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Occurrence of *Streptococcus dysgalactiae* Subsp. *equisimilis* in Masked Palm Civet (*Paguma larvata*)

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Abstract: A number of *Streptococcus* species have been isolated from many animals. However, there have been no reports of infection with *Streptococcus dysgalactiae* Subsp. *equisimilis* (SDSE) in masked palm civets. The first occurrence of SDSE in masked palm civet was reported. Identification of the organism was made by phylogenetic analyses based on partial *16S rRNA* gene sequences. The present result has implications for the ongoing control of SDSE in masked palm civets and evidences a host extension of SDSE.

Key words: Identification, isolation, masked palm civet, *Streptococcus dysgalactiae* subsp. *equisimilis*, organism

INTRODUCTION

The masked palm civet (*Paguma larvata*) is a medium-sized mammal (Carnivore, Viverridae) found throughout tropical and subtropical Asia (Chen *et al*., 2008b). In China, masked palm civets live mainly in the southern provinces but can also be found in northern part of China such as Hebei, Shanxi and Tibet provinces. In recent years, the population of palm civet declines due to habitat destruction, hunting, trade for meat production and several diseases which have led to the masked palm civet becoming an endangered wildlife species and it is now included in the Chinese Protected Animal List (Chen *et al*., 2008b).

Streptococcus species are Gram-positive bacteria that can cause many different types of clinical diseases in humans and animals (Barcelos *et al*., 2010; Slama *et al*., 2009). *Streptococcus dysgalactiae* Subsp. *equisimilis* (SDSE) species can be classified into various groups showing different phenotypic and genotypic characterization: Lancefield Group C β-haemolytic, Lancefield Group G β-haemolytic and Lancefield Group L β-haemolytic SDSE. In recent years, SDSE has increasingly been reported as a cause of invasive disease and causing significant diseases and major socio-economic losses globally (Rantala *et al*., 2010; Takahashi *et al*., 2011). The SDSE infections in animals and humans are responsible for different clinical syndromes including primary bacteraemia, cellulitis, necrotizing fasciitis, arthritis and streptococcal toxic shock syndrome (Preziuso *et al*., 2010; Mcmillan *et al*., 2010). To this knowledge, to date the SDSE has not been isolated from masked palm civet.

MATERIALS AND METHODS

Three masked palm civets which were kept and observed by masked palm civet watchers in pens within a botanical garden in Changsha, China were captured and transferred to the College of Veterinary Medicine, Hunan Agricultural University.

All tests were performed in triplicate with media freshly prepared on separate occasions. In addition, the lung and spleen samples were cultured on blood agar plate and incubated in the broth for 18-24 h at 37°C under aerobic environment. Bacterial species were identified as Streptococcus on the basis of colonial morphology and Gram stain and Beta-hemolysis and fermentation of trehalose. The sorbitol or lactose negative colonies were subcultured for purity (Fig. 1).

The 16S ribosomal RNA (*rRNA*) gene sequences of the isolated bacteria were amplified by PCR with primers which we designed based on conserved regions: 16SFP (5'-AGYGGCGRACGGGTGAGTAA-3')and16SRP(5'-CCATTGTAGCACGTGTGTAGC-3'). PCR reactions (25 uL) were performed in 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM

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$MgCl_2$, 200 mM each of dNTP, 50 pmol of each primer and 2 U Taq polymerase (Takara) in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94°C for 5 min then 94°C for 30 sec (denaturation), 58°C for 30 sec (annealing), 72°C for 30 sec (extension) for 30 cycles followed by 72°C for 5 min (final extension). Each amplicon (5 μL) was examined by agarose gel electrophoresis to validate amplification efficiency. Then, the partial 16S amplicons were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing from both directions by primers used in the PCR amplifications. The sequences of the PCR products were compared with those of closely related species in GenBank by multiple sequence alignment using ClustalX 1.83 (Thompson *et al*., 1997). Phylogenetic relationship among SDSE were performed among the 24 *Streptococcus* species as ingroup plus the sequence obtained in the present study using *Mycoplasma Hyopneumonia*e (GenBank Accession No.: GU227407) as the outgroup, based on nucleotide sequences of 16S rRNA dataset. Three methods, namely Neighbor Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) were used for phylogenetic re-constructions. Standard unweighted MP was performed using package Phylip 3.67 (Felsenstein, 1995). NJ analysis was carried out using the Dayhoff Matrix Model implemented by MEGA 4.0 (Tamura *et al*., 2007) and ML analysis was performed using PAUP 4.0 Beta 10 programme (Swofford, 2002). The consensus tree was obtained after bootstrap analysis with 1000 replications for NJ and MP trees and 100 for ML tree with values >50% reported indicating that the clinical isolate was a strain of SDSE (Fig. 2).

