Toxin typing of *Clostridium perfringens* associated with enterotoxaemia cases of sheep and healthy group in Fars province by PCR and ELISA methods.

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ABSTRACT:

*Clostridium perfringens* is implicated in the etiology of some diseases including fatal enterotoxaemia. Determination of dominant toxin types of this microorganism can be helpful in epidemiologic surveys and formulation of more proper vaccines. To understand the pathogenicity of this bacterium, it seems necessary to describe the toxin and virulence genes content of strains involved in enterotoxaemia and other associated diseases.

The aim of our study was isolation and toxin typing of *C. perfringens* of sheep with suspected enterotoxaemia in Fars province by culture- PCR and ELISA methods and comparison to isolates of healthy group. Samples of intestinal contents from enterotoxaemia cases and healthy group of sheep were collected. The presence of alpha, beta, and epsilon toxins were evaluated by ELISA method. After culture and isolation of *C. perfringens*, toxin typing and screening of isolates for presence of beta-2 and enterotoxin was performed by PCR method. *C. perfringens* was isolated from 102 of 167 suspected enterotoxaemia cases of sheep and from 22 of 50 healthy sheep. The PCR results showed that type A was the most prevalent toxin type in both groups but according to
ELISA type D was the dominant toxin type in clinical group. Enterotoxin gene was detected in 10% of all isolates from healthy and suspected group isolates of types A and D. The beta-2 gene was identified in 35% and 63.6% of enterotoxaemia-associated isolates and isolates not associated with disease respectively. In conclusion, Type D of *C. perfringens* was the dominant causative organism of fatal enterotoxaemia of sheep in Fars province.

**Key words:** *Clostridium perfringens*, toxintyping, PCR, ELISA

1. **BACKGROUND:**

Enterotoxaemia is one of the most important and frequently occurring diseases of livestock in Iran and other countries (Ahsani, 2010). Its causative agent is *Clostridium perfringens*, an anaerobic gram-positive rod-shaped bacterium that produces many toxins among them four major lethal ones including alpha, beta, epsilon and iota are used for typing (Uzal et al, 2010). Beta2 and enterotoxin are two other toxins which their role in pathogenesis of enterotoxaemia remain ambiguous (Uzal et al, 2010). To understand the pathogenicity of this bacterium, it seems necessary to describe the toxin and virulence genes content of strains involved in enterotoxaemia and other associated diseases. Some techniques are used for toxin typing including ELISA and PCR. As PCR is sensitive, specific and rapid test, it is widely used for toxin typing of Clostridium (Meer and Songer, 1997). ELISA is the other helpful method which has been found to be 95% reliable for the detection of *C. perfringens* toxins in intestinal contents of suspected cases of enterotoxaemia (Gökce et al, 2007; Hadimli et al, 2012). In contrast to PCR, in ELISA method expression of toxin genes as the most accepted criterion, is evaluated and positive results of this test confirm the diagnosis of enterotoxaemia (Uzal and Songer 2008).

Because of the high fatality rate of enterotoxaemia which consequently affect the farming industry, this disease and its related researches are so important. In Iran there are few published reports of toxin typing of *C. perfringens* involved in enterotoxaemia in the field (Ahsani, 2010). Furthermore, there has been no research on this issue in Fars province, South of Iran, although there are many reports of this disease annually in this province. On the other hand, formulation of a proper and efficient polyvalent vaccine depends on the knowledge of prevalence of *C. perfringens* toxin types in a region.

The aim of our study was to determine the toxin types of *C. perfringens* isolates of sheep with suspected enterotoxaemia in Fars province by culture-PCR and ELISA methods and comparison to isolates of healthy sheep.

2. **MATERIALS and METHODS:**
Sample collection

A total of 167 samples of intestinal contents of sheep with sudden death suspected to enterotoxaemia from different parts of Fars province were obtained from Shiraz veterinary office, veterinary networks and privet sectors in different parts of Fars province. Indications for enterotoxaemia is usually based on either clinical suspicion of animals showing symptoms of severe and profuse diarrhea or mostly sudden death in fatal cases.

