Clostridium perfringens Enterotoxaemia In Camel (Camelus dromedarius) Calves.

Fayez, M.M1, Suleiman, M. B.2, Al Marzoog, A3, and Al Taweel, H. H.3
1. Serum and Vaccine Research Institute, Cairo, Egypt.
2. Department of Quarantine, Ministry of Animal Resources and Fisheries- Sudan.
3. Veterinary Diagnostic Laboratory – Al Ahsa, KSA.

Manuscript Info
Abstract

Clostridium perfringens produces enteric diseases, generically called enterotoxaemia, in sheep, goats, and other animals. The objective of this work is to conduct a preliminary study for diagnosis of Clostridium perfringens among camel-calf (Camelus dromedarius). A total of 200 fecal samples were collected from camel calf with signs of diarrhea and 120 small intestine (70 from the central slaughter house and 50 after postmortem examination of suddenly dead camel calves). All samples were evaluated by culture for C. perfringens. Cultivation of fecal samples on SFP agar revealed the isolation of 45 C. perfringens isolates from fecal samples and 16 isolates from intestinal contents collected after postmortem examination of dead animals and 14 isolates from intestinal contents of apparently healthy animals. All bacterial isolates exhibited the characteristic features of C. perfringens. Results of pathogenicity test in white mice revealed that out of 75 C. perfringens isolates; 16 (21.33%) were non-pathogenic for mice. Isolates were analyzed by multiplex PCR in order to detect the presence of the toxin genes of C. perfringens. Of these isolates, 25 (33.33%) were type A, 4 (5.3%), were type Aß2, 4 (5.3%), were type B, 13 (17.33%) were type C, 13 (17.33%) were type D and 16 (21.33%) non-pathogenic. In conclusion, C. perfringens type A is the predominant type among camel calves in Al Hasa region, KSA. Its recommended that a vaccination schedule should be implemented to reduce the enterotoxaemia in camel calves.

Introduction

Camels are the most capable animal species in utilizing marginal areas and in survival and production under harsh environmental conditions (Knoess, 1977; Gauthier-Pilters and Dagg, 1981; Hjort and Hussein, 1986; Schwartz, 1992). Many pastoral groups and communities in diverse ecozones throughout the world are depending on camels for their livelihood. They produce about 9%, 24% and 8% of the total meat, milk, and wool, respectively, in the Arab world (Wardeh, 1990). Hamam (1993), reported that camel meat constituted 30% of the meat produced in the Kingdom of Saudi Arabia. In their natural desert habitat, where camels are usually raised particularly during the long dry season, camels are subjected to severe stress conditions which render them susceptible to many diseases and ailments (Abbas et al., 1993; Agab, 1993).

Concerning camel disease, camels were formerly considered resistant to most of the diseases commonly affecting livestock, (Zaki, 1948), but as more research was conducted, it has been proved that camels are susceptible, the same as other livestock or even more, to the common disease causing pathogens affecting other animal species (Abbas and Tilley, 1990; Abbas and Agab, 2002).

Camels are slow reproducers. A female camel is sexually mature at the age of 4-5 years. Pregnancy is just over 12 months and the calving interval in pastoral production systems is normally 24 months or more. Beside this natural productivity limitation, the main factor affecting herd growth is calf mortality, which is high during the postnatal and pre-weaning
stages. In a survey carried out in eastern Sudan, Agaband Abbas (1999), reported a 48% mortality rate among calves under 6 months of age and 14-6% above this age.

Camel calf diarrhea was among the common diseases affecting suckling dromedary calves resulting in high mortality rate among this age group particularly in intensively kept camel herds (Saint-Martin et al., 1992). Etiologically, the disease was caused by mixed infection with numerous microbes, notably Salmonella spp., E. coli and Clostridium perfringens (Abbas et al., 1992; El-Sanousi and Gameel, 1993; Bengoumi et al., 1998).

Clostridium perfringens is a gram-positive anaerobic rod that is classified into 5 toxinotypes (A, B, C, D, and E) according to the production of 4 major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). (Cato et al., 1986). Type A causes food poisoning in human, with diarrhea and cramps (Garmory et al., 2000). Type C strains have been reported to cause enterotoxaemia and necrotic enteritis in sheep, lambs, calves, piglets and fowl whilst type D strains cause lamb dysentery and pulpy kidney disease in sheep and lambs (Hattheway, 1990). The diagnosis of C. perfringens enterotoxaemia is usually based on clinical signs and pathological findings, but identification of toxins in intestinal contents is necessary to confirm the diagnosis.

