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Clostridial myonecrosis clinically resembling black quarter in an Indian elephant (*Elephas maximus*)

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Summary

An Indian elephant (*Elephas maximus*) which died of acute fatal myonecrosis was examined to determine the aetiology of the infection. The causative organism was identified as *Clostridium perfringens* type A. Out of five genes encoding for major toxins (*cpa, cpb, etx, iA*, and *cpe* genes) the isolate was found to harbour the *cpa* gene only, as tested by multiplex polymerase chain reaction. It flanks a 324 base pair segment in the *cpa* gene, indicating the presence of the alpha toxin gene. The organism was sensitive to amikacin, ampicillin, enrofloxacin, gentamicin and norfloxacin but was resistant to bacitracin, oxytetracycline and tetracycline. The acute malignant nature of the myonecrosis and presence of the alpha toxin gene in the isolate suggested that the myonecrosis, although clinically resembling that caused by *C. chauvoei* in cases of black quarter, was caused by *C. perfringens* type A.

Keywords

Black quarter – *Clostridium perfringens* – *cpa* gene – *Elephas maximus* – India – Indian elephant – Myonecrosis – Polymerase chain reaction – Virulence genes – Zoo.

Introduction

Black quarter and malignant oedema (also known as 'false' black quarter, clostridial myonecrosis, and gas gangrene) are highly fatal diseases caused by bacteria of the genus *Clostridium* that affect the muscle and subcutaneous tissues of cattle, sheep, goats and other animal species. Whereas black quarter is an endogenous infection produced by *Clostridium chauvoei*, malignant oedema is considered to be an exogenous infection produced by one or more of the other clostridial organisms: *C. septicum*, *C. novyi, C. sordellii* and *C. perfringens* (19). Black quarter, which is characterised by gangrenous myositis with crepitation, is a common disease of cattle in India, whereas false black quarter or atypical black quarter is a rare disease. Atypical black quarter in cattle caused by *C. perfringens* type A was reported in Manipur in north-eastern India (16). In this paper the authors report a case of fatal myonecrosis in an Indian elephant (*Elephas maximus*), caused by *C. perfringens* type A, in the neighbouring state of Assam.

Materials and methods

History and source of materials

An eight-year-old male Indian elephant calf maintained at the Assam State Zoo became ill with acute myonecrosis.

The elephant calf showed clinical signs of sudden illness characterised by loss of appetite with high fever (39°C) and reluctance to walk. The animal was treated with an intramuscular injection of enrofloxacin, 5 mg/kg body weight. The animal did not respond to treatment and its condition deteriorated rapidly. The animal stopped taking feed and water, became recumbent and finally died within 48 h of the onset of clinical signs. The necropsy was performed and the external body surface was examined for bruises, injuries or any other unusual marks. All the internal organs were thoroughly examined and any macroscopic lesions observed were recorded. Dark, discoloured, swollen muscles with rancid odour from the affected region and intramuscular aspirates were collected aseptically for microbiological investigation.

Bacteriology

The impression smears prepared from the affected tissue were stained with Gram's stain. Tissue samples and aspirated fluid collected from the affected muscles were heated at 80°C for 10 min, inoculated in brain heart infusion (BHI) broth and incubated in a Difco anaerobic jar at 37°C. The heat-treated samples were also inoculated in Robertson's cooked meat medium (RCM) with paraffin overlay and incubated at 37°C for 48 h. The inocula from both the media were transferred onto 10% sheep blood agar and incubated anaerobically for 24 h to 48 h at 37°C. Beta-haemolytic colonies were purified from the blood agar plates and subjected to Gram and malachite spore staining and biochemical tests. In addition to routine biochemical tests, DNase and lecithinase (phospholipase C) activities were detected in DNase medium and egg yolk agar medium, respectively. The isolated organism was identified on the basis of cultural, biochemical and morphological characteristics (10).

Antimicrobial susceptibility was tested by a disc diffusion method using commercially available biodiscs (4). The antimicrobial agents used were amikacin, ampicillin, bacitracin, enrofloxacin, gentamicin, norfloxacin, oxytetracycline and tetracycline.

