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NEW PRELIMINARY RESEARCH IN *Clostridium perfringens* IN DROMEDARIES

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ABSTRACT

In total, 13 *C. perfringens* strains were isolated from dromedaries which died from Clostridiosis. The *C. perfringens* strain 939/06 (No. 5) was identified by ELISA and PCR as a *C. perfringens* A strain and was selected for α-toxin (phospholipase C) and vaccine production from 12 other different *C. perfringens* strains due to its high α-toxin production. The quantative alphatoxin estimation was performed in the lecithovetellin test using culture supernatant from the Trypticase Glucose Yeast Extract broth (TGYEB). The vaccination of dromedaries was carried out according to the recommendation of the vaccine producer (IBT, Dessau, Germany). The results of this vaccination trial clearly showed that dromedaries with low antibody titres to *C. perfringens* (47B, 25, and 68) already produced high antibody levels after the first vaccination. *C. perfringens* antibody levels dropped significantly in almost all 9 tested dromedaries 90 days (in total 120 days after first vaccination) after the first booster, making a third (2nd booster) vaccination necessary. The antibody levels after 3 vaccinations remained high up to at least 12 months. Our investigations showed that *C. perfringens* A is the most prevalent type of *C. perfringens* in dromedaries in the UAE. This was confirmed by ELISA and PCR technology. Approximately 85% of racing dromedaries have natural high levels of antibodies to *C. perfringens* A and are therefore most probably protected against enterotoxaemia. It is proposed to test all racing dromedaries with the *C. perfringens* antibody ELISA and to vaccinate only animals which have no or low levels of *C. perfringens* A antibodies. We prepared a hyperimmunserum alpha toxin in a homologous system which eventually may save lives of racing camels from enterotoxaemia.

Key words: *C. perfringens*, dromedary, toxin, vaccine

Clostridium (*C.*) *perfringens* is an anaerobic Gram-positive bacterial pathogen causing a wide range of diseases. It is widely spread in the soil and gastrointestinal tract of animals, and is characterised by its ability to produce potent exotoxins which are responsible for specific enterotoxaemias in man and various animal species. In the United Arab Emirates (UAE), C. *perfringens* infections have become the most important bacterial disease in dromedaries, hunting falcons and gazelles (Wernery *et al,* 2003; 2004; Gierse, 2001; Wernery and Kaaden, 2002). Predisposing factors to clostridial enterotoxaemias are believed to be concurrent diseases, overtraining, dehydration and management problems.

We here report the following:

- Origin of *C. perfringens* from dromedaries
- Isolation and identification of the bacteria and its toxins
- Efficacy of farm-specific *C. perfringens* toxoid-bacteria vaccine
- Seroprevalence of non-vaccinated randomly selected dromedaries
- Production of a *C. perfringens* alpha hyperimmune serum.

Origin of *C. perfringens* strains

In total, 13 *C. perfringens* strains were isolated from dromedaries which died from Clostridiosis.

The origins of *C. perfringens* strains are summarised in Table 1.

Isolation and identification of the organism

Clostridium (*C.*) *perfringens* bacteria were isolated from dromedaries which died from acute *C. perfringens* enterotoxaemia. Swabs were taken from the haemorrhagic mucosa of the small intestine and other organs and spread onto Zeissler agar containing antibiotic supplements (Oxoid, SR93). Smears were also prepared from the affected organs and stained after Gram.

The Zeissler plates were incubated under anaerobic conditions (Gas generating kit, Oxoid) at 37°C for 24 hours.

