Isolation and characterization of Staphylococcus saprophyticus responsible for death of two six-banded armadillos (Euphractus sexcinctus)

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Research Article

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Abstract

Two six-banded armadillos (Euphractus sexcinctus) suddenly died in a zoo of Nanjing, Jiangsu province, China, after exhibiting weak breath, high temperature, and nasal bleeding. Autopsy revealed dark brown appearance and enlargement of the visceral organs and several granular white necrotic foci in the spleen and liver of the two armadillos. Histological analysis revealed serious congestion, haemorrhage, and multiple necrotic foci in the spleen and liver; lungs exhibited congestion. Moreover, the bacterium suspected to be responsible for the death of the armadillos was observed in the spleen and lung tissue samples. Several gram-positive cocci were observed in the contacting smears of liver tissue samples. Biochemical analysis revealed that the isolated bacterium was Staphylococcus saprophyticus and was designated as S.SNH2021; 16S rRNA and gap gene sequencing of the isolate provided similar findings. Strain S.SNH2021 was resistant only against penicillin. Moreover, the mice challenged with high load of the isolated bacterium died and exhibited histopathological changes similar to those observed in the armadillos. To the best of our knowledge, this is the first report on S. saprophyticus infection in armadillos. This study provides a reference for breeding and management of six-banded armadillos in zoos.

Introduction

Staphylococcus saprophyticus (S. saprophyticus) is a gram-positive, coagulase-negative, and non-haemolytic coccus that is present in the normal human gut flora (Ehlers and Merrill 2022). S. saprophyticus is frequently isolated from food such as beef, pork, and water; therefore, they are considered the primary source of S. saprophyticus infection in humans and animals (Lawal et al. 2021). S. saprophyticus is an opportunistic pathogen that infects when the host is immunocompromised (Nagy et al. 1993; Ehlers and Merrill 2022) and/or during seasonal changes (Kline and Lewis 2016). In young women, S. saprophyticus is a common cause of uncomplicated urinary tract infections (UTIs) (Raz et al. 2005), and some complicated infections including acute pyelonephritis (Matarneh et al. 2021), renal calculi (Hur et al. 2016), and endocarditis (Nishimura et al. 2020). Moreover, once S. saprophyticus enters the human body, it can cause sepsis (Jones et al. 2019) and pneumonia (Hell et al. 1999). In animals, S. saprophyticus has been isolated from the udder of cows with mastitis (Srednik et al. 2017) and the conjunctiva of chickens with conjunctivitis (Wang et al. 2019), however, no reports of S. saprophyticus infection in other animals until now as fa as we know.

Six-banded armadillos (Euphractus sexcinctus) are primarily found in South America and were listed as a low-threat species by the International Union for Conservation of Nature (IUCN) in 2013. As ornamental animals, they are often globally transported to various zoos. Six-banded armadillos have been previously reported to be infected with Mycobacterium leprae (Frota et al. 2012; da Silva Ferreira et al. 2020) and Leptospira (Dalazen et al. 2020), but little is known about their exposure to and/or infection due to other zoonotic pathogens. In the present study, we report a case of S. saprophyticus infection in six-banded armadillos. We isolated and characterised the pathogen from two dead six-banded armadillos. Moreover, we performed histopathological examination of the armadillos, antibiotic-sensitivity analysis of the
isolated pathogen, and bacterial challenge with mice. The results of this study will help us to understand, prevent, and treat infections occurring in six-banded armadillos.

**Materials And Methods**

**Details Of Diseased Six-banded Armadillos**

Two male adult six-banded armadillos were found ill in a zoo in Nanjing city, Jiangsu province on the morning of December 18th, 2021. One of them exhibited shallow breathing, and no movement, and then it suddenly died with nasal bleeding in the morning. The other one exhibited clinical symptoms of respiratory distress, high temperature (35.3°C), and died in the afternoon.

**Autopsy And Sample Collection**

After physical examination, we performed a clinical autopsy on the two armadillos. The gross changes in the organs were carefully examined, including heart, liver, spleen, lung, and kidney tissues. The two livers were placed onto a sterile absorbent paper and a section (approximately 2 cm²) from the surface of each liver was sterilized by searing with a heated metal scissor. Sterile inoculation loops were inserted into the seared flesh, i.e., internal hepatic tissue, and then used to inoculate into blood agar plates (CRmicrobio, Jiangmen, Guangdong), which were incubated at 37°C for 24h. A part of the hepatic tissue was used to prepare contacting smears on microscopic slides, which were stained using Wright’s strain (Kutaish 1982). A part of the heart, liver, spleen, lungs, and kidneys were soaked in 4% paraformaldehyde and fixed for more than 24h for histopathological observation.

