Identification and Molecular Characterization of Serological Group C Streptococci Isolated from Diseased Pigs and Monkeys in Indonesia

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The present study was designed to comparatively investigate 34 beta-hemolytic streptococci isolated from infected pigs and monkeys from various islands in Indonesia. According to the serological and biochemical data, all 34 isolates were Lancefield's serological group C streptococci and could be identified as *Streptococcus equi* subsp. *zooepidemicus*. Of the 34 group C streptococci investigated, 28 grew on solid media in large, mucoid colonies, in fluid media at a uniform turbidity, and in soft agar in diffuse colonies. A decapsulation test with a hyaluronidase-producing *Staphylococcus aureus* strain revealed the hyaluronic acid nature of the capsular material. The remaining six streptococci grew on solid media in small, nonmucoid colonies, in fluid media as sediment with clear supernatant, and in soft agar in compact colonies. Determination of surface hydrophobicity by salt aggregation revealed a hydrophilic surface for the encapsulated bacteria and a hydrophobic surface for the unencapsulated group C streptococci. To further analyze the epidemiological relationships, all 34 mucoid and nonmucoid isolates from pigs and monkeys were subjected to protein and DNA fingerprinting. The latter was performed by pulsed-field gel electrophoresis. The protein profiles of all 34 isolates and the DNA profiles of 32 isolates appeared to be identical, with the DNA profiles of 2 isolates being closely related, indicating that a single virulent clone is responsible for this disease outbreak in Indonesia.

At the beginning of 1994, a disease outbreak among pigs and monkeys was reported on the island of Bali, Indonesia. The diseased animals showed such clinical symptoms as painful swelling of the joints, respiratory disturbances, and diarrhea. Most of the animals died within a few days. The postmortem examinations of the pigs and monkeys revealed signs of polyarthritis, bronchopneumonia, pleuritis, epicarditis, endocarditis, and meningitis (6). Further bacteriological examinations resulted in the isolation of streptococci of serological group C. These bacteria seemed to be one of the major causative agents. These group C streptococci could be isolated from most of the pigs and monkeys in pure cultures.

The first cases were reported among animals of a pig owner in a small village on the island of Bali. In the following weeks and months, the outbreak spread rapidly to the surrounding districts in Bali and into a monkey population. Within 3 months, comparable cases were reported for diseased pigs on the islands of Sumatra and Sulawesi. No comparable disease was reported in Indonesia before 1994.

At present, little is known about the properties and epidemiological relationships of these group C streptococcal isolates from various places in Indonesia. The present study was designed to further characterize pig and monkey isolates from Bali and the islands of Sumatra and Sulawesi.

MATERIALS AND METHODS

Bacterial isolates. A total of 34 beta-hemolytic streptococci were investigated in this study. Thirty streptococci were isolated from diseased pigs from small farms in Bali, Sumatra, and Sulawesi, Indonesia, and four streptococci were isolated from diseased monkeys from two Bali monkey resorts over a time period of 4 months (April to July) in 1994. Before being sent to Germany, the bacteria were stored in liquid nitrogen. The bacteria were cultivated on Columbia sheep blood agar plates (Oxoid, Wesel, Germany) and in Todd-Hewitt broth (Gibco Europe, Karlsruhe, Germany).

Serogrouping. Two commercial grouping kits (Slidex Strepto-Kit; bioMerieux, Nürtingen, Germany; and Streptococcal Grouping Kit; Oxoid) were used.

Biochemical properties. Utilization of sugars was determined with phenol red broth (Merck, Darmstadt, Germany) containing 1% (each) arabinose, inulin, lactose, mannitol, melezitose, raffinose, rhamnose, ribose, saccharose, sorbitol, trehalose, and xylose. The Voges-Proskauer reaction was basically performed by a method described by Collins and Lyne (4). Hydrolysis of sodium hippurate was determined by the method of Hwang and Ederer (11).

Decapsulation test. For the determination of the presence of hyaluronic acid capsular material, the streptococci were cultivated on Columbia blood agar plates in close proximity to a hyaluronidase-producing *Staphylococcus aureus* strain (*S. aureus* Sn 2440) (3).