The classical identification of the toxins is based on neutralization test in mice or in skin of guinea pigs, however, this method is tedious, time-consuming, expensive and monovalent. Furthermore, it is improper and unethical to apply it at the expense of laboratory animals (Yoo et al., 1997). In recent years, enzyme-linked immunosorbent assay (ELISA) kits have been used for the detection of clostridial toxins (El Idrissi and Ward, 1992; Naylor et al., 1997). DNA-based techniques, such as polymerase chain reaction (PCR), have been developed for C. perfringens genotyping and are a reliable alternative method to testing in laboratory animals. Various PCR protocols have been established to genotype C. perfringens isolates with respect to the genes cpa, cpb, etx, iap, cpe, and cpb2, which encode the alpha, beta, epsilon, iota, enterotoxin and β2-toxins, respectively (Petit et al., 1999; Baums et al., 2004). The objective of this work is to conduct a preliminary study for diagnosis of Clostridium perfringens among camel-calf (Camelus dromedarius) in Al-Ahsa region in 26° 35’ 00” N, 48° 10’ 00” E, Saudi Arabia.

### Material and Methods

#### Animals:

Three hundred and twenty camel (Camelus dromedarius) calves of both sexes, 1–10 months of age, raised in different flocks in Eastern Province, Saudi Arabia were included in this study. Two hundred of these animal were had the sings of diarrhea or bloody diarrhea, colic manifested by abdomen distention and vocalization, gas distention in intestinal tract and some nervous sings including convulsions and opisthotonos; 50 camel calves were subjected to postmortem examination after a history of sudden death or death few hours after sings of colic and nervous symptoms and 70 apparently healthy camel calves were examined at slaughter house.

#### Samples:

A total of 200 fecal samples were collected from camel calve with sings of diarrhea and 120 small intestine (70 from the central slaughter house and 50 after postmortem examination of suddenly dead camel calves). Samples were evaluated by culture for C. perfringens.

#### Isolation of Clostridium perfringens:

Fecal samples and intestinal contents were diluted with PBS at a ratio of 1:9. Then, 100 μl aliquots were streaked on Shahidi-Ferguson Perfringens (SFP) agar (Difco) containing 5% egg yolk emulsion (Oxoid) and perfringens (SFP) selective supplement (6 mg kanamycin sulphate, 15000 IU polymyxin B sulphate) (Oxoid), and incubated under anaerobic conditions overnight at 37°C. All isolates were identified biochemically using a commercial kit (API 20A, bioMérieux SA, Marcy-l’Etoile, France).

#### Biological assay for C. perfringens toxin:

Following culture of the C. perfringens isolated strains in cooked meat broth medium, the cells were harvested by centrifugation at 3 000 rpm for 15 min and the cell-free culture supernatants were recovered; one part of the supernatant fluid was treated with trypsin (Sterne and Batty, 1975). White mice (25–40 g) were injected intraperitonealy with 0.3 ml of the culture supernatant and then observed over a period of 3 days for either death or disease symptoms. In the same way, mice were injected with 0.3 ml of intestinal content filtrate. Control group of mice were injected with PBS.

#### Genotyping of Clostridium perfringens Isolates:
DNA from the thawed suspension was prepared with the MagNA pure Compact Nucleic Acid kit I (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. All C. perfringens isolates were PCR-screened for the detection of Alfa (cpa), beta (cpb), epsilon (etx), beta2 (cpb2), and iota (iap), toxin encoding genes, as described by Baums et al., (2004). The multiplex PCR was performed on a 25 μl mixture containing 2.5 mM MgCl2, 250 μM each deoxyribonucleotide triphosphate (dNTP), 0.5 U Platinum Taq DNA polymerase (Invitrogen), and 0.1 μM of each primers listed in Table (1). The thermal cycling was performed as follows: initial denaturation for 2 min 30 sec at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min 20 sec at 72°C and a final extension for 2 min at 72°C. The PCR reaction mixtures were analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of 100-bp DNA ladder (Fermentas Life Science, EU). The agarose gel was supplemented with ethidium bromide in order to visualize the DNA on an UV transilluminator.

