Investigation of haemorrhagic enteritis in pygmy hogs (*Sus salvanius*) from India

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Submitted for publication: 16 August 2007
Accepted for publication: 26 August 2010

Summary

The pygmy hog is a representative of the smallest and rarest wild species of known living Suidae. This paper reports the investigation of haemorrhagic enteritis encountered amongst the pygmy hogs at the Research and Breeding Centre of the Pygmy Hog Conservation Programme, Guwahati, Assam, India. Three out of 68 pygmy hogs died of enteric infection. Post-mortem examination and bacteriological investigation of two out of the three animals that died revealed clostridial infection. The isolates harboured two plasmids of molecular weight 42.8 kilobases (kb) and 51.9 kb. *Clostridium perfringens* Type A positive for the beta2 toxin (*cpb2*) gene was detected by polymerase chain reaction. Sequence analysis of the partial alpha toxin (*cpa*) gene showed 98% to 100% homology with isolates from different geographical locations.

Keywords

Alpha toxin – Beta2 toxin – *Clostridium perfringens* – Haemorrhagic enteritis – India – Polymerase chain reaction – Pygmy hog – *Sus salvanius*.

Introduction

The pygmy hog (*Sus salvanius*) is the smallest and rarest wild member of the Suidae in the world. The International Union for Conservation of Nature (IUCN) (12) has accorded it the highest priority rating among the most endangered mammals – critically endangered (criteria: A1c, B1, +, 2cd, E [visit www.iucnredlist.org for more information about IUCN categories and criteria for listing]). A wild population of this species, containing fewer than 150 individuals, exists only in the Manas Tiger Reserve, north-west Assam, India. Occurrences of salmonellosis in this species have already been reported (15). Clostridial enteritis in captive pygmy hogs appears to be rare; however, it has been reported in captive elephants (1).

Clostridium perfringens is commonly associated with enteric diseases in herbivores. Diseases caused by *C. perfringens* are classified into five different types on the basis of the toxins produced. Association of *C. perfringens* Type A with haemorrhagic enteritis in dogs (16) and with necrotic enteritis and enterocolitis in piglets (6) has been reported. It is difficult to distinguish between different *C. perfringens* isolates in clinical cases. Polymerase chain reaction (PCR) has been used therefore to distinguish isolates and to detect toxin genes (21). This study reports the occurrence of fatal

haemorrhagic enteritis due to *C. perfringens* in pygmy hogs at the Research and Breeding Centre of the Pygmy Hog Conservation Programme in Guwahati, Assam, India, diagnosed by bacterial isolation and PCR.

Materials and methods

History

A total of 68 pygmy hogs were maintained in a protected area at the Research and Breeding Centre to avoid all mammalian and avian contact. Nine out of 68 animals showed anorexia, pyrexia, rough hair coat and prolonged episodes of haemorrhagic diarrhoea. The atmospheric temperature and humidity were recorded at 30ºC to 35ºC and 80% to 90% respectively, with heavy rainfall during the study period. These animals did not respond to treatment with mebendazole orally (Wormin 500; Cadila) and oxytetracycline parenterally (Oxytetracycline injection; Sarabhai Zydus), and three out of the nine animals died. On post-mortem examination of two animals, severe haemorrhages were observed throughout the intestines. Samples from the small and large intestines were collected aseptically and processed for the isolation of bacteria.

Bacterial isolation and biochemical tests

Bacterial isolation was attempted under both aerobic and anaerobic conditions. For aerobic isolation, samples were inoculated onto nutrient agar plates after 4 h of enrichment in nutrient broth at 37ºC. Bacterial colonies were purified and identified separately following standard bacteriological procedures. For anaerobic isolation, samples of intestine were preheated and inoculated in Robertson's cooked meat (RCM) medium with neutral oil overlay and incubated at 37ºC for 48 h. The inocula from the RCM media were seeded onto 10% goat blood agar and incubated anaerobically for 24 h at 37ºC (3). Bacterial colonies were purified individually on the basis of the size, shape, colour and pattern of haemolysis on blood agar and were subjected to Gram and malachite spore staining. A set of biochemical tests, including the litmus milk, gelatinase, deoxyribonuclease (DNase) and lecithinase tests, and fermentation of glucose and lactose were performed. The isolates were identified as described in the established scheme (9).