Growth properties in soft agar. One loopful of the freshly cultivated bacteria was mixed with 10 ml of NaCl at 0.14 mol/liter. One loopful of this suspension was inoculated into 10 ml of soft agar medium (brain heart infusion [Gibco] containing 0.15% agar) and incubated for 18 h at 37°C. The morphology of the bacterial colonies was evaluated as described previously (25).

SAT. A salt aggregation test (SAT) was performed by a method described by Jonsson and Wadström (12), with the bacteria being adjusted photometrically (10% transmission at 620 nm) in 0.002 mol of sodium phosphate buffer (pH 6.8) per liter. The bacteria were mixed with equal volumes of ammonium sulfate at various concentrations (0.2 to 3.2 mol/liter of distilled water). A visible aggregation was regarded as positive. To exclude the possibility of self-agglutination, a bacterial suspension in the buffer mentioned above was used as a control.

Whole-cell protein profiles. The bacteria were cultivated, centrifuged, and resuspended in distilled water to a protein concentration of approximately 2 mg/ml. The suspension was heated (95°C for 5 min) in the presence of a mixture containing sodium dodecyl sulfate (SDS; 0.625 mol of Tris-HCl [pH 6.8] per liter, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromphenol blue). Approximately 20 μl of the solubilized proteins was subsequently examined by SDS–11% polyacrylamide gel electrophoresis (PAGE) with a Mini Protean II apparatus (Bio-Rad Laboratories, Munich, Germany) and stained with 0.25% Coomassie blue (R 250; Serva, Heidelberg, Germany).

Pulsed-field gel electrophoresis. The preparation and digestion of genomic DNA were performed according to the methods of Maslow et al. (16) and Thiele et al. (23). The cultivated bacteria (in 40 ml of Todd-Hewitt broth) were centrifuged, washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), resuspended in TE buffer (5% transmission at 620 nm), and mixed with the same volume of 1% low-melting-point and low-gelling-point InCert agarose (FMC Bio Products, Rockland, Maine), and then this mixture was solidified for 10 min on ice in special molds. The cells were lysed by subsequent incubation of the blocks in 200 μl of ES buffer (0.5 M EDTA [pH 8.0], 1% *N*-lauroylsarcosine [Sigma,

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Deisenhofen, Germany]) for 30 min at room temperature and then by the addition of 10 μl of mutanolysin (10 U/μl; Sigma) for 1 h at 37°C. After overnight treatment with proteinase K (final concentration, 0.5 μg/ml; Boehringer, Mannheim, Germany) in 200 μl of TE buffer at 56°C, the agarose blocks were washed twice for 30 min each time in TE buffer, incubated with phenylmethylsulfonyl fluoride (final concentration, 1.0 mM phenylmethylsulfonyl fluoride in 200 μl of TE buffer; Sigma) twice for 1 h each time at 56°C, and washed in TE buffer twice for 30 min each time. The genomic DNA of the isolates was digested by incubation of the agarose block with 6 μl of SmaI (48 U; Stratagene, Heidelberg, Germany) suspended in 40 μl of 10× universal buffer (Stratagene) and 354 μl of twice-distilled water for 5 h at 25°C.

Samples were electrophoresed at 14°C through 1% chromosomal-grade agarose (Bio-Rad) (in a 13-by-14-by-0.55-cm gel) containing 0.5 μg of ethidium bromide (Sigma) per ml in 0.5× TBE buffer (45 mM Tris, 45 mM borate, 1.0 mM EDTA [pH 8.3]) in a CHEF-DR II PFGE electrophoresis cell (Bio-Rad).

Lambda Mix (MBI Fermentas, St. Leon-Rot, Germany) and a 50-kb ladder (λ DNA concatemers at 50 to 1,000 kb; Sigma) served as standards.

The running conditions were as follows. Switch time ramping was for 0.1 to 11 s for 8 h at 5 V/cm and then for 9 to 45 s for 17 h at 6 V/cm. For control purposes, *Streptococcus equi* subsp. *zooepidemicus* 500 and W60 were added. Both strains were kindly obtained from J. F. Timoney (University of Kentucky at Lexington).

RESULTS

All 34 isolates used in the present investigation were beta-hemolytic and could serologically be classified into Lancefield's serological group C. Both serological grouping kits gave identical results.