C. perfringens colonies grown on Zeissler agar were subjected to identification and classification by:

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a) Typical growth with double zone haemolysis and production of green pigment after exposure to oxygen for several hours at room temperature

b) Typical appearance in Gram stain of pure colonies from Zeissler agar and direct smear from haemorrhagic intestine

c) ELISA (Enterotoxaemia ELISA, Cypress Diagnostics, Belgium). Thse ELISA works with culture supernatants derived after sub-cultivation of *C. perfringens* suspicious colonies from Zeissler agar, and further incubation under anaerobic conditions for 4 hours in Trypticase Glucose Yeast Extract broth (Merck Cat. No. 1.05459; Wernery *et al*, 2003). The Cypress ELISA detects alpha (α), beta (β) and epsilon (ε) toxins of *C. perfringens* as well as identifies the bacteria itself (id).

d) Duplex and Multiplex PCR according to Tansuphasiri *et al* (2002) and Gkiourtzidis *et al* (2001). The Duplex PCR identifies 2 genes of *C. perfringens*: the alpha toxin (plc or phospholipase gene α) and the enterotoxin (ENT or CPE), whereas the Multiplex PCR identifies 6 genes, which are: α, β, β2, ε, L and enterotoxin. PCR results and results of the enterotoxaemia ELISA are shown in Table 2.

Results of both the duplex and the multiplex PCRs are shown in Figures 6a and 6b

Table 1. *C. perfringens* strains isolated from different camel organs.

	Id of strains	Collection site
1	1331/06	Small intestine
2	1245/06	Small intestine
3	1330/06	Small intestine
4	663/06	Small intestine
5	**939/06**	**Small intestine**
6	1478/06	Small intestine
7	1484/06	Small intestine
8	1559/06	Kidney
9	1559/06	Lung
10	1573/06	Small intestine
11	1584/06	Small intestine
12	1616/06	Lung
13	1529/06	Small intestine

Selection of *C. perfringens* strain for alpha toxin and vaccine production

The *C. perfringens* strain 939/06 (No. 5) was identified by ELISA and PCR as a *C. perfringens* A strain and was selected for α-toxin (phospholipase C) and vaccine production from 12 other different *C. perfringens* strains due to its high α-toxin production. The strain originated from a 2 year-old female dromedary racing camel which had died from an acute clostridial enterotoxaemia. Toxin and vaccine production was carried out by IDT Biologika, Dessau, Germany, website: www.idt@idt-biologica.de. The method of the vaccine production cannot be released due to the company's policy. The designation of the alpha toxin is batch FLB 37/07 and of the vaccine is BESTVAC® 5890807. Strain 939/06 produced only the α-toxin. The vaccine produced in Dessau is an alpha-toxoid bacterial vaccine.

Table 2. ELISA and PCR results of 13 *C. perfringens* strains isolated from dromedaries.

	Strain	Enterotoxaemia ELISA(identification, α, β, ε toxins)				Duplex PCR (α and ENT genes)		Multiplex PCR (α, β, β2, ε, L, ENT genes)					
		id	α	β	ε	α	ENT	α	β	β2	ε	L	ENT
1	1331-06	+	+	-	+	+	-	+	-	-	+	-	-
2	1245-06	+	+	-	-	+	-	+	-	-	-	-	-
3	1330-06	+	+	-	-	+	-	+	-	-	-	-	-
4	663-06	+	+	-	-	+	-	+	-	-	-	-	-
5	**939-06**	**+**	**+**	**-**	**-**	**+**	**-**	**+**	**-**	**-**	**-**	**-**	**-**
6	1478-06	+	+	-	-	+	-	+	-	-	-	-	-
7	1484-06	+	+	-	-	+	-	+	-	+	-	-	-
8	1559-06	+	+	-	-	+	-	+	-	-	-	-	-
9	1559-06	+	+	-	-	+	-	+	-	+	-	-	-
10	1573-06	+	+	-	-	+	-	+	-	-	-	-	-
11	1584-06	+	+	-	-	+	-	+	-	-	-	-	-
12	1615-06	+	+	-	-	+	-	+	-	-	-	-	-
13	1529-06	+	+	-	+	+	-	+	-	-	+	-	