Detection of plasmids

Plasmid DNA was detected by the alkali lysis method (2). The plasmid DNA was subjected to electrophoresis in 0.7% agarose in 1× TAE (Tris–Acetate–EDTA; pH 8.0) buffer. The gel was stained with 0.4 µg/ml ethidium bromide. The approximate molecular weight of the plasmids was determined by comparison with a super-coiled DNA ladder (GENEI, Bangalore). The separated plasmid DNA bands were visualised and photographed in a gel doc system (Image Master® VDS, Pharmacia Biotech, Sweden).

Polymerase chain reaction

After overnight incubation, bacterial colonies were picked separately from the agar plate and placed in 100 µl of Milli-Q water, vortexed gently, and boiled at 100ºC for 10 min in a water bath. The clear supernatant (after centrifugation at 8,000 *g* for 5 min at 4ºC) was used as the source of template DNA.

The PCR was done in three stages. In the first stage, a multiplex PCR for the *cpa, cpb, iA* and *etx* genes was carried out. The PCR amplification was carried out in 25 µl reaction volumes containing 12.5 µl of 2× PCR master mix (4 mM $MgCl_2$; 0.4 mM of each deoxynucleotide triphosphate [dNTP]; 0.5 U of *Taq* DNA polymerase; 150 mM Tris–HCl PCR buffer), primers and 2.5 µl of template DNA. After initial denaturation at 94ºC for 4 min, the amplification cycles comprised denaturation, annealing and extension at 94ºC, 55ºC and 72ºC for 1 min each, respectively. Final extension was done at 72ºC for 10 min. The second stage was a PCR for the *cpb*2 gene and the third was for the *cpe* gene, with the same cycling conditions. The primer pairs for the *cpa, cpb, etx, iA*, *cpb*2 and *cpe* genes were synthesised commercially (GENSET K K, Tokyo, Japan) and the details are shown in Table I.

Culture CP1 of *C. perfringens* Type A, which was positive for alpha and beta2 toxins and was isolated from an

Table I
Primers used for the detection of the *cpa, cpb, etx, iA, cpb2* and *cpe* genes of *Clostridium perfringens*

Toxin genes	Primer sequences	Primer concentration (µM)	Amplicon size (bp)	Reference
cpa	For 5/-gctaatgttactgccgttga-3/	0.5	324	20
	Rev 5/-cctctgatacatcgtgtaag-3/			
cpb	For 5/-gcgaatatgctgaatcatcta-3/	0.5	180	10
	Rev 5/-gcaggaacattagtatatcttc-3/			
etx	For 5/-gcggtgatatccatctattc-3/	0.5	655	11
	Rev 5/-ccacttacttgtcctactaac-3/			
iA	For 5/-actactctcagacaagacag-3/	0.34	446	14
	Rev 5/-ctttccttctattactatacg-3/			
cpb2	For 5/-agattttaaatatgatcctaacc-3/	0.36	567	6
	Rev 5/-caatacccttcaccaaatactc-3/			
cpe	For 5/-ggagatggttggatattagg-3/	0.36	233	4
	Rev 5/-ggaccagcagttgtagata-3/			

atypical black quarter from an infected cow maintained in the laboratory (typed at the Diagnostic Bacteriology Laboratory of the United States Department of Agriculture, Ames, Iowa), was used as a positive control (17). For the negative control, a vaccine strain of *C. septicum* obtained from the Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore, India, was used. The PCR amplicons (5 µl) were separated by electrophoresis in a 1.5% agarose gel with TAE as the running buffer. The gel was stained with ethidium bromide, visualised, and photographed in the gel doc system (Pharmacia Biotech, Sweden). The PCR amplicons were sequenced using a commercial sequencing service (Microsynth, Switzerland and Genei, Bangalore, India) and were deposited in GenBank with accession numbers DQ838708 and DQ838709. The sequences of the partial alpha toxin gene (292 base pairs [bp] fragment) were compared with 22 reference sequences of *C. perfringens* from different geographical locations available in GenBank. A phylogenetic tree was constructed using the ClustalW method in MegAlign software (DNA star, United States).

Results

Aerobic isolations yielded a mixed culture of both Gram-positive and Gram-negative bacteria that did not provide any conclusive evidence for an aerobic causative agent in the disease. However, on anaerobic culture, pure colonies showing alpha and beta haemolysis were observed on goat blood agar. The bacterial colonies were flat with an irregular thin border. On malachite green staining, Gram-positive rods and sub-terminal oval endospores were observed. The isolates produced stormy fermentation, acidity, reduction and coagulation in litmus milk and liquefied gelatin. They showed both DNase and lecithinase enzyme activities and fermented glucose and lactose. The biochemical profile led the authors tentatively to identify the bacterium as *C. perfringens*. The isolates harboured identical double plasmid DNA of molecular weight 42.8 kb and 51.9 kb.

