Studies on Diarrhea in Calves with Emphasis on the Role of Clostridium Perfringens and Escherichia Coli

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Abstract: Bovine enterotoxaemia is an acute, peracute syndrome occurring mainly in calves and characterized by the sudden or very rapid death of the calf, with colics, convulsions and nervous disorders as clinical signs, if any. The presence of clostridium perfringens and Escherichia coli were examined in 28 fecal samples collected from diarrheic calves less than 2 months age from different governorates in Egypt. The percentage of recovery of C.perfringens and E.coli were 85.7% and 78.5% respectively. C.perfringens was tested for the production of their major toxin using I/D neutralization in guinea pig. 83.3% of the isolated C.perfringens was toxigenic while 16.7% didn't yield toxins. The most prevalent toxin type of C.perfringens found was type D representing 75% of the isolated strains while C.perfringens type A was isolated in 8.3%. 22 isolates of Escherichia coli was serotyped as O157 (n=4) and non O157 (n=18) including O111, O114, O45, O122, O28 and O165. 27.3% of E.coli isolates were identified as enterotoxigenic strain by using sucking mouse bioassay. C.perfringens and E.coli were isolated either in single infection (23.1%) or in mixed infection (16.9%). 2 C.perfringens isolates were typed using PCR assays for the detection of α, β and ε – toxin genes isolates showed the presence of α and ε- toxin genes but were devoid of the β-toxin gene.

Keywords: - Calves, Diarrhea, Enterotoxaemia, Clostridium perfringens, Escherichia coli.

INTRODUCTION

Clostridium perfringens is a Gram positive sporulated anaerobe responsible for necrotic and hemorrhagic enteritis and enterotoxaemia in humans and in different animal species, this bacterial species can produce up to 17 different toxins, five of which (the α, β, ε, τ toxins and the enterotoxin) are responsible for the tissue lesions and the death of the hosts[1,2,3].

Bovine enterotoxaemia is characterized by a high case fatality rate, sudden death, lesions of necrotic and hemorrhagic enteritis of the small intestine and most often, an absence of other clinical signs. The infections etiology of bovine enterotoxaemia has not been formally identified yet, but C.perfringens is often regarded as responsible[4,5,6].

A recent survey of more than 70 typical cases in Belgium confirms toxin type A C.perfringens as putative etiology[7,8].

However, if the α-toxin is highly pathogenic for all species the reproduction of typical necrotic and hemorrhagic lesions has been prefermed only in chickens[1,2,3].

Diarrhea can be attributed to infection with a single or multiple agents[5]. It is evidence that the infections agents capable of causing diarrhea in calves are numerous; the most important bacterial enteropathogens are Enterotoxigenic E.coli (ETEC) producing directly detectable toxin, salmonella, Y. enterocloctica and campylobacters as well C.perfringens may be the most widely occurring pathogenic bacterium and is certainly the most important cause of clostridial enteric disease in domestic animals[2].

The major animal pathogens E.coli, Salmonella species and Yersinia species can cause both enteric and systemic diseases[8]. Several forms of enteric diseases attributed to E.coli[9], isolation of E.coli as main cause of diarrhea in calves was reported by[10,11].

The main object of the present study was to determine the association between enteropathogens causing calf diarrhea and using a polymerase chain reaction (PCR) for the detection of the genes encoding the different C.perfringens toxins as a diagnostic method for specific identification of C.perfringens types in calf enterotoxaemia. As PCR technique become a first – choice tool for identification and typing of C.perfringens strains that cause disease[12].

MATERIALS AND METHODS

Fecal samples were collected from 28 diarrheic calves up to 6 week old calves. At Minia El-Kamh (Sharkia) and Ashmon (Monufaya), Egypt. These animals either died after showing depression, diarrhea,
growth overnight in 5 ml volumes of brain heart toxin genes: PCR analysis for the presence of alpha and epsilon genes was calculated. A ratio of less than 0.083 was determined.

Isolation and Confirmation of C. perfringens: Samples were cultured on cooked meat broth (CMB) and incubated anaerobically at 37°C for 18 hrs. A loopful from the overnight CMB were cultured onto blood agar plates with 5% sheep blood supplemented with 200mg/ml neomycin sulphate according to the method described by. The plates were read after 24 and 48 hrs from growth of C. perfringens. Typical colonies were identified by characteristic colony morphology, hemolytic, Gram staining and biochemical tests. Isolates identified as C. perfringens were lecithinase-positive and caused glucose, lactose, sucrose and fructose to ferment. Starch was hydrolyzed.

