Salmonellosis in pigmy hogs (Sus salvanius) – a critically endangered species of mammal

H. Rahman (1), P. J. Deka (2), A. Chakraborty (3) & G. Narayan (2)

(1) Division of Animal Health, ICAR Complex for NEH Region, Umroi Road, Umiam-793103, Meghalaya, India
(2) Pigmy Hog Conservation Programme, Basistha, Guwahati-781029, Assam, India
(3) Department of Pathology, Assam Agricultural University, Guwahati-781022, Assam, India

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Summary
The pigmy hog (Sus salvanius) is the smallest and the rarest wild suid in the world. This species is on the verge of extinction and the World Conservation Union has rated it among the most endangered of all mammals. This paper reports the investigation into an outbreak of salmonellosis among captive pigmy hogs at the Research and Breeding Centre of the pigmy hog conservation programme, Guwahati, Assam, India. Of 75 pigmy hogs (38 males and 37 females) maintained at the Centre, seven (9.3%) died within five days. The causative organism associated with the outbreak was identified as Salmonella Typhimurium (syn. S. enterica serovar Typhimurium). All the isolates of S. Typhimurium belonged to phage type DT193. The isolates harboured multiple plasmids. Five isolates harboured four (65.0 MDa, 4.2 MDa, 3.0 MDa, 1.3 MDa), while two isolates carried three plasmids (65.0 MDa, 4.2 MDa, 3.0 MDa). All strains showed resistance to amikacin, ampicillin, streptomycin and sulfamerazine; five strains were resistant to oxytetracycline and trimethoprim. All the strains were sensitive to chloramphenicol, ciprofloxacin, enrofloxacin and gentamicin. All seven isolates of S. Typhimurium were found to harbour str, sopB and pelA genes. However, none of them was found to carry sec C and sopE genes.

Keywords

Introduction
The pigmy hog (Sus salvanius) is the smallest and the rarest wild suid in the world, and the smallest: ten to 20 times lighter than the wild boar (Sus scrofa). Pigmy hogs measure about 650 mm in length and 250 mm in height, and weigh 8 kg to 9 kg. Females are a little smaller, and the newborn piglets weigh only 150 g to 200 g.

Today, this species is on the verge of extinction and the World Conservation Union (IUCN) has accorded it the highest priority rating (status category 6: critically endangered), putting it among the most endangered animals of all mammals. Although pigmy hogs were formerly widespread in the tall grasslands of the Himalayan foothills, from Uttar Pradesh to Assam, through the Nepali ‘terai’ and North Bengal ‘duars’, they are currently restricted to a few pockets along Assam’s border with Bhutan and Arunachal Pradesh. A viable wild population of the species exists only in the Manas Tiger Reserve in Assam, India (10). The main threats to the survival of pigmy hogs are the loss and degradation of habitat caused by human settlement, agricultural encroachment, flood control schemes and inappropriate management.
In 1996, six wild hogs (two males and four females) were caught in the Manas National Park and transferred to the Research and Breeding Centre of the pigmy hogs conservation programme at Guwahati, Assam, India. These six foundation animals were kept in captivity and bred, so that the population had risen to 75 by the time of the salmonellosis outbreak. The hogs are maintained in a protected area to avoid all mammalian and, so far as possible, avian contact, and form the only captive population of pigmy hogs in the world (11). Information on diseases in this species is very scanty (15, 16). This paper reports an outbreak associated with Salmonella Typhimurium in these captive pigmy hogs, along with data on selected potential virulence determinants of the organisms involved.

Materials and methods

History and source of materials

An outbreak of salmonellosis among the captive pigmy hogs maintained at the Research and Breeding Centre of the pigmy hog conservation programme was investigated. A total of 75 pigmy hogs (38 males and 37 females) were maintained in the Centre at the time of the outbreak. Of these animals, 62 were adults (above one year old, 30 male, 32 female) and 13 were young (below one year old, eight male, five female). Five animals, including three adults (two males, one female) and two young animals (both male), died at the onset of the outbreak without showing any detectable clinical signs.

Another five adult animals (two males, three females) showed symptoms of clinical illness. These animals were isolated and were treated with 5 mg per day of enrofloxacin (En) per kg body weight delivered intramuscularly for three days, and then with an oral solution of norfloxacin (animals refused to take oral enrofloxacin due to its bitter taste) with feed for five days (at 200 mg to adult and 100 mg to young animal twice daily). The apparently healthy animals were also treated with an oral solution of norfloxacin with feed for five days, at the same dosage.

In every case where animals died, necropsy was performed within two hours to six hours after death. External body surfaces were examined for bruises, injuries or other harm, if any, on the body. All the internal organs were thoroughly examined and any macroscopic and gross lesions observed were recorded. The heart-blood, liver, spleen, lymph nodes and intestinal contents were collected during the post-mortem examination for microbiological investigation. Samples from feed and water used for the animals were also collected aseptically for microbiological investigation.