**Histopathological Examination**

Tissue samples fixed in 4% paraformaldehyde were serially dehydrated using alcohol, clarified in xylene, and embedded in paraffin. The fixed samples were sliced into 4µm sections and stained with haematoxylin and eosin. The stained sections were examined under a light microscope (Carl Zeiss, Gottingen, Germany).

**Morphological And Biochemical Characterisation**

The strains isolated from the two armadillos were initially identified based on morphology (shape, colour, mobility, and Gram’s staining) and catalase and coagulase activity. Catalase activity of the isolates was determined using 3%H₂O₂, and 3% H₂O₂ with PBS was used a negative control. Coagulase activity was determined using rabbit plasma (Hopebil, Qingdao, China), and rabbit plasma with Staphylococcus aureus ATCC25923 was used as a positive control. Biochemical characterisation of strains was performed using the Vitek 2 Compact system (BioMe'rieux, Lyon, France).
Bacterial 16s Rrna And Gap Gene Sequencing

In *Staphylococcus* spp., the 16S rRNA gene and the gap gene that encodes glyceraldehyde-3-phosphate dehydrogenase are highly conserved (Ghebremedhin et al. 2008); in this study, these genes were amplified using the primers reported in previous studies (Weisburg et al. 1991; Yugueros et al. 2000). The genomic DNA of the isolated bacterial strains was extracted according to the instructions of TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (Takara Bio, Beijing, China). PCR products were subjected to agarose gel electrophoresis and then purified and sequenced by Tsingke Biotechnology Co., Ltd. The 16S rRNA and gap gene sequences were compared with those published in the nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/).

Antimicrobial Susceptibility Tests

Minimum inhibitory concentrations (MICs; µg/mL) of 14 antibiotics (penicillin, gentamicin, moxifloxacin, clindamycin, daptomycin, vancomycin, rifampicin, oxacillin, levofloxacin, erythromycin, linezolid, teicoplanin, yigecycline, and cotrimoxazole) were determined against the isolated strain using the Vitek 2 Compact system. *Escherichia coli* ATCC25922 and *S.aureus* ATCC25923 were used as the control strains for drug sensitivity testing. The results were inferred as per the guidelines set by the Clinical and Laboratory Standards Institute (CLSI).

Bacterial challenge with *S. saprophyticus* in vivo

Twenty clean-grade Balb/c mice were obtained from Nanjing ShengMin Research Animal Farm and were provided with sterilized water and feed without antibiotics ad libitum. After 3 d of adaptation, the mice were randomly divided into four groups, with five mice in each group. The control mice were intraperitoneally injected with 200 µL of PBS, whereas the other three groups were intraperitoneally injected with $1 \times 10^9$ CFU/mouse (high dose infection group), $1 \times 10^7$ CFU/mouse (middle dose infection group), and $1 \times 10^5$ CFU/mouse (low dose infection group) of the isolated bacterium with the same volume (200 µL), respectively. All mice were monitored daily for 7 d for their clinical signs. Mice that died during the experiment were immediately dissected and their organs were collected, and other mice were euthanised after 7 d and their organs were collected. Bacterial loads in the heart, liver, spleen, lungs, kidneys, and brain were assessed. The organs were weighed and approximately 50% of tissue from each organ was removed aseptically and homogenized in 1 mL PBS in homogenizer. The homogenates were serially diluted in PBS, and Luria-Bertani (LB) agar plates were inoculated with appropriate dilutions ($10^{-3}$ to $10^{-7}$), which were incubated at 37°C for 24 h prior to enumeration. Bacterial counts were expressed as CFU/g tissue. A section of the heart, liver, spleen, lungs, and kidneys were fixed in 4% paraformaldehyde for histopathological observation as described in section Histopathological examination.

Statistical analysis
The differences of the bacterial load in organs were analyzed by Student’s t-test, and variables are expressed as the mean ± standard deviation (SD). P < 0.05 was considered statistically significant, and P < 0.01 was considered to indicate a high degree of significance.

Results

Examination Of Gross Lesions

Physical examination of the two armadillos revealed no trauma (Fig. 1a); however, autopsy revealed that the heart, liver, spleen, and lungs were enlarged and dark brown in appearance (Fig. 1b-e). Moreover, slight swelling was observed in the kidneys (Fig. 1f), and several granular white necrotic foci were observed in the liver and spleen (yellow arrow) (Fig. 1c-d). Both armadillos exhibited similar gross lesions.

No lesions were found on body surface during examination (a); Congestion and enlargement were observed in the heart (b), liver (c), spleen (d), and lungs (e); White necrotic foci were found in the liver (c) and spleen (d); Slight swelling was observed in the kidneys (f).

