobial susceptibility pattern of E. coli serotypes from diarrheic calves

Concerning antimicrobial susceptibility testing in this study, tested E. coli strains showed sensitivity against ciprofloxacin, enrofloxacin, polymixin and amoxicillin/clavulanic acid with percentage 81.25%, 75%, 75% and 62.5%, respectively. Meanwhile, streptomycin showed complete resistance (100%);

The high incidences of E. coli isolates to tetracycline in our study were in contrary to Kwon et al., (2011). However, (Schröder et al., 2002; Rigobelo et al., 2006; Alberto et al., 2011 and Hang et al., 2019). In our study, the screening of six virulence genes in isolates from diarrheic calves to tetracycline (63.21%) and susceptible to chloramphenicol, gentamycin (96.33%) and imipenem (99.06%) in addition to 69.81% of these strains expresses multidrug resistance. Furthermore, (Hang et al., 2019) found that tetracycline was detected as the most resistant antibiotic by E. coli strains followed by sulfamethoxazole, ampicillin, trimethoprim, and ciprofloxacin.

Another investigation in Egypt by (El-Seedy et al., 2016) demonstrated that E. coli isolates had high susceptibility to marbofloxacin, spectomycin, and neomycin. Otherwise, (Ortman and Svensson, 2004) discussed that trimethoprim/sulfa and enrofloxacin were considered an effective treatment for diarrhea in young calves.

The high resistances of E. coli isolates to tetracycline in our study were previously supported by many authors (Schröder et al., 2002; Rigobelo et al., 2006; Alberto et al., 2011 and Hang et al., 2019). However, higher resistance to streptomycin, gentamycin, tetracycline, chloramphenicol, sulfamethoxazole/trimethoprim and neomycin in our study was in contrary to other reports by (Wani et al., 2013; Rehman et al., 2014 and Srivani et al., 2017), that reported STEC isolates from diarrheal calves showed high susceptibility to gentamycin and chloramphenicol.

The high incidence of resistant or multi-resistant E. coli isolates in the present study may be attributed to the wide and haphazard using of antimicrobials in animals for treatment, prevention, and control of infectious diseases, and as growth promoters for potential livestock production (Marshall and Levy, 2011). In Egypt, the random using of these traditional antibiotics without veterinarian prescription and the overuse or misuse of antimicrobials in the veterinary field by dairy farmers, lead to emerging of their resistance genes which are of public concern as it could be passed on to humans (WHO, 2014).

Molecular detection of E. coli virulence genes

 Regarding E. coli strains, their pathogenicity mainly relies on the existence of powerful toxins and certain virulence determinants, of those shiga toxins (ST) and intimin (eaeA) that facilitate the adhesion and colonization of E. coli strains in the host cells (Oswald et al., 2000). Intimin (eaeA gene) is an essential genetic element for attaching/effacing (A/E) lesion formation. Moreover, numerous fimbrial genes are proposed to have critical roles in the adhesion, attachment, and colonization of E. coli (Mainil, 2013). Shiga toxins are responsible for binding to the glycolipids on (Gb3) sites on the cell surface, resulting in the stoppage of protein synthesis and causing cell death (Kaper et al., 2004).

In our study, the screening of six virulence genes in E. coli isolates (iss, fimH, eaeA, stx2, tsh and iutA) was carried out using specific primers sets in PCR assay. The results revealed that iss and fimH were the most frequently detected genes in all tested isolates (Fig. 1A-B), followed by the intimin (eaeA) gene in 31.25% (Fig. 1C). Our results related to the study reported by (Kwon et al., 2008) who explaining the vital role of fimbrial genes such as F5 (K99) in E. coli colonization in the mucosa of the small intestine of calves and by studying the frequency of the iss gene he reported that iss gene ranged from 80 to 100%. Other fimbrial genes (F41 and F17) were identified in ETEC in neonatal calves (Mellata et al., 2003). Another study reported by (Lynne et al., 2007) clarified the function of the iss and hor genes in the pathogenicity of E. coli strains and reported that these genes believed to prevent the effect of inhibitory mediators generated by the host complement and resist the process of phagocytosis, and therefore, enhance the pathogenic influence of E. coli strains on the target host. Likewise, the incidence of the stx2 gene in our investigation was low as existed in only one isolate (6.25%) (Fig. 1D). Our results are in agreement with (Wilson et al., 1992), who revealed a lower incidence of the stx2 gene (3.5%) in E. coli strains of young calves in Canada. On contrary, in Egypt (Abotalip et al., 2017) recorded a high prevalence (43.75%) of the stx2 gene. Interestingly, neither the tsh nor the iutA gene was detected in any tested samples in the current investigation, which attributed to variation in the results obtained by the researchers. In accordance with the previous study, (Saidenberg et al., 2013) illustrated that the frequency of the tsh gene in E. coli avian strains was 85.3%. Another investigation in Egypt by (Abdulgaiyed et al., 2015) revealed that the prevalence of the tsh gene in E. coli of diarrheic calves was 100%. This difference requires further investigation regarding the role and prevalence of this gene in animal species and the possibility of interspecies transmission and the environmental risk factor.
bp), while lanes 1, 2, 3, 4, 6, 8, 11, 13, 14, 15 and 16 were negative. (D) Only one strain (Lane 13) was positive for the stx2 gene (779 bp), whereas 15 strains (lanes 1-12 and 14-16) were negative for this gene. Lane M: 100 bp DNA marker; lane CtN: Negative control; and lane CIP: Positive control.