To study the virulence, *C. perfringens* was tested by multiplex polymerase chain reaction (PCR) for the presence of *cpa* (alpha toxin), *cpb* (beta toxin), *etx* (enterotoxin), *iA* (iota toxin) and *cpe* (epsilon) genes using their specific primers (9, 18). The primer pairs for *cpa* (20), *cpb* (11), *etx* (12), *iA* (14) and *cpe* (7) genes were commercially synthesised (Table I). Bacterial cells from overnight cultures were suspended in sterile distilled water and boiled at 100°C for 10 min. After boiling, the cell suspensions were cooled in an ice bath and cell debris was removed by centrifugation. The supernatant was used as a source of template DNA. Supernatants from tissue aspirates as well as RCM broth culture that was clarified by centrifugation and boiled were also used as a source of template DNA. The amplification reaction was carried out in a final volume of 15 µl containing 7.5 µl of 2× master mix (4mM $MgCl_2$, 0.4 mM of each dNTPS), 0.05 units/ml of *Taq* DNA polymerase, 1.5 µl Tris-HCl PCR buffer (MBI Fermentas, United States of America [USA]), primers, nuclease-free distilled water and 1.5 µl of template DNA. Amplification was performed in a thermocycler (iCycler, BioRAD, USA) with thermal cycling programmes of 4 min initial denaturation at 94°C followed by 35 cycles of 1 min each at 94°C, 1 min at 55°C and 1 min at 72°C. Final extension was allowed for 10 min at 72°C. Polymerase chain reaction products (amplicons) were electrophoretically separated in agar gel (1%) containing 0.5 µl ethidium bromide per ml. The separated bands were visualised and analysed under an ultraviolet transilluminator (300 nm) and photographed using the Gel Doc 2000 documentation system (BioRAD, USA).

Histopathology

The affected muscle samples were subjected to histopathological examination. The tissues were fixed in

Table I
Primers used for the major toxin genes of *Clostridium perfringens*

Name of gene	Primer sequence	Primer concentration (mM) each	Amplicon size (bp)	Reference
cpa	For-gctaatgttactgccgttga			
	Rev-cctctgatacatcgtgtaag	0.25	324	20
cpb	For-gcgaatatgctgaatcatcta			
	Rev-gcaggaacattagtatatcttc	0.36	180	11
etx	For-gcggtgatatccatctattc			
	Rev-ccacttacttgtcctactaac	0.44	655	12
iA	For-actactctcagacaagacag			
	Rev-ctttccttctattactatacg	0.50	446	14
cpe	For-ggagatggttggatattagg			
	Rev-ggaccagcagttgtagata	0.35	233	7

10% formal saline. After proper fixing, the tissues were trimmed to a suitable size (1 × 1.5 cm) and thoroughly washed. The washed tissues were dehydrated in ascending grades of ethyl alcohol and cleared in benzene. The dehydrated tissues were embedded in paraffin and sections were cut at 5 µm thickness and stained with haematoxylin and eosin.

Results

Necropsy findings

There was no sign of injury, trauma or history of injection prior to the infection. At necropsy, diffuse haemorrhage with reddish to bluish discoloration of the muscles of the neck, tongue (Fig. 1), back region (Fig. 2) and left shoulder to carpel region were observed. Haemorrhage was noticed in these areas and crepitation could be felt on palpation of the shoulder and back muscles. On incision of these muscles, sero-sanguineous fluid oozed out from the emphysematous tissues (Fig. 3). There was diffuse haemorrhagic and necrotic enteritis in both the small and large intestines. Discrete areas of necrosis were present on the walls of the caecum and colon. The liver was enlarged and showed haemorrhagic infarction and necrotic foci. Mesenteric lymph nodes were enlarged and haemorrhagic. The spleen was markedly enlarged and showed degenerative changes. Petechial haemorrhage was present on both the endocardium and epicardium. The cortical surface of the kidneys was congested and showed petechial haemorrhages. The meninges showed mild congestion.

Histopathological findings

Microscopically, the affected muscles showed diffuse haemorrhage in the bundles of muscle and in the intramuscular spaces. Focal necrosis in the muscle bundles was present. At many points, empty spaces within the muscle bundles could be seen, indicating the presence of gas bubbles (Fig. 4).