Samples of intestinal contents for control group were collected from 50 healthy Sheep in slaughterhouses. The samples were transferred on ice to the microbiology laboratory. A portion of samples was inoculated onto blood agar supplemented with 40 μg/ml neomycin and tryptose sulphite cycloserine agar (Oxoid, Germany) containing 400 mg/l cycloserine (SR88, Oxoid). The plates were incubated anaerobically at 35°C. The suspected colonies of *C. perfringens* which were gray, smooth, sometimes rhizoid with double zone of hemolysis on blood agar and black colored on TSC agar were picked, gram stained, purified and submitted to biochemical tests (Jouseimies-Somer et al. 2002). Further confirmation and toxin typing was done by PCR.

PCR:

The PCR tests were carried out using the primer pairs for each toxin gene including α, β, ε, ι, β2, enterotoxin and the species-specific primer of 16SrRNA. The primer sequences are listed in Table 1. Suitable reference strains for toxin types were included as positive controls in the PCR tests. The PCR program was set up and conducted for 30 cycles of 95 °C, 60 °C and 72 °C for 1 min each. The exception was annealing temperature which decreased to 55 °C for CPE and CPB2 primers.

ELISA:

Screening of intestinal contents and body fluids for the presence of *C. perfringens* toxins was carried out by the related ELISA kits (Cypress Diagnostics Co., Belgium). Alpha, beta and epsilon toxin *C. perfringens* ELISA kits were used for evaluation of α, β and ε toxin respectively. Each kit contains 96-well microtitration plates sensitized by specific monoclonal antibodies to allow a specific capture of the corresponding antigen (toxin), which is present in the samples. The tests were performed according to the kit protocol.

3. RESULTS:
C. perfringens was isolated from 102 of 167 suspected enterotoxemic cases of sheep and from 22 of 50 healthy sheep and confirmed by biochemical tests. The isolates were non-motile, catalase negative, sucrose and lactose fermentation positive, urease and indole negative, gelatinase and lecithinase positive and lipase negative.

The PCR results showed that all isolates were positive for the 279 bp segment of 16SrRNA gene, confirming these isolates as C. perfringens strains. PCR products of toxin typing for alpha, beta and epsilon toxins were 324 bp, 196 bp, 655 bp respectively (Figure 1a).

Typing of C. perfringens isolates by PCR showed that 46, 18, 6 and 32 of isolates from enterotoxemic group were type A, B, C and D respectively. In control group 16 of isolates were type A and 6 were type D. There was no type E in isolated strains of both groups (Table 2).

The results of ELISA showed that all intestinal content samples from healthy group were negative for the presence of 3 major toxins except one sample which was positive for alpha toxin. In suspected cases 68 samples were positive for at least one of alpha, beta or epsilon toxins. The number of different toxin types based on ELISA method are listed in Table 2.

The screening of isolates for the presence of enterotoxin and beta-2 toxin genes by PCR (Figure 1, b and c) revealed that nine isolates out of 102 from suspected group (8.8%) and four out of 22 isolates from healthy group (18.1%) were positive for enterotoxin. A total of 36 (53.2%) and 14 (63.6%) of isolates were identified as positive for beta-2 gene from suspected and healthy group respectively. Toxin types of these isolates are listed in Table 2.

**DISCUSSION:**

Clostridium perfringens is an under-studied pathogen with the most prolific toxins including alpha, beta and epsilon as major ones (Kiu and Hall, 2018). It has been well established that detection of these toxins in intestinal contents and body fluids is a definitive evidence of enterotoxaemia disease (Uzal and Songer, 2008). The fatality rate of this disease in sheep is high and ranges between 58 and 100% mostly presenting as sudden death (Omer et al., 2020).