The isolates did not ferment mannitol or produce indole. Toxigenicity of C. perfringens isolated was evaluated for the presence of lethal toxins by intravenous injection in mice and their pathogenicity by d ermonecrotic testing in guinea pigs according to (16, 10).

Isolation and Confirmation of E. coli: Fecal samples were enriched is Gram negative (GN) broth at 37°C for 24hrs. A loopful of GN broth is streaked onto MacConkey agar and eosin methylene blue (EMB). The inoculated plates were incubated at 37°C for 24 hrs. Suspected colonies were picked up and subcultured onto nutrient agar slopes and incubated at 37°C for 24 hrs for further identification. The suspected isolates were subjected to oxidative and catalase test (10). Oxidative negative and catalase positive colonies were biochemically identified according to (10).

Biochemically identified isolates were serologically investigated by the use of a commercial slide agglutination kit (SEUKEN, Japan) according to (20). All isolates were assayed for the detection of enterotoxin by the infant mouse test (21).

Infant albino mice (1-2 gm, 1-4 days old) were subjected to intragastric inoculation with 0.1 ml of the crude culture filtrate. After 4 hours they were necropsied for the small intestine weights and distention the gut weight to the remaining body weight was calculated. A ratio of less than 0.083 was considered negative.

PCR Analysis for the Presence of Alpha and Epsilon Toxin Genes: From pure culture, C. perfringens was grown overnight in 5 ml volumes of brain heart infusion (BHI; oxoid) supplemented with 1% (w/v) sodium thioglycolate (Gibco) under anaerobic conditions one milliliter of culture was centrifuged at 5,000 x g for 15 min, and the cell pellet was washed twice with sterile saline and resuspended in 200 ml of high-pressure liquid chromatography-grade water and then placed in boiling water bath for 20 min for cell lyses. After centrifugation, 10 ml of supernatant fluid was used as the template for PCR. The sequences of the primers for the C. perfringens alpha toxin gave (cpa), were selected from the sequence published by (23).

- CPALPHATOXIN1-L (18-mer: 5’-AGATT TGT AAG GCC CTT-3’)
- CPALPHATOXIN1-R (18-mer: 5’-ATT TCC TGA AAT CCA CTC-3’).

The sequences of the primers for Beta-toxin gene (cpb) were selected from the sequence published by (23).

- CPBETATOXIN1-L (19-mer: 5’-AGG TTT TAT TAT GAA G-3’)
- CPBETATOXIN1-R (22-mer: 5’-TCT ATA TAG CTG TTA CTT TGT G-3’)

The sequences of the primers for epsilon-toxin gene (etx) were selected from the sequence published by (14).

- CPETOXIN1-L (30-mer: 5’-CTC ATC TCC CAT AAG TGC ACT ATT TCC-3’)
- CPETOXIN1-R (30-mer: 5’-TAC TCA TTAC GTG GGG AAC TTC CAT ACA AGC)

PCR Amplification: The PCR was performed in a touch-down thermocycler (Hybaid) in a total reaction volume of 50 µl containing 5 µl of 10x PCR buffer (10 ml M tris – HCL [pH 9.0], 50 mM KCL, .005% tween, 0.1% triton x-100), 5 µl of 25 mM Mg CL2, 250Mm each deoxynucleotide triphosphate, 2U of tag DNA polymerase, 1µM of each primer and 5ml of template DNA. Amplification was obtained with 35 cycles following an initial denaturing step at 94°C for 30 sec. Each cycle involved denaturation at 94°C for 1 min, annealing at 56°C for 1 min for cpa-gene and 59.6°C for etx-gene, and extension at 72°C for 2 min. Specific amplification of the beta-toxin gene was achieved by 35 cycles consisting of 30 sec denaturation at 94°C, 30 sec primer annealing at 39°C and 30 sec chain extension at 72°C. The ramping time from the annealing temperature to 72°C was adjusted to 1min 30 sec.

The results were determined by electrophoresis of 20 µl of PCR products in a 1% agarose gel for 30 min at 80V and staining with ethidium bromide. The 1167,
Enterotoxaemia usually occurs in bovine when prophylactic measures are not observed. The presence of *C. perfringens* is also a public health problem since human can be intoxicated by ingesting contaminated meat. *C. perfringens* is divided into five toxin type (A, B, C, D and E), on the basis of the production of four major lethal toxins, alpha, beta, epsilon and iota.[24, 25] Out of 28 examined fecal samples obtained from diarrheic calves 24 *C. perfringens* isolates were recovered (85.7%) . the recovered *C. perfringens* isolates were classified into toxigenic isolates (83.3%) and belonged to type D (75%) which produce both α and ε toxins , type A (8.3%) which produce α toxin only and non-toxigenic ones (16.7%) . as shown in table (1) . These results is in parallel with[26] who isolated both *C. perfringens* types A and D from diarrheic calves with an incidence of 9.85% and 17.42 respectively[27] could identified *C. perfringens* type A (31%), B (7.2%), C (3.5%) and D (48.3%) from diarrheic calves.