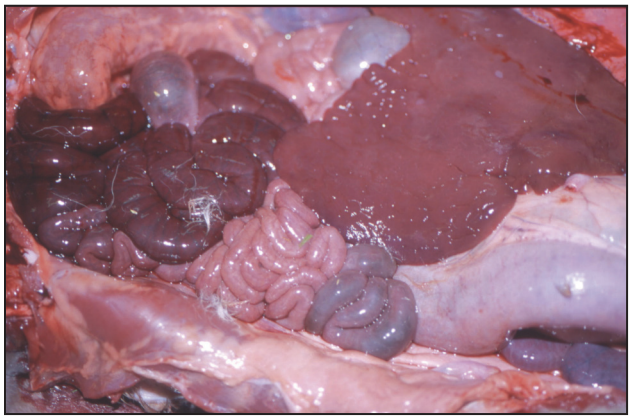

Fig 1. Haemorrhagic enteritis caused by *C. perfringens*.

Fig 2. Incubation of Zeissler agar in anaerobic jars.

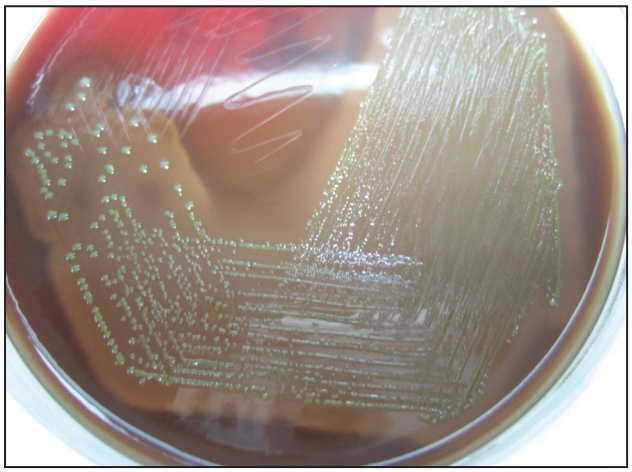

Fig 3. *C. perfringens* colonies on Zeissler agar with haemolysis and green pigmentation of colonies.

Alpha toxin estimation for immunisation

The quantative alphatoxin estimation was performed in the lecithovetellin test using culture supernatant from the Trypticase Glucose Yeast Extract broth (TGYEB). The TGYEB supernatant was diluted two-fold (1:2 to 1:1409) usisng 0.5 ml supernatant and 0.5 ml 0.9% NaCl. To every dilution 0.5 ml egg yolk suspension was added which was prepared as follows:

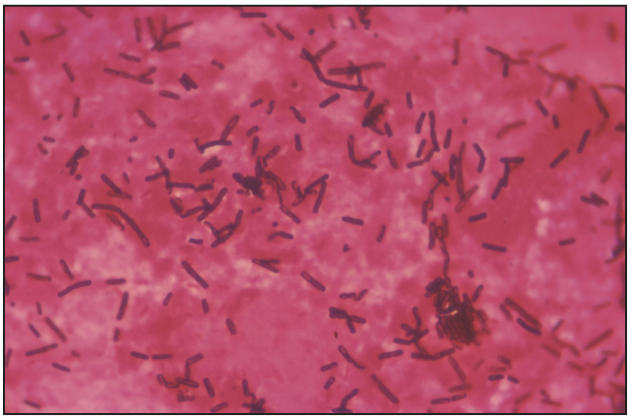

Fig 4. High numbers of Gram positive rods from direct smear of a dromedary intestinal mucosa.