**Result and Discussion**

**Clinical sings and Postmortem examination:**
The common clinical sings among the examined animals were diarrhea (watery and bloody), rectal temperatures were subnormal, recumbency with the head stretched forward, eyes closed, ears directed backward, and legs stretched, colic. The abdomen was distended, with gas tympany in the intestinal tract. Nervous signs including signs convulsions, circling, prostration with opisthotonos and paddling, posterior paralysis, and coma. sudden death. Postmortem examination revealed hyperemia and petechia of the subcutaneous tissues. The small intestine, particularly the jejunum and ileum, were congested, with blood-stained intestinal contents and distended with gas.

**Bacteriological analysis of collected samples:**
Cultivation of fecal samples on SFP agar revealed the isolation of 45 C. perfringens isolates from fecal samples and 16 isolates from intestinal contents collected after postmortem examination of dead animals and 14 isolates from intestinal contents of apparently healthy animals. All bacterial isolates exhibited the characteristic features of C. perfringens. The colonial characters on SFP agar are shown in Fig (1), on blood agar, showed dew drops smooth greyish convex colonies with a double zone of haemolysis. Microscopic characters revealed Gram positive non motile rods. Biochemical identification of the isolates showed catalase, lecithinase positive and a haemolytic activity on sheep blood agar showing double zone of haemolysis. Gas and acid from glucose, fructose, lactose sucrose and mannitol were seen, urease negative and gelatinase positive. The distribution of C. perfringens among different age groups of calf camels were illustrated in Tables (2 & 4).

**Biological assay for C. perfringens toxin:**
Results of pathogenicity test in white mice were observed during 3 days which revealed that out of 75 C. perfringens isolates; 16 were non-pathogenic for mice. Table (4).

**Genotyping Of toxigenic Clostridium perfringens isolates:**
As shown in Table (3), a total of 75 C. perfringens isolates were genotyped by PCR. Of these isolates, 25 (33.33 %) were type A, 4 (5.3 %), were type A β2, 4 (5.3 %), were type B, 13 (17.33 %) were type C, 13 (17.33 %) were type D and 16 (21.33 %) non-pathogenic. The distribution of genotypes according to age group was illustrated in Table (4). PCR products of cpa (900 bp), cpb (612 bp), etx (396 bp) and cpb2 (200 bp) were shown in (Figure 2).

<table>
<thead>
<tr>
<th>Toxin gene</th>
<th>Toxin</th>
<th>Sequence 5′→3′</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa alpha</td>
<td>5′- AGT CTA CGC TTG GGA TGG AA -3′&lt;br&gt;5′- TTT CCT GGG TTG TCC ATT TC -3′</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>cpb beta</td>
<td>5′- TCC TTT CTT GAG GGA GGA GGA TAA A -3′&lt;br&gt;5′- TGA ACC TCC TAT TTT GTA TCC CA -3′</td>
<td>612</td>
<td></td>
</tr>
<tr>
<td>etx epsilon</td>
<td>5′- GGG GAA CCC TCA GTA GTT TCA -3′&lt;br&gt;5′- ACC AGC TGG ATT TGA GTT TAA TG -3′</td>
<td>396</td>
<td></td>
</tr>
<tr>
<td>iap Iota</td>
<td>5′- AAA CGC ATT AAA GCT CAC ACC -3′&lt;br&gt;5′- CTG CAT ACC CTG GAA TGG CT -3′</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>cpb2 b2</td>
<td>5′- CAA GCA ATT GGG GGA GTT TA -3′&lt;br&gt;5′- GCA GAA TCA GGA TTT TGA CCA -3′</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>
Table (2) Results of isolation of C. perfringens from fecal and intestinal content samples of camel calves.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Samples</th>
<th>Fecal samples</th>
<th>Small intestine after postmortem</th>
<th>Small intestine after slaughtering</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 2 month</td>
<td></td>
<td>17/70 (24.28%)</td>
<td>10/22 (45.45%)</td>
<td>0</td>
</tr>
<tr>
<td>3 – 4 month</td>
<td></td>
<td>16/72 (22.22%)</td>
<td>5/24 (20.83%)</td>
<td>2/12 (16.66%)</td>
</tr>
<tr>
<td>5 – 6 month</td>
<td></td>
<td>3/27 (11.11%)</td>
<td>1/4 (25%)</td>
<td>3/21 (14.28%)</td>
</tr>
<tr>
<td>7 – 8 month</td>
<td></td>
<td>6/18 (33.33%)</td>
<td>0</td>
<td>5/22 (22.72%)</td>
</tr>
<tr>
<td>9 – 10 month</td>
<td></td>
<td>3/13</td>
<td>0</td>
<td>4/15 (26.66%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>45/200 (22.5%)</td>
<td>16/50 (32%)</td>
<td>14/70 (20%)</td>
</tr>
</tbody>
</table>