Fig. 1
Severe haemorrhage in the tongue
Note necrosis of muscle in the incised area

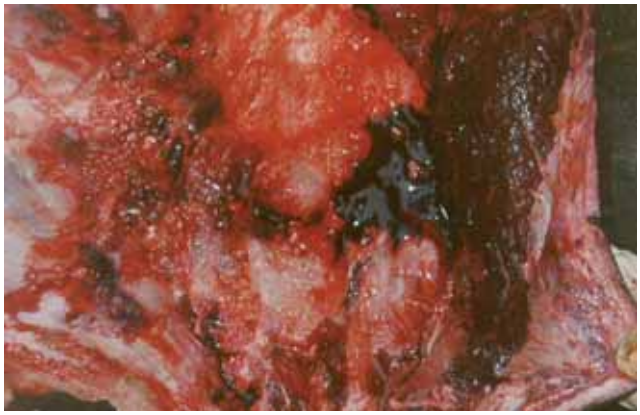

Fig. 2
Haemorrhages and necrosis on the back of the muscle

Fig. 3
Haemorrhages and sero-sanguineous fluid in the shoulder muscle

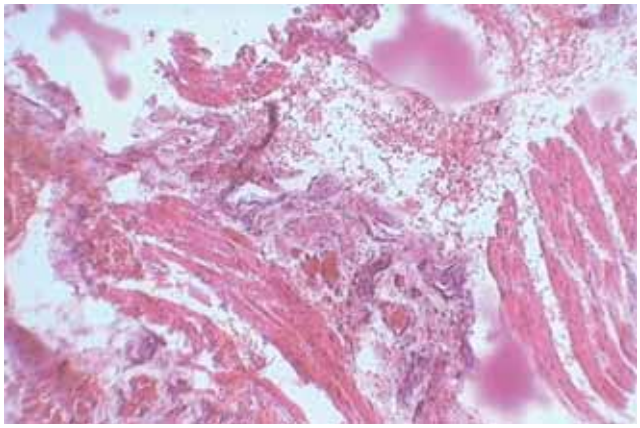

Fig. 4
Affected muscles showing diffuse haemorrhages in the bundles of muscle and the intramuscular spaces with focal necrosis and empty spaces indicating the presence of gas bubbles

Bacteriological findings

The impression smear prepared from the affected tissue showed the presence of Gram-positive thick rods. The heat-treated pieces of affected muscle and intramuscular aspirates inoculated in RCM resulted in the production of gas after 16 h to 18 h of incubation. The isolates grew luxuriantly on BHI broth and BHI agar under anaerobic conditions. Beta-haemolytic, rough, large, flat, opaque and spreading types of colonies on sheep blood agar were observed. These cultures were Gram-positive thick rods and showed sub-terminal spores on Gram and malachite spore staining, respectively. The isolate showed DNase and lecithinase activities and was identified as *C. perfringens*. It was sensitive to amikacin, ampicillin, enrofloxacin, gentamicin, and norfloxacin but was resistant to bacitracin, oxytetracycline and tetracycline. The isolate was found to harbour *cpa* gene as detected by PCR. It gave rise to a 324 base pair (bp) segment in the *cpa* gene indicating the presence of the alpha toxin gene. Thus the isolate was confirmed as *C. perfringens* type A.

Discussion

The clinical manifestations and pathological alterations observed at necropsy were suggestive of clostridial myonecrosis. The haemorrhagic and necrotic enteritis observed in this particular case were indicative of primary damage in the intestine from where the disease has extended to the muscles to cause secondary lesions. The organism might have reached its site of predilection in the muscles from the intestine to cause haemorrhages and necrosis by its toxins. Moreover, the haemorrhages observed in various organs, such as the liver, kidneys, heart, lymph node and spleen, are suggestive of the septicaemic nature of the disease. The diagnosis of clostridial myonecrosis was further confirmed by the isolation and characterisation of *C. perfringens* type A. *Clostridium perfringens* is the most common cause of clostridial myonecrosis. Two membrane-active toxins produced by *C. perfringens*, alpha toxin and perfringolysin O, are considered to be the most important virulence factors in the initiation of muscle infection (17) and prevent entry of phagocytic cells at the site of infection (13). In addition to their enzyme activities, the toxins are also known to have lethal haemolytic and necrotic activities. Damage to the tissue is attributed to the production of exotoxin lecithinase or alpha-toxin-phospholipase C phosphatidyl–choline phosphohydrolase rather than to host inflammatory mechanisms. *C. perfringens* produces a number of exotoxins and enzymes, which play a major role in myonecrosis, and attacks muscle cells, leukocytes and platelets (1, 15).