As well as[28] detected *C. perfringens* type C(41.7%) and D(33.3%) from diarrheic calves , while[29] isolated *C. perfringens* type A and C only among diarrheal calves . on the other hand *C. perfringens* type B,C,D and E were not isolated by[30,6,31] among diarrheic calves.

In the present study the highest incidence was recorded by *C. perfringens* type D (75%) among diarrheic calves[32,33] claimed that type D enterotoxaemia is important in calves and[34] reported that type D enterotoxaemia rarely occur in adult cattle. Determination of the toxin types of *C. perfringens* isolated from fecal samples from diarrheic calves supports the etiological diagnosis of enterotoxaemia in calves. The standard method for confirmation of enterotoxaemia has so far been the mouse neutralization assay, this toxin – typing technique requires continues supply of laboratory animals and use of monovalent diagnostic sera , which is increasingly difficult to find and extremely expensive . While 22 cases of diarrheic calves were positive for *E. coli* with an incidence of 78.5%, the obtained result agree with that of[24] as the isolated *E. coli* with an incidence of (50.76%) . lethal *E. coli* was foundin6/32 samples[13]

### RESULTS AND DISCUSSIONS

Enterotoxaemia usually occurs in bovine when prophylactic measures are not observed. The presence of *C. perfringens* is also a public health problem since human can be intoxicated by ingesting contaminated meat. *C. perfringens* is divided into five toxin type (A, B, C, D and E), on the basis of the production of four major lethal toxins, alpha, beta, epsilon and iota. Out of 28 examined fecal samples obtained from diarrheic calves 24 *C. perfringens* isolates were recovered (85.7%) . The recovered *C. perfringens* isolates were classified into toxigenic isolates (83.3%) and belonged to type D (75%) which produce both α and ε toxins, type A (8.3%) which produce α toxin only and non-toxigenic ones (16.7%) . as shown in table (1) . These results is in parallel with[26] who isolated both *C. perfringens* types A and D from diarrheic calves with an incidence of 9.85% and 17.42 respectively[27] could identified *C. perfringens* type A (31%), B (7.2%), C (3.5%) and D (48.3%) from diarrheic calves.

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### Table 1: typing of *C. perfringens* isolates recovered from the examined calves:

<table>
<thead>
<tr>
<th>No of examined samples</th>
<th>No of <em>C. perfringens</em> isolates</th>
<th>A</th>
<th>D</th>
<th>Non toxigenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>24</td>
<td>2(8.3)</td>
<td>18(75)</td>
<td>4(16.7)</td>
</tr>
</tbody>
</table>

No = number

% was calculated according to the number of *C. perfringens* isolates.

### Table 2: characterization of the different isolates of *E. coli* isolated from diarrheic calves and examined for enterotoxin

<table>
<thead>
<tr>
<th>Number</th>
<th>% **</th>
<th>Serotype</th>
<th>Suckling mouse bioassay *</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>18.8</td>
<td>O157</td>
<td>2/4</td>
</tr>
<tr>
<td>4</td>
<td>18.8</td>
<td>O111</td>
<td>1/4</td>
</tr>
<tr>
<td>6</td>
<td>27.3</td>
<td>O111</td>
<td>2/6</td>
</tr>
<tr>
<td>2</td>
<td>9.1</td>
<td>O105</td>
<td>0/2</td>
</tr>
<tr>
<td>2</td>
<td>9.1</td>
<td>O142</td>
<td>1/2</td>
</tr>
<tr>
<td>2</td>
<td>9.1</td>
<td>O26</td>
<td>0/2</td>
</tr>
<tr>
<td>2</td>
<td>9.1</td>
<td>O142</td>
<td>0/2</td>
</tr>
</tbody>
</table>

* For detection of enterotoxin

** The percentage was calculated according to the number of positive samples for *E. coli*.

### Table 3: prevalence rate of *C. perfringens* and *E. coli* in examined diarrheic calves

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Bacterial isolates</th>
<th>No of positive samples</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single infection</td>
<td><em>C. perfringens</em></td>
<td>4</td>
<td>15.4</td>
</tr>
<tr>
<td>Single infection</td>
<td><em>E. coli</em></td>
<td>2</td>
<td>7.7</td>
</tr>
<tr>
<td>Mixed infection</td>
<td><em>C. perfringens</em> + <em>E. coli</em></td>
<td>20</td>
<td>76.9</td>
</tr>
</tbody>
</table>