Table (3) Genotyping of C. perfringens isolates from fecal and intestinal content samples of camel calves.

<table>
<thead>
<tr>
<th>C. perfringens type</th>
<th>Positive genes</th>
<th>Positive isolates</th>
<th>Source of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fecal samples</td>
</tr>
<tr>
<td>A</td>
<td>cpa</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>A ß2</td>
<td>cpa , pb2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>cpa, cpb</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>cpa, cpb, etx</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>cpa, etx</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>E</td>
<td>cpa, iap</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Non toxigenic</td>
<td></td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>75</td>
<td>45</td>
</tr>
</tbody>
</table>
Table (4) Distribution of C. perfringens Genotype in relation to age group of camel calves.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>No. of isolates</th>
<th>C. perfringens Genotype</th>
<th>Type A</th>
<th>Type A β2</th>
<th>Type B</th>
<th>Type C</th>
<th>Type D</th>
<th>Non toxigenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 2 month</td>
<td>27</td>
<td></td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3 – 4 month</td>
<td>23</td>
<td></td>
<td>9</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5 – 6 month</td>
<td>7</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>7 – 8 month</td>
<td>11</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>9 – 10 month</td>
<td>7</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td></td>
<td>25</td>
<td>(33.33%)</td>
<td>4</td>
<td>(5.33%)</td>
<td>4</td>
<td>(5.33%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>(17.33%)</td>
<td>13</td>
<td>(17.33%)</td>
</tr>
</tbody>
</table>

Fig (1) Camel calves with a history of sudden death (A), small intestine with bloody stained intestinal contents and gas distention (B & C) and isolated C. perfringens colonies on Shahidi-Ferguson Perfringens (SFP) agar.
**Discussion**

Infectious diseases play a prominent role in the production of camelids. Prevention, diagnosis, and treatment are vital to the management of these animals. (Fowler, 1989; Griner, 1983). Neonatal calf diarrhea is considered one of the most serious constraints of animal production. Schwartz and Dioli, (1992) reported that morbidity and mortality rates due to camel calf diarrhea could reach up to 30% and 100%, respectively. Abbas et al., (1992) reported that camel calf diarrhea affects about 33% of the neonates causing 23% mortality in Sudan. Clostridium perfringens is considered to be one of the most widespread pathogenic organisms in animals (Hatheway, 1990). This organism causes acute enterotoxaemia in ruminants including cattle, sheep, goats, buffalos and camelds (Uzal and Songer, 2008).

In the present work, a total of 200 fecal samples were collected from camel calves with signs of watery or bloody diarrhea, subnormal rectal temperatures, colic, recumbency, and nervous signs. The postmortem examination of dead animals was congestion in the small intestine Fig (1) with bloody stained intestinal contents and gas distention. These clinical sings and postmortem findings represent the sings of enterotoxaemia as described by (Songer, 1996). Enterotoxaemia caused by Clostridium perfringens is a major cause of mortality in neonatal llamas (Whitehead and Anderson, 2006).

During this work, a total of 75 clostridial isolates were recovered. The distribution of these isolates were 45 (22.5%) out of 200 fecal samples, 16 (32%) from intestinal contents collected during the postmortem of dead animals and 14 (20%) out of 70 intestinal contents of apparently healthy animals Table (2). Based on biochemical tests, and in consultation with Bergey's Manual of determinative bacteriology (Holt et al., 1994), the isolates were identified as C. perfringens. These results come parallel with the results obtained by (Naser et al., 2008, Mohamed et al., 2010) where they isolated C. perfringens from diarrheic and apparently healthy camel calves.