C. perfringens and other clostridia have also been reported to be the causative organism in a variety of other disease conditions in humans and animals. A 22-year-old female African elephant (*Loxodonta africana*) died as a result of diarrhoea caused by *C. perfringens* which produced beta2 toxin and the alpha toxin (2). Two cases of fatal enteritis caused by *C. difficile* in captive Asian elephants were reported from an outbreak affecting five females in a zoo (5). The isolation of *C. perfringens* type A was also reported from cases of gas gangrene in humans (3). Recently, *C. perfringens* type A secreting alpha and beta toxin was isolated from the muscles and lungs of a 13-year-old horse suffering from acute haemorrhagic myonecrosis (6). *Clostridium perfringens* type A was also found to be associated with clostridial enterotoxaemia (84% of the cases) in lambs and kids (8).

Songer and Meer (18) reported the use of multiplex PCR using six primers specific for the major toxin genes of *C. perfringens*, (*cpa, cpb, etx, iA, cpe, cpb*2), which allowed classification into genotypes as follows:

– A (positive for *cpa*)
– B (positive for *cpa, cpb* and *etx*)
– C (positive for *cpa* and *cpb*)
– D (positive for *cpa* and *etx*)
– E (positive for *cpa* and *iA*).

Molecular typing of the isolate by multiplex PCR using five toxin-specific primers in the present study amplified only the primer specific for *cpa* (324 bp), indicating the presence of the alpha toxin gene.

The isolation of highly virulent *C. perfringens* type A from a case of fatal myonecrosis in an Indian elephant warrants further study. In India, fatal myonecrosis simulating black quarter caused by *C. perfringens* in elephants has not been recorded before and the epidemiology of the infection should be investigated further.

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Un cas de myonécrose clostridienne aux manifestations cliniques proches du charbon symptomatique chez un éléphant d'Asie (*Elephas maximus*)

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Résumé

Les auteurs décrivent les résultats d'un examen réalisé sur un éléphant d'Asie (*Elephas maximus*) décédé après avoir été atteint de myonécrose aiguë mortelle, en vue de déterminer l'étiologie de l'infection. L'agent causal a pu être identifié comme étant *Clostridium perfringens* de type A. L'épreuve d'amplification en chaîne par polymérase multiplexe a révélé que l'isolat ne possédait qu'un des cinq gènes codant pour la production des principales toxines (gènes *cpa, cpb, etx, iA*, et *cpe*), à savoir le gène *cpa*. Il était flanqué d'un fragment de 324 paires de bases, ce qui indique la présence du gène de la toxine alpha. Le micro-organisme s'est révélé sensible à l'amikacine, à l'ampicilline, à l'enrofloxacine, à la gentamicine et à la norfloxacine ; en revanche, il était résistant à la bacitracine, à l'oxytétracycline et à la tétracycline. Le caractère malin et aigu de cette myonécrose et la présence du gène de la toxine alpha dans l'isolat permettent de conclure que malgré un tableau clinique similaire à celui du charbon symptomatique dû à *C. chauvoei*, l'infection est dans le cas présent causée par *C. perfringens* de type A.

Mots-clés

Amplification en chaîne par polymérase – Charbon symptomatique – *Clostridium perfringens* – Éléphant d'Asie – *Elephas maximus* – Gène codant pour des facteurs de virulence – Gène *cpa* – Inde – Myonécrose – Parc zoologique.

Mionecrosis clostridial, clínicamente semejante a la pierna negra, en un elefante indio (*Elephas maximus*)

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Resumen

Los autores describen los resultados del examen practicado a un elefante indio (*Elephas maximus*) que murió de mionecrosis aguda fatal a fin de determinar la etiología de la infección. Se pudo identificar el organismo causante, que resultó ser un *Clostridium perfringens* de tipo A. Mediante reacción en cadena de la polimerasa (PCR) múltiple, se observó que, de los cinco genes que codifican las principales toxinas (genes *cpa, cpb, etx, iA* y *cpe*), el microorganismo aislado contenía únicamente el gen *cpa*, flanqueado por un segmento de 324 pb indicativo de la presencia del gen de la toxina alfa. El microorganismo era sensible a la amicacina, la ampicilina, la enrofloxacina, la gentamicina y la norfloxacina, pero resistente a la bacitracina, la oxitetraciclina y la tetraciclina.

Del carácter agudo y maligno de la mionecrosis y de la presencia de toxina alfa en el microorganismo aislado se dedujo que la infección, pese a su semejanza clínica con la causada por *C. chauvoei* en los casos de pierna negra, se debía a *C. perfringens* de tipo A.

Palabras clave
Clostridium perfringens – Elefante indio – *Elephas maximus* – Gen *cpa* – Genes de virulencia – India – Mionecrosis – Pierna negra – Reacción en cadena de la polimerasa – Zoológico.

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