In this study, collected samples of suddenly dead cases which were suspected to enterotoxaemia from different flocks and veterinary departments in various regions of Fars province in Iran were transferred to our lab for isolation of C. perfringens and confirmation of disease by ELISA method, typing of isolates and comparison of these isolates with the isolates from healthy flocks. Results of this study showed that from 167 suspected cases
61% were positive for isolation of *C. perfringens* whilst only 40% definitively were demonstrated to be positive for the presence of at least one of alpha, beta or epsilon toxins in their intestinal contents and body fluids. It is indicating that most of suspected cases were not diagnosed as enterotoxaemia by ELISA and evaluation of other diseases and agents should be considered. There are some reports of enterotoxaemia prevalence and typing of *C. perfringens* isolates in different parts of the world. In Iran Ahsani has reported the isolation and typing of *C. perfringens* from non-vaccinated sheep in Kerman province. According to his results type C was the most and type A was the least prevalent isolates in Kerman province (Ahsani, 2010). In contrary, the results of our survey in Fars province showed that type A was the most prevalent isolate in two groups of clinical and non-clinical isolates by culture and PCR method. This result is consistent with the results of Meer and Songer, 1997, Gokcci et al. 2007, Hadimli et al. 2012, Tutuncu et al. 2018 and Omer et al.2020.

Gokcci et al reported that from 220 samples of suspected cases of enterotoxaemia from sheep in Turkey 58.6% and 84.6% were positive for the presence of typing toxins with the type A as a dominant type by latex and ELISA method respectively (Gokcci et al., 2007).

According to other research in Turkey by Hadimli et al. the most common type of *C. perfringens* was type A which determined by both ELISA and PCR methods. They isolated this bacterium from 8.66% of lambs suspected of enterotoxaemia (Hadimli et al.,2012).

In this study the dominant type detected by ELISA method was type D in suspected group, which reveals the important role of this type in pathogenesis of the disease.

As it is obvious that isolation of *Clostridium* is not enough for confirmation of the disease, the bacteria were isolated from about 20% of clinical cases with negative ELISA results and 44% of healthy ones. This can be indicative of the role of *C. perfringens* type A as a dominant normal flora in sheep intestine. Based on PCR results, beta toxin gene was not detected in healthy group and iota gene, representing the uncommon type E, was not present in any groups.

According to published data some toxin genes are located on conjugative plasmids (Hughes et al., 2007). It can be suggested as previously postulated by others that type B, C, D and E can be derived from type A by acquiring the toxin plasmids or vice versa (Hughes et al., 2007; Li et al., 2013).

PCR experiments showed that the other two nontypings toxins, beta-2 and enterotoxin were present in some isolates of both groups.
It has been reported before, that enterotoxin is associated with food-borne and non-foodborne gastroenteritis and may be produced by some type A, C, D, and E but not by any known type B isolates (Li et al, 2013; Azimirad et al, 2019). This chromosomally or plasmid encoded toxin is considered as one of the pathogenesis factors which induces histological damages (Uzal et al, 2007; Kiu and Hall, 2018; Heikinheimo, 2008; Li et al, 2013).

According to previous publications less than 5% of all \textit{C. perfringens} isolates in the world carry enterotoxin gene (Freedman et al, 2016). In this study enterotoxin gene was detected in 10% of all isolates from healthy and suspected group isolates of types A and D. Based on new classification described by Rood et al in 2018, these nine isolates of type A which harbor \textit{cpe} gene can be reclassified as type F, meaning 5% of isolates belonged to this type (Rood et al, 2018).

The other toxin, beta-2, was detected in 63.6% and 35.2% of healthy and clinical isolates respectively. Presence of this toxin was seen in all isolates of types A, B, C and D.

It has been stated in previous studies that beta-2 toxin can be produced by all \textit{C. perfringens} types. The exact role of beta-2 toxin in pathogenesis has not yet been determined, although its cytotoxicity for CHO cells and induction of hemorrhagic necrosis in Guinea pig intestine and enterocolitis in foals have been reported (Uzal et al, 2010; Kiu and Hall, 2018; Li et al, 2013). Enterotoxin is another toxin which is associated with sporulation and has been implicated in food-borne and non-foodborne gastroenteritis (Afshari et al, 2015; Kiu and Hall, 2018).

Our results showed that not only presence of the two accessory enterotoxin and beta2 genes were not higher in clinical isolates but it was less prevalent in this group in comparison to healthy group.