In the PCR assay, the isolates showed amplification products of 324 and 567 bp with the primers specific for alpha toxin and beta2 toxin genes, respectively, similar to those of the positive control. The other toxin-specific primers for the *cpb, etx, iA* and *cpe* genes did not show any amplification with the clinical isolate.

Partial sequencing of the *cpa* gene of the *C. perfringens* isolates showed 98% to 100% homology with representative *C. perfringens* sequences available in GenBank (Table II). More than one sequence from a single geographical location was included to give significant sequence variation. The isolates showed only three nucleotide substitutions, at positions $12_{G\rightarrow A}$, $156_{A\rightarrow G}$ and $229_{T\rightarrow C}$ respectively. A radial phylogram was constructed, as shown in Figure 1, on the basis of the partial sequences and using Tree View software (www.ebi.ac.uk/clustalw).

Discussion

Reports of infectious diseases in pygmy hogs are rare, owing to the limited population of these mammals. The only captive population, in Assam, India, gives an insight into the susceptibility of these animals to various diseases. Heavy rainfall, high humidity, the feeding habits of the animals and the lack of response to antiparasitic drugs and tetracyclines led to the suspicion of clostridial toxins as a probable cause of the syndrome observed. The role of *C. perfringens* in this disease was made clear by isolation of the organism under anaerobic conditions, without any other species of clostridia. Aerobic isolation did not yield any dominant bacterial type suggestive of enteric infection. The involvement of *cpb*2 strains of *C. perfringens* Type A in enteric diseases of piglets (13) and African elephants (1) has been recorded.

The isolates showed double plasmids of molecular weight 42.8 kb and 51.9 kb. An identical plasmid pattern in *C. perfringens* strains isolated from different disease outbreaks (5) and a single plasmid of molecular size 42.8 kb have been reported earlier (19).

The PCR assay showed amplification specific for the alpha toxin gene (*cpa*) and the beta2 toxin gene (*cpb*2) but not for the *cpb, etx, iA* and *cpe* genes. On the basis of the PCR results, the isolates were typed as *C. perfringens* Type A with the *cpb*2 gene. Bacteriological identification methods are expensive, time-consuming and have low sensitivity, whereas PCR is very useful in the diagnosis of clinical diseases, molecular typing and epidemiological surveys of *C. perfringens* (18).

The differences within the 292 bp region between various isolates showed a maximum of five nucleotide substitutions. The nucleotide substitutions at positions 12, 156 and 229 in the partial sequence of the *C. perfringens* isolates from the pygmy hog (DQ838708 and DQ838709) were strikingly similar to those of a few isolates from Japan (D32123), China (AY823400), Denmark (AF477009) and the United States (DQ184079), and hence these were placed as a single cluster in the phylogram. However, various substitutions were also noticed among other isolates. The positions 12, 77, 85, 255 and 156 within the 292 bp region showed a high rate of nucleotide substitution. Other substitutions were also noticed, at lower frequencies, among isolates from France (positions 24, 30 and 56), Japan (105), the United States (105 and 220), China (24, 30 and 56) and Denmark (27 and 210). Substitutions at positions 56 (cytosine to thiamine),

Table II
Nucleotide substitutions in the partial *cpa* gene sequences of *Clostridium perfringens* isolates from different geographical locations

Partial *cpa* gene sequences

1 GTTAAGTTTGAGACTTTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAG 75
76 GATTTTTATGCTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAA 150
151 ACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGATTGGGATTATGCAGCAAAGGTA 225
226 ACTTTAGCTAACTCTCAAAAAGGAACAGCAGGATATATTTATAGATTCTTACACGATGTATCAGAGG 292