Histopathological Examination

We observed obvious histopathological changes in the spleen, liver, and lungs of the two armadillos. The spleen was structurally destroyed, and serious haemorrhaging and multiple necrotic foci (yellow arrow) and bacteria (white arrow) were observed in the red pulp (Fig. 2a-b). Congestion, hepatocyte degeneration and necrosis and inflammation cells around the blood vessel (yellow arrow) were found in the liver (Fig. 2c-d). The lungs exhibited serious congestion in the alveolar wall, oedema, and presence of some cocci (white arrow) in the alveoli (Fig. 2e-f). Moreover, slight congestion in the heart and kidneys was observed (Fig. 2g-i).

Identification Of The Isolated Bacterium

Several blue-stained cocci were observed among the hepatocytes in contacting smears of liver tissue after Wright’s staining (Fig. 3a). The isolated bacteria grew on blood agar producing whitish, non-haemolytic colonies after incubation at 37°C for 24h (Fig. 3b). Gram staining revealed that the bacteria were gram-positive coccus (Fig. 3c). The bacteria were positive for catalase and negative for coagulase (Fig. 3d-f). These results indicated that the isolated bacteria were both coagulase negative staphylococcus. The Vitek 2 Compact system also identified the isolated strains as \textit{S. saprophyticus}. Molecular analysis of the staphylococcal isolates via BLAST revealed that the nucleotide sequences of 16S rRNA and gap genes of the isolated bacteria had maximum identity with those of \textit{S. saprophyticus}, i.e., more than 99% and 90% identity, respectively. The isolated microorganisms were identified as \textit{S. saprophyticus} and designated as \textit{S.SNH2021}. 
Several cocci were observed in the contacting smears of liver tissue samples of the dead armadillos after Wright’s staining (white arrows) (a). Morphology of the isolated bacteria on blood agar (b). Gram-positive cocci were observed after Gram staining of the isolated strains (c). Strain S.S NH2021 produced bubbles during in the presence of hydrogen peroxide during the catalase test but not with PBS (d). *Staphylococcus aureus* ATCC25923 induced plasma coagulation in the coagulase test (e) but strain S.S NH2021 did not coagulate plasma (f).

**Antimicrobial Sensitivity Of The Isolated S. Saprophyticus**

Based on the CLSI guidelines, we observed that the strain S.S NH2021 was sensitive to all antibiotics, except penicillin, as per their MICs.

**Animal Experiment**

During the bacterial challenge, the behavior and growth of mice in the PBS group were consistently natural and steady, respectively. However, lethargy, rough hair, and reduction in food and water intake were observed in three mice of the high dose infection group 2 d post infection (dpi), and one mouse died 3 dpi; similar symptoms were observed in one mouse in the middle dose infection group 3 dpi, which died at 4 dpi; no obvious symptoms and no dead mice were found in the low dose infection group (Fig. 4a). Autopsy revealed obvious lesions in the liver and spleen of the mice; the liver isolated from three mice each from both high and middle dose infection groups were enlarged and exhibited massive necrotic foci (red arrow), whereas spleen isolated from five and four mice from high and middle dose infection groups, respectively, were swollen and exhibited necrotic foci (yellow arrow; Fig. 4b). However, no such pathological changes were found in the control and low dose infection groups. Moreover, slight local congestion in the lungs and slight swelling in the kidneys were observed in the high and middle dose infection groups (Fig. 4b). High bacterial load was observed in the liver and spleen, followed by the kidneys, heart, and brain, which exhibited low bacterial load in all infected mice (Fig. 4c).

Histopathological examination in high dose infection group, revealed congestion, thrombosis (red arrow), and necrosis (yellow arrow) in the liver and spleen; renal tubular epithelial cell necrosis and inflammatory cell infiltration in the kidneys; and cardiomyocyte necrosis and inflammatory cell infiltration in the heart and congestion in the alveolar wall; however, no such significant pathological changes were observed in the brain (Fig. 4d–i).

The survival curve of the mice after incubation with different doses of strain S.S NH2021 (a). Gross changes observed in the liver, spleen, lungs, kidneys, heart, and brain (b). Bacterial load of different tissues of mice incubated with S.S NH2021 (c). Histopathological changes of the liver (d–e), spleen (f–g), and heart (h–i) in the dead mice.