Collectively, based on ELISA results, type D of \textit{C. perfringens} was the dominant cause of fatal enterotoxaemia of sheep in Fars province. ELISA results for estimation of prevalence and typing of \textit{C. perfringens} differ from culture and PCR method in enterotoxaemia cases. The use of ELISA method for reporting the disease in a region and evaluating other diseases in negative cases is recommended.

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Declaration of competing interest:
The authors declare no conflict of interest

References:


Table1. List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene specificity</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrRNA</td>
<td>Species specific</td>
<td>AAAGATGGCATCATCATTCAAC TACCTCATATTATCTTCCCCAAA</td>
<td>279</td>
<td>Wang et al. 1994</td>
</tr>
<tr>
<td>CPA</td>
<td>α toxin</td>
<td>GCTAATGTTACTGCCGTTGGA CCTCTGATACATCGTGTAAG</td>
<td>324</td>
<td>Meer &amp; Songer 1997</td>
</tr>
<tr>
<td>CPB</td>
<td>β toxin</td>
<td>GCAGAAATGCTGGAATACATCTA GCAGGAACATTAGTATATCTTC</td>
<td>196</td>
<td>Meer &amp; Songer, 1997</td>
</tr>
<tr>
<td>ETX</td>
<td>ε toxin</td>
<td>GCAGTGATATCCCATCTCTATCCACTTTACCTTCTACTAAC</td>
<td>655</td>
<td>Meer &amp; Songer, 1997</td>
</tr>
<tr>
<td>Ia</td>
<td>t toxin</td>
<td>ACTACTCTCAGACAAGACAG CTTTCCTCTATTACTATACG</td>
<td>446</td>
<td>Meer &amp; Songer, 1997</td>
</tr>
<tr>
<td>CPE</td>
<td>enterotoxin</td>
<td>GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA</td>
<td>233</td>
<td>Meer &amp; Songer, 1997</td>
</tr>
<tr>
<td>CPB2</td>
<td>β2 toxin</td>
<td>AGATTTTAAATATGATCTTAACC CAATACCTCACCACAAATACTC</td>
<td>567</td>
<td>Bueschel et al. 2003</td>
</tr>
</tbody>
</table>
Table 2. Number of different toxin types in two suspected enterotoxemic and healthy groups

<table>
<thead>
<tr>
<th>Number of Toxin types</th>
<th>Type A</th>
<th>Type B</th>
<th>Type C</th>
<th>Type D</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of isolates in suspected group (ELISA Method)</td>
<td>17</td>
<td>18</td>
<td>6</td>
<td>27</td>
<td>68 (40.71%)</td>
</tr>
<tr>
<td>Number of isolates in healthy group (ELISA Method)</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Number of isolates in suspected group (PCR Method)</td>
<td>46</td>
<td>18</td>
<td>6</td>
<td>32</td>
<td>102 (61.07%)</td>
</tr>
<tr>
<td>Number of isolates in healthy group (PCR Method)</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>22 (44%)</td>
</tr>
<tr>
<td>Number of enterotoxin positive isolates in suspected group</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>9 (8.82%)</td>
</tr>
<tr>
<td>Number of enterotoxin positive isolates in healthy group</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>4 (18.18%)</td>
</tr>
</tbody>
</table>
Number of beta-2 positive isolates in suspected group

<table>
<thead>
<tr>
<th></th>
<th>7</th>
<th>10</th>
<th>1</th>
<th>18</th>
<th>36 (35.29%)</th>
</tr>
</thead>
</table>

Number of beta-2 positive isolates in healthy group

|          | 5 | -  | - | 9  | 14 (63.63%) |

Figure 1. Agarose gel electrophoresis of PCR assays for detection of a: *C. Perfringens* 16srRNA, a band of 279 bp (right) and toxin typing of *C. perfringens* isolates (left), bands of 196 bp, 324 bp and 655 bp are amplicons of beta, alpha and epsilon toxin genes respectively. Lane 2 is PCR negative control, lane 3, 4, 5 and 6 are representative of type A, type C, type D and type B of *C. perfringens* respectively. The marker (M) is a 100 bp gene ruler, RTU, cat No. PR911653 (cinnagen, Iran). b: detection of beta-2 toxin gene (567bp) and c: detection of enterotoxin gene (233bp).