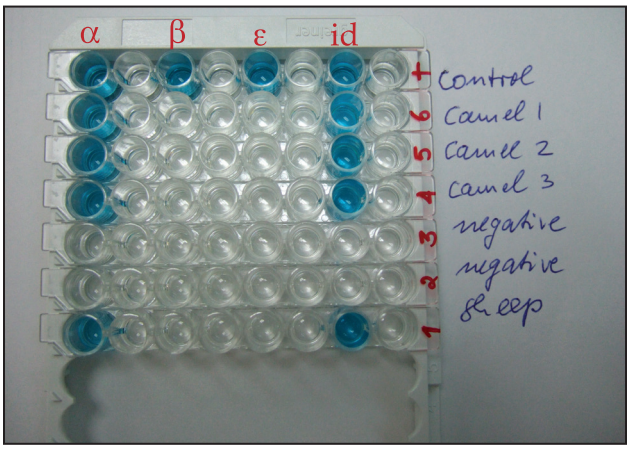

Fig 5. *C. perfringens* ELISA test results. The ELISA identifies 3 major toxins and the isolated strains.

The egg yolk of 5 fresh chicken eggs was resuspended in 500 ml 0.9% NaCl, kept overnight at 4°C and centrifuged for 20 minutes at 7000 rpm. The supernatant was then mixed with an equal volume of 0.4% $CaCl_2$ solution and sterile filtered. This solution was then added to the twofold diluted phospholipase C suspension. The phospholipase C activity in the TGYEB is measured in minimal phospholipase C units (MPU). The MPU is the unit which produces turbidity. If, for example, a turbidity of the egg yolk emulsion is observed at a dilution of 1:1024, 1 MPU is detected. Correspondingly, 1 ml of the TGYEB supernatant consists of 1024 phospholipase C units (MPU). The *C. perfringens* strain 939/06 (No. 5) used for manufacturing the toxoid FLB 37/07 possessed 5I2 MPE/ml.

Vaccination with the 939/06 *C. perfringens* alpha-toxoid bacterial vaccine

The vaccination of dromedaries was carried out according to the recommendation of the vaccine producer (IBT, Dessau, Germany). The vaccine

was applied twice within a 3 week time period, and the dose was 10 ml given subcutaneously. A 3rd vaccination followed 6 months after the booster dose was given. According to the manufacturer, annual vaccinations are then sufficient. Before vaccination and several times after vaccination the *C. perfringens* antibody level was tested using the BIO-X Diagnostics ELISA BIO K221. This test is designed to monitor the animal's serological response after immunisation by a vaccine or after natural contact with *C. perfringens*. It is a competitive ELISA and therefore can be used in all animal species. The results are expressed in per cent inhibition which is calculated from the mean optical densities of positive and negative sera using special formulas.

With our investigations we also explored antibody development after vaccination with the *C. perfringens* toxoid-bacteria vaccine over a period of 12 months. The results of these investigations are shown in Table 3.

The results of this vaccination trial clearly showed that dromedaries with low antibody titres to *C. perfringens* (47B, 25, and 68) already produced high antibody levels after the first vaccination. Dromedaries which already possessed high levels before vaccination showed no further significant rise in antibody levels to the *C. perfringens* vaccine.

Table 3. *C. perfringens* antibody ELISA development of 9 dromedaries before and after vaccination.

No. of day	0 day	30 day	60 days	120 days	12 months
Vaccination history	1st dose of vaccine given	1st booster dose		2nd booster dose	
Identification	% inhibition				
47B	37	94	95	82	95
A6F	63	95	95	83	94
6B I	93	96	96	88	93
5B F	95	96	96	79	95
Tag # 20	66	85	75	49	90
Tag # 25	31	82	75	43	91
Tag # 68	16	86	91	38	90
Tag # 294	89	88	94	77	89
Tag # 331	94	94	95	76	93

Calculated value	Degree of positivity
% inhibition <20	0
% inhibition 20 – 40	+
% inhibition 40 – 60	++
% inhibition 60 – 80	+++
% inhibition > 80	++++

C. perfringens antibody levels dropped significantly in almost all 9 tested dromedaries 90 days (in total 120 days after first vaccination) after the first booster, making a third (2nd booster) vaccination necessary, which was also recommended by the vaccine producer.