Isolation and identification of Salmonella

The technique of Dusch and Altwegg (5) was adopted with slight modifications to isolate Salmonella organisms. The collected materials were inoculated into tetrahionate broth (10 ml) and incubated at 43°C for 24 hours. Six drops of tetrahionate broth were then inoculated discretely onto modified semisolid Rappaport Vassiliadis (MSRV) agar as spot inoculation and incubated at 37°C for 24 hours. Swarming growth from the MSRV plates was streaked on triple sugar iron agar and brilliant green agar (BGA) plates and incubated at 37°C for 24 hours. Positive colonies in BGA were identified by biochemical reactions (6) and confirmed by a slide agglutination test with O-polysaccharide antiserum.

Sero-typing and phage typing of the organisms

The isolates were sero-typed at the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India, and were phage typed at Robert Koch Institute, Germany.

Antimicrobial sensitivity test

Antimicrobial susceptibility was tested by a disc diffusion method using commercially available biodiscs (7). The antimicrobial agents used were amikacin (Ak), ampicillin (Am), chloramphenicol (Cm), ciprofloxacin (Cf), enrofloxacin, gentamicin (Gm), oxytetracycline (Ox), streptomycin (S1), sulfamerazine (Sn) and trimethoprim (Tm).

Detection of plasmid

Plasmid deoxyribonucleic acid (DNA) was detected by means of the method described by Kado and Liu (9). The organisms were grown on trypticase soy agar overnight at 37°C. A well-isolated colony was suspended in 100 μl of lysis buffer (10 mM ethylenediamine tetra-acetic acid, pH 8.0). The cells were lysed by adding 250 μl of lysis buffer (1% sodium dodecyl sulphate in 0.1 M NaOH, pH 12.6). The solution was heated at 65°C for 25 min in a water bath. After incubation, 500 μl of phenol-chloroform solution (1:1, v/v) was added and emulsified by brief shaking. The suspension was centrifuged (12,000 rpm for 10 min at 4°C). The plasmids were then subjected to electrophoresis in 0.7% agarose in TE buffer and stained with ethidium bromide. The separated DNA bands were visualised on an ultraviolet (UV) transilluminator and photographed. The approximate molecular weight of the plasmids was determined by comparison with the known DNA ladder.
Detection of stn, sef, pef, sopB and sopE genes by polymerase chain reaction

To study the virulence of the organisms, all the seven S. Typhimurium strains were tested by polymerase chain reaction (PCR) for the presence of stn, sef, pef, sopB and sopE genes, using their specific primers. Bacterial cells from overnight cultures were suspended in sterile distilled water and boiled at 100°C for ten minutes. After boiling, the cell suspensions were cooled in an ice bath and were immediately tested for the presence of stn, sef, pef, sopB and sopE genes by the PCR amplification technique, using their specific primers. The PCR mixture (25 µl) contained 10 × PCR buffer, 1 µ/mol of each primer, 200 µ/mol each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate, 1 µ/mol of MgCl₂ solution, 0.25 U of AmpliTag polymerase and 2.5 µl of template (target) DNA preparation from the test organism. The PCR incubation was performed in a thermal cycler in 30 cycles. Then a 15 µl aliquot of each PCR product was electrophoretically separated in agar gel (1%) containing 0.5 µl ethidium bromide per ml. The separated bands were visualised and analysed under a UV transilluminator (300 nm) and photographed using the Gel Doc 2000 documentation system. Strains of S. Typhimurium (DT96) and S. Enteritidis (RK-172) were used as positive controls for pef and sef genes, respectively, while a strain of S. Dublin (2229) was used as a positive control for stn, sopB and sopE genes. A strain of Escherichia coli (C-600) was used as a negative control for all the five genes.

Results

Seven (9.3%) of the 75 animals maintained at the Research and Breeding Centre died within five days of the onset of the disease outbreak. Three adults (two male and one female) and two young animals (both male) died at the onset of the outbreak without showing any detectable clinical signs (and without receiving any specific treatment). Another five adult hogs (three males and two females) were also affected. Two of these (one male, one female) died, but the rest of animals responded to antibiotic treatment and no more deaths were observed. Both young and adult hogs showed similar symptoms. All the affected animals showed the same progression of the disease: a general loss of condition, decrease in food intake and high fever (40°C to 41°C) with loose faeces. The faeces contained mucous and blood. Severe dehydration, loss of weight, abdominal breathing and recumbancy before death were observed, and so too were nervous signs including tremor and weakness. The 65 apparently healthy animals showed no sign of infection/illness. The ratio of the clinical illness was 1:7.5 (ten animals affected out of 75 in the population).