**Discussion**
In this study, we report *S. saprophyticus* infection in two armadillos. The two armadillos exhibited acute septicaemia with enlargement, congestion, and necrosis in the liver and spleen. *S. saprophyticus* mainly exists in food and water (Hedman et al. 1990; Basso et al. 2014), it seldom causes serious diseases except when a large number of the bacterium enters the blood (Ishihara et al. 2001; Matarneh et al. 2021; Ehlers and Merrill 2022). In the current study, no wounds were observed on the two armadillos and there were no changes in their living environment; therefore, the most probable origin of *S. saprophyticus* was hypothesised to be food. Moreover, the temperature of their habitat suddenly dropped 2 d before the onset of the disease, which may have contributed to the death of armadillos. *S. saprophyticus* is an opportunistic pathogen (Nagy et al. 1993; Kline and Lewis 2016; Ehlers and Merrill 2022), and the cold stress may have lowered the immunity of the armadillos, making them susceptible to bacterial invasion. The antimicrobial sensitivity test revealed that the isolated *S. saprophyticus* was sensitive to most antibiotics except penicillin; this could be because penicillin resistance is related to β-lactamases (Latham et al. 1984) which are secreted into the surrounding medium and can act on hydrolysable penicillin (Latham et al. 1984; Bruns and Keppeler 1987). The result indicates that the bacterium is not multidrug resistant because only a few antibiotics have been used to treat its infection till now, which could be the reason behind fewer reports. Hence, the treatment of *S. saprophyticus* infection should be manageable if the infection is diagnosed in time.

Animal experiment revealed that *S. saprophyticus* caused tissue lesions including congestion and necrosis of the liver and spleen, which were similar to those observed in the armadillos, and death in the high and middle dose infection groups. The result demonstrates that *S. saprophyticus* can cause lesions and death at high-dose bacteria (1 × 10⁹ CFU/mouse), indicating that the the pathogenicity of *S. saprophyticus* is low. The liver and spleen of the mice were the main damaged organs, which was in agreement with the high bacterial load detected in these tissues. However, more serious liver lesions were observed in mice than that in the armadillos, which may be related to differential effect of the bacterium based on species. Moreover, high bacterial load, tubular epithelial cells necrosis, and inflammatory cell infiltration was found in kidneys of the mice infected with *S. saprophyticus*, which is in agreement with the kidney lesions reported in humans (Matarneh et al. 2021). A previous study reported *S. saprophyticus* can locate in the kidneys of a urease-induced UTI rat model that exhibited inflammation in the kidneys (Gatermann et al. 1989; Kline et al. 2010; Matarneh et al. 2021). Altogether, these findings suggest that *S. saprophyticus* probably infection in two armadillos, but the detailed mechanism needs to be further unmasked.

**Conclusion**

An *S. saprophyticus strain*, designated S.SNH2021, was isolated from dead armadillos, which mainly caused lesions in the spleen, lungs, and liver. The bacterium was sensitive to most antibiotics except penicillin, and the mice challenged with the bacterium exhibited histopathological changes similar to those observed in the armadillos. To the best of our knowledge, this is the first report on armadillos being
infected with S. saprophyticus. The results of this study provide a reference for breeding and management of armadillos in zoos.

**Declarations**

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**Data availability**

The data that support this study will be shared upon reasonable request to the corresponding author.

**Author Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Chuangen Guo, Weibo Sun, Wangkun Cheng, Nan Chen, Changlin Deng, Haoran Xu, Congyu Wu, and Yingjun Lv. The first draft of the manuscript was written by Chuangen Guo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Ethics approval**

Animal experiments were performed in compliance with Chinese legislation and were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

**Consent to participate**

Not applicable.

**Consent for publication**

All authors give consent for publication.

**References**


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No lesions were found on body surface during examination (a); Congestion and enlargement were observed in the heart (b), liver (c), spleen (d), and lungs (e); White necrotic foci were found in the liver (c) and spleen (d); Slight swelling was observed in the kidneys (f).
Figure 2

Histopathological changes observed in the spleen (a–b), liver (c–d), lungs (e–f), heart (g), and kidneys (h–i) of the dead armadillos
Figure 3

Identification of the isolated bacteria

Several cocci were observed in the contacting smears of liver tissue samples of the dead armadillos after Wright's staining (white arrows) (a). Morphology of the isolated bacteria on blood agar (b). Gram-positive cocci were observed after Gram staining of the isolated strains (c). Strain *S. SNH2021* produced bubbles during in the presence of hydrogen peroxide during the catalase test but not with PBS (d). *Staphylococcus aureus* ATCC25923 induced plasma coagulation in the coagulase test (e) but strain *S. SNH2021* did not coagulate plasma (f).
Figure 4

Analysis of mice incubated with the isolated strain S.S NH2021

The survival curve of the mice after incubation with different doses of strain S.S NH2021 (a). Gross changes observed in the liver, spleen, lungs, kidneys, heart, and brain (b). Bacterial load of different
tissues of mice incubated with S.S NH2021 (c). Histopathological changes of the liver (d–e), spleen (f–g), and heart (h–i) in the dead mice.