The antibody levels after 3 vaccinations remained high up to at least 12 months. In future, ongoing investigations about the duration of antibodies to a *C. perfringens* A vaccination will continue. However, from our investigation, it is obvious that 3 vaccinations are necessary to maintain high levels of antibodies for at least one year against a *C. perfringens* A toxoid-bacteria vaccine, which may protect animals from disease.

Field investigations for the detection of *C. perfringens* antibodies

Fifty non-vaccinated, randomly selected racing dromedaries were tested for their *C. perfringens* antibodies, the results of which are shown in Table 4.

Our findings show that approximately 15% of tested dromedaries had low levels of antibodies (+ degree of positivity) to *C. perfringens* which may explain our observations in the UAE that only a few racing dromedaries succumb to the disease.

Fifty percent of the examined dromedaries possessed high antibody levels, which may protect them from disease.

Immunisation of 2 dromedaries with a-toxin from *C. perfringens* strain 939/06

The alphatoxin FLB 37/07 produced by IDT, Dessau, Germany, from strain 939/06 was used for

Table 4. *C. perfringens* antibody ELISA levels of 50 non-vaccinated randomly selected dromedaries.

	No. of dromedaries each group	% inhibition	Degree of Positivity	Percentage
Group 1	26	92	++++	50
Group 2	6	68	+++	12
Group 3	20	48	++	20
Group 4	8	20	+	16
Total	50			100

Calculated value	Degree of positivity
% inhibition <20	0
% inhibition 20 – 40	+
% inhibition 40 – 60	++
% inhibition 60 – 80	+++
% inhibition > 80	++++

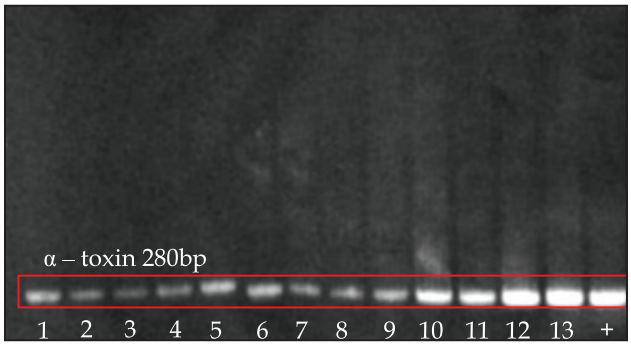

Fig 6a. Duplex PCR detects α and enterotoxin genes.

All 13 samples positive for alpha genes, not for enterotoxin genes.

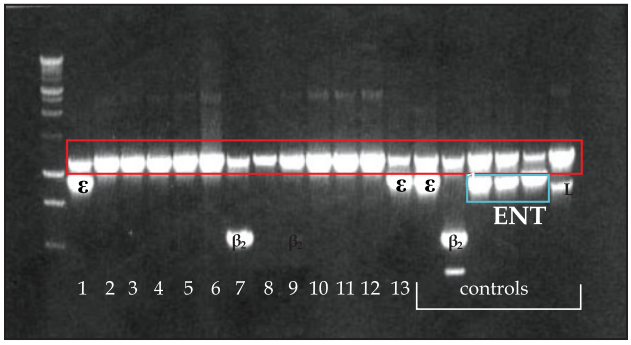

Fig 6b. Multiplex PCR detects α, β, β2, ε, L and enterotoxin genes.

Samples 1331/06 (No. 1) and 1529/06 (No. 13) are positive for alpha and epsilon toxin genes. Samples 1484/06 (No. 7) and 1559/06 (No. 9) are positive for alpha and beta 2 toxin genes.

Sample 939/06 (No. 5) positive for alpha toxin gene only (See also Table 2).

the immunisation of 2 three-year old male castrated dromedaries kept at CVRL. For this purpose, 5 ml PBS containing 0.4 g toxin were mixed with 5 ml of Montanide adjuvant. The mixture was subcutaneously (s/c) given at the base of the neck. In total 4 immunisations were carried out, one each week. The camels were bled regularly and the serum

Fig 7. After a small incision is made above the *Vena jugularis*, a 4 mm gauge needle is inserted for the blood letdown. Before this procedure, the camel received 1.5 ml of Rompun intravenously.

tested with Bio-X Diagnostics *C. perfringens* alphatoxin serological ELISA kit BIOK221 for *C. perfringens* antibodies. The results of this investigation are shown in Table 5.