Isolation of C. perfringens from both apparently healthy as well as clinically affected birds and mammals confirmed the ubiquitous nature of the organism. Small numbers of C. perfringens may be found in the gastrointestinal tract of healthy individuals. The organism proliferates only if the environment in the intestine deteriorates (Harbola and Arora, 1994).

Regarding to the results of pathogenicity to mice as shown in Table (4), a total of 16 (21.33%) C. perfringens isolates were nonpathogenic for mice. Where 10 (25%) and 6 (42.85%) were recovered from fecal samples and intestinal contents of apparently healthy animals respectively. All isolates from intestinal contents of dead animals were pathogenic to mice.

In its role in the intestinal flora, C. perfringens becomes associated with various forms of disease under certain favorable conditions. Therefore, detection of virulence factors including identification of toxin genes in the isolates of C. perfringens plays a significant role in the differentiation of pathogenic from non-pathogenic organisms and in establishing its role in the disease condition (Yoo et al., 1997).

PCR-based technology is considered to be a convenient and highly reliable tool for molecular...
detection of all the major toxin genes of C. perfringens (Meer and Songer, 1997).

In this work, multiplex PCR was used for genotyping of C. perfringens isolates. Fig (2) showed the PCR products of cpa (900 bp), cpb (612 bp), etx (396 bp) and cpb2 (200 bp). Concerning the results of PCR as shown in Table (3), a total of 75 C. perfringens isolates were genotyped. Of these isolates, 25 (33.3%) were type A, 4 (5.33%), were type A β2, 4 (5.33%), were type B, 13 (17.33%) were type C, 13 (17.33%) were type D and 16 (21.33%) were nonpathogenic.

The predominant type in this study was C. perfringens type A; these results come in agree with (Mohamed et al., 2010). Type A enterotoxaemia is the most serious disease of neonate alpacas in Peru and death losses from type A enterotoxaemia occurred in crias from three to eighty days of age, with more than 85% of the losses occurring between eight and thirty-five days. (Moro Sommo, 1963; Huaman et al., 1981). Toxinotype A is the C. perfringens type found most frequently in the intestine of healthy sheep and goats (Uzal and Marcellino, 2002). However, the zoonotic characteristic of C. perfringens type A should be taken in account.

The β2 toxin producing C. perfringens type A has also recently been linked to disease in several animal species, including sheep and goats. However, most evidence implicating β2 in pathogenesis of ovine or caprine infections is based on isolation of β2 positive C. perfringens from sick animals. The condition has not been reproduced experimentally with β2 producing strains, and the importance of including the gene encoding for β2 production detection (in vivo or in vitro) in the diagnostic definition of type A infections is unknown. (Dray, 2004; Bueschel et al., 2003).

Concerning to C. perfringens type C and as shown in Table (4) Most type C infections occur in neonatal calves and are referred to as hemorrhagic enteritis. Clostridium perfringens beta toxin is considered the main virulence factor in type C infections (Uzal and Songer, 2008); it is very sensitive to trypsin digestion, and animals with low levels of intestinal trypsin (such as newborns) are usually the most susceptible to infection by C. perfringens isolates producing CPB (Songer, 1996). In contrast, C. perfringens type D was not detected in the young age group where ETX requires proteolysis via trypsin, or other intestinal or bacterial proteases, to become completely active. (Songer, 1996).

There were geographical differences in the prevalent types of the bacterium. Also, the type could be different depending on the animals species in the area. (Yoo et al., 1996).

In conclusion, C. perfringens type A is the predominant type among camel calves in Al Hasa region, KSA. Beta 2 toxin gene was detected in 4 C. perfringens type A isolates. isolation of toxigenic C. perfringens strains from intestine of apparently healthy animals has a public health significance.

The multiplex PCR can be used to type C. perfringens isolates in epidemiological studies an alternative to conventional procedures. Its recommended that a vaccination schedule should be implemented to reduce the enterotoxaemia in camel calves. Further research is needed to study the molecular relationship between C. perfringens from camels and human strains to trace the source of infection.

References