All the seven animals that died showed diffused haemorrhagic and necrotic enteritis in both 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 haemorrhages were present on both the endocardium and epicardium. The cortical surface of the kidneys was congested and showed petechial haemorrhages. Meninges showed mild congestion.

Salmonella organisms were isolated from all seven samples of liver, heart-blood, spleen, lymph nodes and intestinal contents. The isolated salmonellae were identified as S. enterica serovar Typhimurium (S. Typhimurium). Seven isolates, one each from each animal, were utilised for

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Phage type</th>
<th>Plasmid profile (MDa)</th>
<th>Resistance pattern</th>
<th>Virulence genes as detected by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0, 1.3</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + + –</td>
</tr>
<tr>
<td>2</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0, 1.3</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + + –</td>
</tr>
<tr>
<td>3</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + –</td>
</tr>
<tr>
<td>4</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0, 1.3</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + –</td>
</tr>
<tr>
<td>5</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0</td>
<td>Ak Am St Sn</td>
<td>+ – + + –</td>
</tr>
<tr>
<td>6</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0, 1.3</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + –</td>
</tr>
<tr>
<td>7</td>
<td>DT193</td>
<td>65.0, 4.2, 3.0, 1.3</td>
<td>Ak Am Ox St Sn Tm</td>
<td>+ – + + –</td>
</tr>
</tbody>
</table>

Ak = amikacin  
Am = ampicillin  
Ox = oxytetracycline  
St = streptomycin  
Sn = sulfamerizin  
PCV = polymerase chain reaction  
+ = detected  
– = not detected
detailed studies (Table I). All these isolates of S. Typhimurium belonged to phage type DT193 (Anderson scheme). They harboured multiple plasmids. Five isolates harboured four plasmids (65.0 MDa, 4.2 MDa, 3.0 MDa, 1.3 MDa), while two isolates carried three (65.0 MDa, 4.2 MDa, 3.0 MDa). All strains showed resistance to Ak, Am, St and Sn, and five strains to Ox and Tm. All the strains were sensitive to Cm, Cf, En and Gm. All these seven isolates of S. Typhimurium were found to harbour strn, sopB and pefA genes (Table I). However, none of them was found to carry sefC and sopE genes. No Salmonella was isolated from the feed or water used for the animals.

**Discussion**

The clinical manifestations and pathological alterations observed at necropsy were suggestive of salmonellosis. (This was further confirmed by the isolation and characterisation of S. Typhimurium.) Pigs affected with S. Choleraesuis usually develop a dark red to purple discolouration of the skin, especially on the abdomen and ears (13). In this outbreak, none of the affected animals showed such discolouration. However, very little is known about the diseases of pigmy hogs, and this might be due to a peculiarity of this particular species of suid. The first ever reported salmonellosis in this species of animal was found to be associated with S. Enteritidis and affected only adult animals (15). The other report of salmonellosis in these animals was associated with S. Choleraesuis, and in that case both adult and young animals were found to be affected (16).

Although all the seven salmonellae were recovered from the same outbreak, they showed a variation in their sensitivity patterns to different antimicrobial agents. This variation was similar to one observed with S. Enteritidis isolated from an earlier outbreak of salmonellosis (13). The animals at the Centre had been treated with enrofloxacin and norfloxacin for salmonellosis and hoglet diarrhoea as early as 1997. No antibiotic treatment was given to these animals until the outbreak reported here. As the isolates were from different animals, variability in their susceptibility to antibiotic is to be expected. However, none of the animals received any treatment with Ak, Am, Ox, St or sulphamethazine, to which the isolated Salmonella showed resistance. Indiscriminate use of antimicrobials is thought to underlie the origin of bacterial strains that demonstrate multiple antimicrobial resistances.

Livestock are important vectors for dissemination of Salmonella, but human travel, wildlife migration and the global trade in meat and other foods and animal feed-stuffs are also involved in the dissemination of resistant strains (19). Use of antimicrobials on farms leads to the local amplification and persistence of resistant strains. Subtherapeutic antimicrobial use in livestock also emerges as an important factor in the rise of multi-resistant strains (14).

The pathogenic process of salmonellosis is a complex phenomenon and is dictated by an array of virulence factors that act in tandem and are ultimately manifest in the typical symptoms of the disease. Virulence genes encode products that assist the organisms in expressing their virulence in the host cells. Some genes of Salmonella are known to be associated with:

- adhesion of the organism to the small intestine: *pef* (2, 19), *sef* (4, 20)
- invasion of the epithelial cells of the intestine: *sopE* (8, 17)
- the actual manifestation of pathogenic processes: *sopB* (8, 12, 21), *strn* (3).

The *strn* and *sopB* genes are associated with gastroenteritis and diarrhoea in man and animals (3, 8, 12).