Accession no.	Country	Source and year	Nucleotide substitutions observed: Position of substitutions 12	21	24	30	56	77	85	105	156	210	229	255
AF475144	Denmark	Chicken, 2002	A	*	*	*	*	*	*	*	*	C	C	A
AF477009	Denmark	Chicken, 2002	A	*	*	*	*	*	*	*	G	*	C	*
AF477010	Denmark	Chicken, 2002	*	G	*	*	*	C	A	*	*	*	*	*
AY277724	China	Spotted deer, 2003	*	*	A	G	T	*	*	*	G	*	*	A
AY823400	China	Spotted deer, 2003	A	*	*	*	*	*	*	*	G	*	C	*
D32123	Japan	Spotted deer, 1995	A	*	*	*	*	*	*	*	G	*	C	*
D32124	Japan	Spotted deer, 1995	*	*	*	*	*	*	*	*	G	*	C	A
D63911	Japan	Soil, 1996	*	*	*	*	*	*	*	*	G	*	C	A
DQ183950	USA	Bovine, 2006	*	*	*	*	*	*	*	*	*	C	C	A
DQ184014	USA	Bovine, 2006	*	*	*	*	*	*	*	*	*	*	*	A
DQ184033	USA	Bovine, 2006	*	*	*	*	*	*	*	*	*	*	*	A
DQ184040	USA	Bovine, 2006	A	*	*	*	*	*	*	G	*	*	*	A
DQ184053	USA	Bovine, 2006	*	*	*	*	*	C	A	*	*	*	*	A
DQ184074	USA	Bovine, 2006	*	*	*	*	*	C	A	*	*	*	*	*
DQ184079	USA	Bovine, 2006	A	*	*	*	*	*	*	*	G	*	C	*
DQ184151	USA	Bovine, 2006	*	*	*	*	*	C	A	*	*	C	C	*
DQ838708	India	Pygmy hog, 2006	A	*	*	*	*	*	*	*	G	*	C	*
DQ838709	India	Pygmy hog, 2006	A	*	*	*	*	*	*	*	G	*	C	*
L43546	Belgium	Soil, 1996	*	*	*	*	*	*	*	*	*	*	*	A
L43547	Belgium	Soil, 1996	*	*	*	*	*	C	A	*	*	*	*	*
M24904	Japan	Soil, 1989	*	*	*	*	*	C	A	*	*	*	*	*
NC003366	Japan	Soil, 2002	A	*	*	*	*	*	*	G	*	*	*	A
X13608	England	Chicken, 1989	*	*	*	*	*	C	A	*	*	*	*	*
X17300	France	Strain 8-6 (mutant of NCTC8798, isolated from human), 1989	*	*	A	G	T	*	*	*	G	*	*	A

* indicates no nucleotide substitution

77 (adenine to cytosine) and 85 (guanine to adenine) resulted in a change of amino acid from alanine to valine, aspartic acid to alanine, and alanine to threonine respectively. The nucleotide substitutions identified in the *C. perfringens* isolates from pygmy hogs did not result in amino acid substitutions.

The role of the alpha toxin in the pathogenesis of intestinal disease is still not clear; however, diseased animals generally harbour alpha toxin and an additional toxin gene. Similarly, *C. perfringens* with at least one other toxin gene in addition to the *cpa* gene was observed in lamb dysentery (7). Furthermore, a recent study of diarrhoeic piglets revealed that the associated strains of *C. perfringens* contained the *cpa* gene and the beta2 toxin gene (13), and similar strains were also found in horses with intestinal disorders (8).

On the basis of these observations, the authors consider that the alpha toxin by itself, although it may be needed as a basic virulence factor for successful infection, is probably not sufficient to cause disease. Although haemorrhagic enteritis associated with *C. perfringens* appears to be rare, further experiments are needed to assess the role of the alpha toxin and beta2 toxin genes and their potential synergies in the pathogenesis of this enteric disease.