Relationship between phenotypic resistance and virulence determinant genes among E. coli serotype

The results in Table 3 showed a correlation between phenotypic resistance and virulence genes among different E. coli serotype, our finding emphasizes that the E. coli serotype which exhibits more phenotypic resistance carried more virulence genes. Moreover, E. coli O26 and O157 were the most E. coli serovars which exhibit multidrug resistance to more than five antimicrobial agents and carried three virulence determinant genes. Other serovars (O78, O146, O125, O18, and O44) observed in our result also displayed multidrug resistance to three or more antimicrobial agents. The proper association between drug resistance and virulence genes determinants is still poorly unclear to understand. However, several evidence pointed out a correlation between phenotypic-resistance and virulence determinant genes (Orden et al., 2000; Rasko et al., 2008; Badri et al., 2009 and Bonyadian et al., 2014) clarified that many of the E. coli strains showed multidrug resistance to several antimicrobial groups and thus enhanced more virulence to these strains, which may lead to complicate the treatment of some urinary tract and enteric infections in animals. Additionally, (Srivani et al., 2017) observed that 69.81% of shiga toxinogenic E. coli serovars from diarrheic calves expressed multidrug resistance and serovars which carried hlyA and eaeA genes may possess a zoonotic threat and high prevalence of multidrug resistance. A similar finding in Brazil was conducted by (Rigobelo et al., 2006) who described that STC isolates of diarrheal calves showed 100% multidrug resistance. Meanwhile, (Kumar et al., 2013) explained that the indiscriminate and widespread use of antimicrobial drugs act as the main reason of prevalent multidrug resistance among bacteria and obstacle in the treatment of many bacterial diseases.

Table 3 Relationship between antimicrobial resistance and virulence factor genes among E. coli serotype

<table>
<thead>
<tr>
<th>Isolates no.</th>
<th>Serotype</th>
<th>Phenotypic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O26</td>
<td>S, TET, SXT, C, N, AMC</td>
</tr>
<tr>
<td>2</td>
<td>O26</td>
<td>S, TET, SXT, C, N, PB</td>
</tr>
<tr>
<td>3</td>
<td>O26</td>
<td>S, TET, SXT, C, N, AMC</td>
</tr>
<tr>
<td>4</td>
<td>O26</td>
<td>S, TET, SXT, C</td>
</tr>
<tr>
<td>5</td>
<td>O157</td>
<td>S, TET, SXT, C, N, ENR, CIP, AMC</td>
</tr>
<tr>
<td>6</td>
<td>O157</td>
<td>S, TET, SXT, C, N, ENR</td>
</tr>
<tr>
<td>7</td>
<td>O157</td>
<td>S, TET, SXT, C, N, PB, AMC</td>
</tr>
<tr>
<td>8</td>
<td>O157</td>
<td>S, TET, SXT, C, N, PB</td>
</tr>
<tr>
<td>9</td>
<td>O157</td>
<td>S, TET, SXT, C</td>
</tr>
<tr>
<td>10</td>
<td>O125</td>
<td>S, TET, SXT, C</td>
</tr>
<tr>
<td>11</td>
<td>O125</td>
<td>S, TET, SXT, C, AMC</td>
</tr>
<tr>
<td>12</td>
<td>O146</td>
<td>S, TET, SXT, C, AMC</td>
</tr>
<tr>
<td>13</td>
<td>O146</td>
<td>S, ENR, PB</td>
</tr>
<tr>
<td>14</td>
<td>O146</td>
<td>S, ENR, PB</td>
</tr>
<tr>
<td>15</td>
<td>O146</td>
<td>S, CIP, ENR</td>
</tr>
<tr>
<td>16</td>
<td>O18</td>
<td>S, CIP, ENR</td>
</tr>
</tbody>
</table>

Legend
- S: Streptomycin
- TET: Tetracycline
- SXT: Sulfamethoxazole/Trimethoprim
- Ne: Neomycin
- AMC: Amoxicillin/Clavulanic acid
- PB: Polymyxin B
- ENR: Enrofloxacin
- CIP: Ciprofloxacin

CONCLUSION

Given from the above-mentioned results, concluded the high importance of diarrheogenic E. coli which constitute a potential problem in neonatal calves as well as it acts as a source of infection dissemination in animal population. The problem is more dangerous with the existence of multi-antimicrobial resistant strains as well as E. coli associated virulence genes that could contribute to the capability of E. coli to cause diarrhea in calves. Furthermore, it is significant to provide valuable knowledge about the virulence ability of the circulating E. coli strains in Egypt and the ideal methods for prevention and control of such problems in the animal industry.

Ethics statement: This study was approved by the Ethics Committee and current legislation on research and ethical approval of the Faculty of Veterinary Medicine (Local ethical approval), Sohag and Sadat City Universities.

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

REFERENCES