Fig. 1: Gram stain of a pure culture of *Streptococcus dysgalactiae* subsp. *equisimilis*, isolated from the liver of a diseased masked palm civet

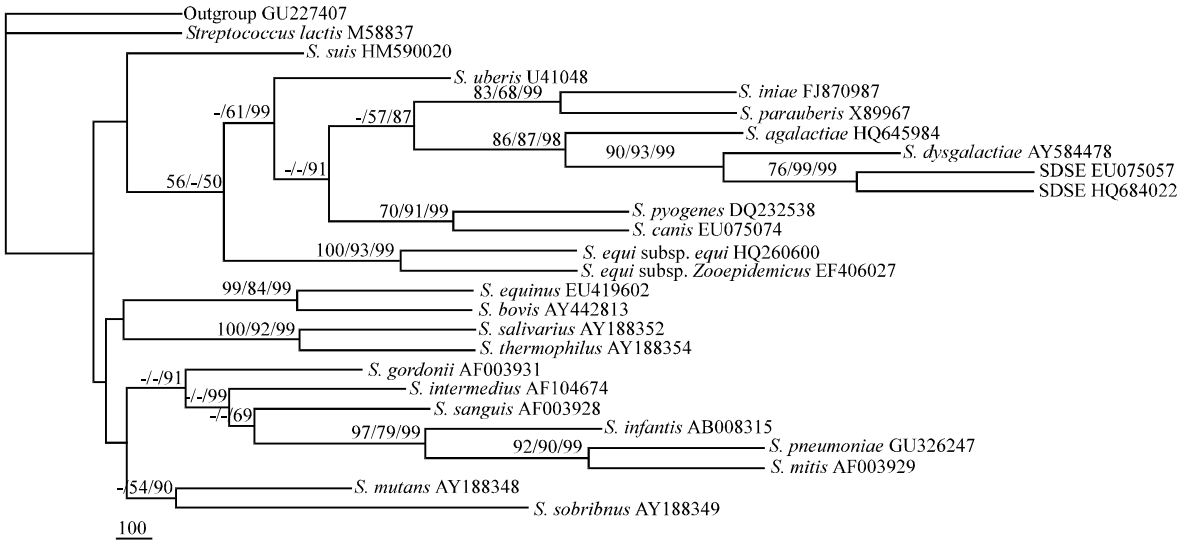

Fig. 2: Inferred phylogenetic relationship among *Streptococcus* species based on analyses of 16S rRNA dataset utilizing Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbour Joining (NJ) using one species (*Mycoplasma hyopneumoniae*) as outgroup. The numbers along branches indicate bootstrap values resulting from different analyses in the order: MP/ML/NJ. Values <50 are given as "-"

RESULTS AND DISCUSSION

Physical examination of these masked palm civets revealed emaciation, severe fever, deep lethargy, diarrhea, hard pad, ocular and nasal discharge. All diseased masked palm civet were examined after intravenous injection of glucose and sodium chloride (5%) due to the severity of bad general condition. All the masked palm civets died 3 h later. At necropsies, histopathologic lesions were observed and characterized by interstitial pneumonia with congestion and hemorrhage and edema, focal emphysema. The spleens had necrosis, kidneys hemorrhagic and joint swelling and increased synovial fluid were present. In the central nervous system, moderated demyelination of white matter and focal areas having gitter cells associated with malacia in the cerebellum were observed. The spinal cord had demyelination and Wallerian degeneration.

The partial 16S rRNA sequences of SDSE determined in the present study was 1146 bp in size which was identical to that of SDSE isolated recently from Australia (GenBank Accession No.: EU075057). The present sequences have been deposited in the GenBank under the Accession No.: HQ684022 (16S rRNA). Previous studies revealed the occurrence of SDSE in humans, foals, horses, swine and marine mammals (Hong *et al*., 1993; Imai *et al*., 2009; Kawata *et al*., 2003; Preziuso *et al*., 2010; Rantala *et al*., 2010). As the lack of 16S rRNA sequences or the sizes of the known 16S rRNA sequences of SDSE of different hosts are so small (only approximately 400 bp, except humans) (Imai *et al*., 2009; Preziuso *et al*., 2010) and the phylogentic analysis based on these short sequences may not give enough evolutionary information so, the present study didn't compare the sequence with the sequences from other hosts. However, this report is believed to be the first documenting for the presence of SDSE in masked palm civets. The masked palm civets may have become infected through secondary infections occurred after canine distemper virus and immunocompromised hosts is likely to have played an important role in this case because Canine Distemper Virus (CDV) was detected by CDV antibody detection kit from the collected sample of masked palm civets. In addition, CDV was also detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) from fresh brain and lung tissues of samples. The CDV was diagnosed in masked palm civets in this case and it is not surprising because CDV infections were found in many areas and they may represent reservoirs of CDV (Chen *et al*., 2008a). It is believed that dogs or other carnivores infected with the virus were the most likely source, because canine distemper is a very common disease in suburban and farm dogs in China because of the absence of vaccination. Although, the SDSE may sometimes be iaolated as normal flora from the skin, gastrointestinal and genitourinary tracts (Kawata *et al*., 2003; Takahashi *et al*., 2011), the investigation found that SDSE infections cause the histologic changes in all masked palm civets. Addition, all patient masked palm civets was treated with penicillin, kanamycin and sulfamethoxazole after the first symptoms but theirs symptoms haven't gone. It is believed that masked palm civets were infected SDSE through secondary infections.

CONCLUSION

In this study, the present study demonstrated for the first time the occurrence of SDSE in masked palm civet which indicates palm civet may be a new reserve of SDSE and has implication for the ongoing control of SDSE in animals and human beings.

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