Thirty days after the fourth immunisation, when both dromedaries showed high levels of antibodies to the a-toxin, approximately 6 litres of blood were withdrawn (Fig 7) from both camels from which 2.8 litres of serum were gained. From this serum, IgG were extracted for the production of an anti-*C. perfringens* alpha hyperimmune serum.

C. perfringens IgG extraction method

The clinical cause of clostridial enterotoxaemia is too acute for an effective treatment with antibiotics. Therefore hyperimmune serum is an efficient short-term prophylactic method. The antitoxin given intravenously will neutralise the toxin circulating in the sick animal. In sheep the administration of epsilon antitoxin 20 IU/kg body weight will save the animal's life. Antitoxins will circulate for more than 20 days in the animal's body.

Table 5. *C. perfringens* antibody ELISA development of 2 dromedaries before and after immunisation with alpha toxin.

Weeks	0	1 week	2 weeks	3weeks	4 weeks	Blood taken
	Immunisation done	1st immunisation	2nd immunisation	3rd immunisation	4th immunisation	
Identification	inhibition					
C6C	95	94	96	95	95	96
DBO	56	24	79	73	70	89

Calculated value	Degree of positivity
% inhibition <20	0
% inhibition 20 – 40	+
% inhibition 40 – 60	++
% inhibition 60 – 80	+++
% inhibition > 80	++++

For the IgG extraction we followed a method described by Harrison (2008, personal communication):

1. Thaw frozen dromedary serum, pipette 100 ml into a glass beaker 3 times the size of the serum volume
2. Place the serum on a stirrer plate, add the sterilised magnetic stirrer, turn the stirrer on high speed until there is a vigorous stirring
3. Add dropwise caprylic acid (octanoic acid, Sigma) to an end volume of 5%, and stir vigorously for 2 h – the non-IgG components will precipitate into a pale white mass
4. Pipette the entire suspension into centrifuge tubes and centrifuge at 4°C for at least 20 min at 8000 g in large volume centrifuge tubes
5. Immediately dialyse the IgG which is in the supernatant overnight at 4°C against 0.1 M NaH_2PO_4 for 24 h and store the IgG at -20°C.

In experimental trials it must now be calculated how much of the hyperimmuneserum must be intravenously administered into dromedaries suffering from enterotoxaemia to save their lives. This is a time consuming project as the disease cannot be experimentally produced and relies entirely on field cases.

Conclusion

Our investigations showed that *C. perfringens* A is the most prevalent type of *C. perfringens* in dromedaries in the UAE. This was confirmed by ELISA and PCR technology.

We also found that approximately 85% of racing dromedaries have natural high levels of antibodies to *C. perfringens* A and are therefore most probably protected against enterotoxaemia. This may explain why only single dromedaries succumb to this disease.

We proved that 3 subcutaneous vaccinations with a toxoid-bacteria *C. perfringens* A vaccine are necessary to obtain high level of antibodies for at least one year. It will be seen if vaccinated dromedaries are protected against *C. perfringens* A enterotoxaemia and for how long. Dromedaries that already possessed high levels of antibodies did not show any further significant antibody rise after vaccination. Therefore, it is proposed to test all racing dromedaries with the *C. perfringens* antibody ELISA and to vaccinate only animals which have no or low levels of *C. perfringens* A antibodies.

Finally, we prepared a hyperimmuneserum alpha toxin in a homologous system which eventually may save lives of racing camels from enterotoxaemia. It is anticipated that this hyperimmuneserum is given intravenously to dromedaries suffering from acute clostridial enterotoxaemia.

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