Studies of the virulence determinants of the strains of S. Typhimurium indicated that they harboured several pathogenic factors. All the isolates were enterotoxigenic, as indicated by the presence of the *strn* and *sopB* genes. The organisms also carried the fimbrial gene (*pefA*) essential for colonisation on the intestinal tract (18). These virulence determinants have also been reported in Salmonella isolated from humans and some domestic animals (8, 12, 14). Although there have been few studies of the phage typing and plasmid profiles of S. Typhimurium isolated from this species of animal, a similar phage type and plasmid profiles have been reported for S. Typhimurium isolated from man and animals (14).

As salmonellae were not isolated from the feed or water used for the animals, the involvement (if any) of these items as the source of infection could not be demonstrated. Pests and rodents such as mice act as reservoirs of Salmonella, so infections may typically come from mice or pests present in the environment.

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La salmonellosse chez des sangliers nains (*Sus salvanius*) – une espèce de mammifères particulièrement menacée d’extinction

H. Rahman, P.J. Deka, A. Chakraborty & G. Narayan

**Résumé**
Le sanglier nain (*Sus salvanius*) est le plus rare et le plus petit des suidés de la planète. L’espèce étant en voie d’extinction, l’Union mondiale pour la nature l’a classée parmi les espèces de mammifères les plus menacées. Cet article présente les résultats d’une étude réalisée sur un foyer de salmonellosse survenu chez des sangliers nains vivant en captivité dans le centre de recherche et de reproduction du Programme de conservation du sanglier nain à Guwahati, Assam, Inde. Sept des 75 sangliers nains (38 mâles et 37 femelles) vivant en captivité dans le centre (soit 9,3 %) sont morts dans les cinq jours. L’agent causal s’est révélé être *Salmonella Typhimurium* (syn. *S. enterica* sérovar Typhimurium). Les isolats de *S. Typhimurium* étaient tous de phage type DT193. Les isolats portaient différents plasmides : cinq d’entre eux en portaient quatre (à savoir : 65,0 MDa, 4,2 MDa, 3,0 MDa et 1,3 MDa), et deux autres isolats en portaient trois (65,0 MDa, 4,2 MDa et 3,0 MDa). Toutes les souches se sont révélées résistantes à l’amikacine, l’ampicilline, la streptomycine et la sulfamerazine ; en outre, cinq souches étaient résistantes à l’oxytétracycline et au triméthoprim. Toutes les souches étaient sensibles au chloramphénicol, à la ciprofloxacine, à l’enrofloxacine et à la gentamicine. Les sept isolats de *S. Typhimurium* possédaient les gènes *sln*, *sopB* et *pefA*. En revanche, les gènes *sefC* et *sopE* étaient absents des sept isolats.

**Mots-clés**

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Salmonellosis en el jabalí enano (*Sus salvanius*), un mamífero en situación crítica

H. Rahman, P.J. Deka, A. Chakraborty & G. Narayan

**Resumen**
El jabalí enano (*Sus salvanius*) es el más pequeño y poco común de los suidos salvajes del mundo. Se encuentra al borde de la extinción, y la Unión Mundial para la Naturaleza lo tiene catalogado dentro del grupo de mamíferos más amenazados. Los autores dan cuenta de la investigación de un brote de salmonellosis que afectó a jabalíes enanos en cautividad del Centro de Investigación y Reproducción del programa de protección del jabalí enano, en Guwahati, Assam (India). De los 75 ejemplares del Centro (38 machos y 37 hembras), siete (9,3%) murieron en cinco días. Pudo determinarse que el agente causal asociado con el brote era *Salmonella Typhimurium* (Sin. *S. enterica* serovar Typhimurium). Todas las colonias aisladas de *S. Typhimurium* pertenecían al tipo fago DT193 y albergaban múltiples plásmidos. En cinco de las cepas había cuatro plásmidos (65,0 MDa, 4,2 MDa, 3,0 MDa, 1,3 MDa), y en dos de ellas había tres (65,0 MDa, 4,2 MDa, 3,0 MDa). Todas eran resistentes a la amikacina, la amplicilina, la streptomicina y la sulfametaxazina, y cinco lo eran además a la oxitetetraciclina y el trimetoprim. En cambio, eran sensibles en su
totalidad al cloranfenicol, la ciprofloxacina, la enrofloxacina y la gentamicina. Se comprobó que las siete cepas de *S. Typhimurium* albergaban los genes *stn*, *sopB* y *pefA*, pero ninguna era portadora de los genes *sefC* o *sopE*.

**Palabras clave**

Antibiograma — Gen de virulencia — Jabalí enano — Plásmido — Reacción en cadena de la polimerasa — *Salmonella Typhimurium* — Salmonelosis — *Sus salvanius*.

References