Percentage was calculated according to the total number of positive samples (26 isolates)

while[37] found that 25% of the examined calves were positive for beta-hemolytic *E. coli* and less than 1% of the *E. coli* isolates were verotoxigenic . Table (2) illustrated that Serological identification of the isolated *E. coli* revealed O157, O111, O145, O142, O16, O115 and O145[30] mentioned that O111 was implicated epidemiologically as associated agent of calf diarrhea[37] serotype *E. coli* type O126, O126, O146, O147, O158, O1, O1, O144, O144 among diarrheic calves . Concerning enterotoxin in the examined isolates using infant mice bioassay 27.3% were enterotoxigenic. Various studied indicated the relationship between enterotoxigenicity and serovars is difficult to elucidate because enterotoxin production is a plasmid mediated[38, 39, 40].

Finally *E. coli* was almost certainly associated with the enteric lesion and further investigation was needed to identify virulence attributes among the isolates. The recovery of *C. perfringens* and *E. coli* was in single infection pattern in 6 cases (31.2%) and in mixed infection pattern is 20 cases (76.9%) Table 3.

In this aspect[41,42,43] suggested that the role of toxigenic *E. coli* and salmonella species can not be formally excluded in isolated cases of sudden death

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1025 and 402-bp PCR products of cpa, cpb and etx, respectively, were observed. PCR markers (Biotecnoogy Department Bio Basic Inc) consisting of nine DNA fragments ranging from 0.5 to 10.0 Kilobases (KB) were used as the standard.

Amplified bands were visualized by UV illumination and photographed on high-density thermal paper film.
The diagnosis of enterotoxaemia remains a balance between a series of factors, including environmental circumstances, clinical signs, lesions and bacterial analysis. Two *Clostridium perfringens* isolates obtained from diarrheic calves were analyzed by PCR for *cpa*, *cpb* and *etx* genes. Amplification conducted with destroyed culture from *C. perfringens* isolates yielded in the predicted 1167-bp fragment for *α* – toxin gene and 402-bp fragment for *ε* – toxin gene while the *β*-toxin gene was not found in the tested isolates (figure -1). Moreover, toxin-typing results are obtained in less than 24 or even 48 h observation\(^{[44,46]}\), to avoid the use of experimental animals PCR analysis of different genes for the toxin *α*, *β*, *ε* and *θ* allows a clear differentiation of the five toxin type of *C. perfringens* reference strains\(^{[44,45]}\) and was previously successfully used diagnosis of *C. perfringens* type C enteritis in pigs\(^{[45]}\). The use of DNA amplification method is particularly suitable in predicting the toxigenic potential of *C. perfringens* and for identification of type D which is known to produce the lethal *α* and *ε*-toxin.

The *α*-toxin gene was found in all tested *C. perfringens* isolates, it is therefore suggested as a diagnostic method for confirmation of the species *C. perfringens*. The identification of *C. perfringens* type D based on the *ε*-toxin gene. The presence of the *ε*-toxin gene in *C. perfringens* in the tested isolates and the absence of the *β*-toxin gene in these isolates allowed us to identify them as *C. perfringens* type D\(^{[46]}\).

**Summary:** Diarrhea remain one of the most important causes of calf mortality and the economic significance varies among herds, it has complex aetiological agents as several infectious agents, environmental, nutritional, immunological and genetic factors. The aim of the present study was to determine the association between *C. perfringens* and *Escherichia coli* (one of the most important enteropathogenic bacteria causing calf diarrhea). So the bacteriological examination carried out on 28 fecal samples collected from diarrheic calves to determine the prevalence rate of *C. perfringens* and *E. coli*. Among 24 isolated *C. perfringens* (85.7%) *C. perfringens* type D was the predominant (75%) followed by *C. perfringens* type A (8.3%) *E. coli* was isolated in a percentage of (78.5)

Serotyping of *E. coli* were identified in percentage of (27.3) for O\(_{11}\), (18.8) for each of O\(_{15}\) and O\(_{17}\) and (9.1) for each of O\(_{16}\), O\(_{17}\), O\(_{2}\) and O\(_{16}\). Mixed infection of *C. perfringens* and *E. coli* was detected in (76.9%) from diarrheic calves. PCR analyses of *C. perfringens* isolates for *cpa*, *cpb* and *etx* genes yielded in the predicted 1167-bp fragment for *α*–toxin gene and 402-bp fragment for *ε*–toxin gene while the *β*-toxin gene was not found in the tested isolates.

**REFERENCES**