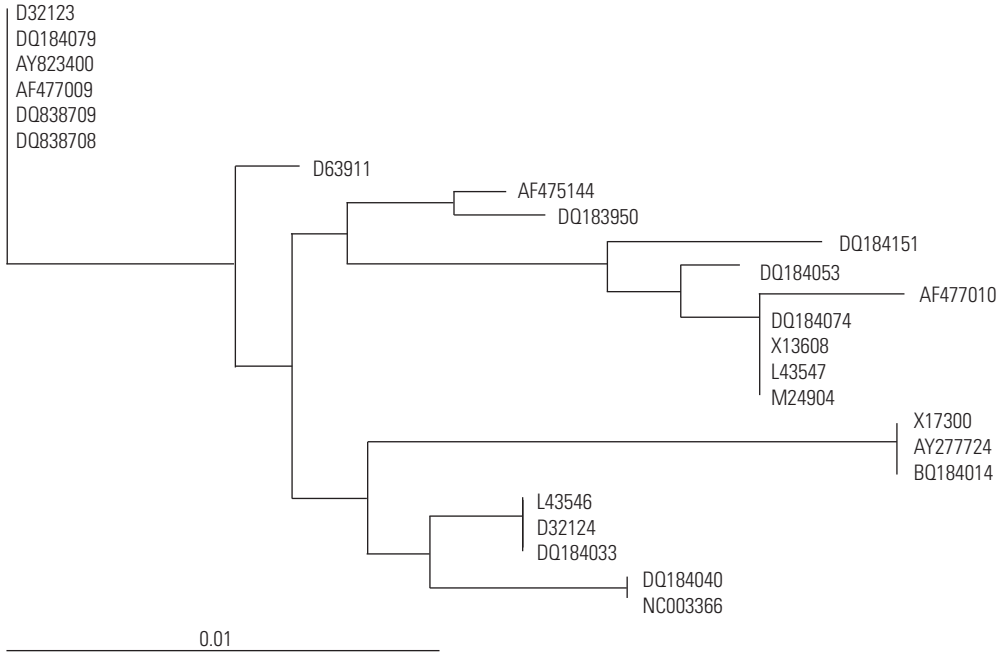

Fig. 1
Phylogenetic tree constructed using the Clustal W method for partial *cpa* gene sequences of *Clostridium perfringens* isolates

Acknowledgements

The authors gratefully acknowledge the members of the Research and Breeding Centre of the Pygmy Hog Conservation Programme, Guwahati, Assam, the Forest Department of the Government of Assam, and the Ministry of Environment and Forests for their cooperation. The authors also wish to thank all the technical staff and laboratory workers of the Division of Animal Health of the Research Complex for the North East Hill Region of the Indian Council of Agricultural Research, Meghalaya for their help.

Étude sur des cas d'entérite hémorragique survenus chez des sangliers nains (*Sus salvanius*) en Inde

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Résumé
Le sanglier nain représente l'espèce la plus petite et la plus rare parmi toutes les espèces de Suidés connues. Les auteurs décrivent une étude réalisée suite à l'observation de cas d'entérite hémorragique chez des sangliers nains du Centre de recherches et d'élevage du Programme de protection du sanglier nain à Guwahati, Assam, Inde. Trois des 68 sangliers nains du Centre avaient succombé à une infection entérique. L'autopsie et l'examen bactériologique réalisés sur deux de ces animaux ont conclu à une infection clostridienne. Les isolats possédaient deux plasmides de respectivement 42,8 kilobases (kb) et 51,9 kb de poids moléculaire. L'amplification en chaîne par polymérase a identifié le gène codant pour la toxine Beta2 (*cpb*2) de *Clostridium perfringens* de type A. L'analyse de la séquence du gène codant pour une partie de la toxine Alpha (*cpa*) a révélé une homologie de 98 % à 100 % par rapport aux bactéries isolées dans d'autres régions géographiques.

Mots-clés
Amplification en chaîne par polymérase – *Clostridium perfringens* – Entérite hémorragique – Inde – Sanglier nain – *Sus salvanius* – Toxine Alpha – Toxine Beta2.

Investigación sobre la enteritis hemorrágica en jabalíes enanos (*Sus salvanius*) de la India

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Resumen
El jabalí enano es un representante de la especie más pequeña y rara que se conoce de entre los suidos vivos. Los autores describen un estudio de la enteritis hemorrágica observada en ejemplares de jabalí enano del Centro de investigación y reproducción del Programa de protección del jabalí enano de Guwahati, en el estado de Assam (India). Tres de 68 jabalíes enanos murieron a resultas de una infección entérica. El examen post-mortem y el análisis bacteriológico de dos de los tres animales muertos pusieron de manifiesto una infección clostridial. En las bacterias aisladas había dos plásmidos con sendos pesos moleculares de 42,8 kilobases (kb) y 51,9 kb. Por reacción en cadena de la polimerasa se detectó la bacteria *Clostridium perfringens* de tipo A, positiva para el gen de la toxina beta2 (*cpb*2). El análisis de la secuencia de una parte del gen de la toxina alfa (*cpa*) reveló una homología de entre el 98% y el 100% con bacterias aisladas en distintos puntos geográficos.

Palabras clave
Clostridium perfringens – Enteritis hemorrágica – India – Jabalí enano – Reacción en cadena de la polimerasa – *Sus salvanius* – Toxina alfa – Toxina beta2.

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