The biochemical properties of the 34 group C streptococci isolated from pigs and monkeys appeared to be identical. All group C streptococci utilized lactose, ribose, saccharose, and sorbitol but failed to utilize arabinose, inulin, mannitol, melezitose, raffinose, rhamnose, trehalose, and xylose. They were Voges-Proskauer and sodium hippurate negative and could be identified as *S. equi* subsp. *zooepidemicus.*

Upon cultivation of the bacteria on Columbia blood agar, 24 of the group C streptococcus isolates from pigs and all 4 isolates from monkeys grew in large mucoid colonies. The remaining six group C streptococci grew in small, nonmucoid colonies on solid media. Cultivation of the bacteria in the presence of a hyaluronidase-producing *S. aureus* strain caused a change in colony morphology. All mucoid colonies cultivated in proximity to the hyaluronidase-producing *S. aureus* strain appeared to be small and nonmucoid. The growth of the six unencapsulated group C streptococcal strains remained unchanged.

All 24 group C streptococci isolated from pigs and all 4 group C streptococci that were isolated from monkeys and that had mucoid growth on solid media grew to a uniform turbidity in liquid media and exhibited a broad, diffuse colony morphology in soft agar. In addition, these cultures did not react in the SAT, even at an ammonium sulfate concentration of 3.2 mol/ liter. In contrast, all six group C streptococci showing nonmucoid growth on solid media grew in liquid media, producing sediment and clear supernatant, grew in soft agar in compact colonies, and aggregated in SATs at an ammonium sulfate concentration of 1 mol/liter. The protein fingerprints of all 34 of the *S. equi* subsp. *zooepidemicus* cultures tested revealed identical protein profiles. A typical protein fingerprint of the isolates is shown in Fig. 1.

In addition, digestion of the chromosomal DNAs of 32 of the isolates with endonuclease SmaI revealed identical DNA patterns for these isolates. Digestion of the chromosomal DNAs of the remaining *S. equi* subsp. *zooepidemicus* isolates yielded DNA patterns with a difference in two fragments (Fig. 2). The DNA restriction patterns of the *S. equi* subsp. *zooepidemicus* control strains appeared as unrelated DNA profiles (Fig. 3).
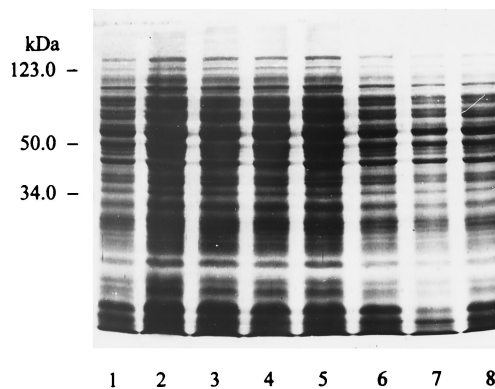

FIG. 1. Typical SDS-PAGE patterns of protein extracts of eight *S. equi* subsp. *zooepidemicus* isolates from pigs and monkeys.

DISCUSSION

All streptococcal isolates used in the present investigation were isolated from diseased pigs and monkeys in Indonesia.

On the basis of their serological and biochemical properties, all 34 isolates from the islands of Bali, Sumatra, and Sulawesi belonged to Lancefield's serological group C and could be identified as *S. equi* subsp. *zooepidemicus*. All 34 cultures, regardless of their place of isolation, had identical biochemical profiles.

S. equi subsp. *zooepidemicus* is well known from infections of a wide variety of animals, including pigs, sheep, cows, goats, foxes, birds, rabbits, guinea pigs, and monkeys (21). All of these animals might be potential reservoirs for infections of humans. Cases of human infections with *S. equi* subsp. *zooepidemicus* have been reported, and such infections are frequently associated with the consumption of homemade cheese or unpasteurized milk (2, 5, 7). In addition, the isolation of *S. equi* subsp. *zooepidemicus* from humans has been described in cases of endocarditis (15), pneumonia (17), meningitis (9, 14), septic arthritis (1, 10) and cervical lymphadenitis (13). However, pig infections with *S. equi* subsp. *zooepidemicus* are rare.

To our knowledge, this is the first report of *S. equi* subsp. *zooepidemicus* infections in pigs with bacteria being isolated from a large number of infected animals.

In the past decade, the application of chemotaxonomic criteria and the use of techniques based on nucleic acid hybrid-
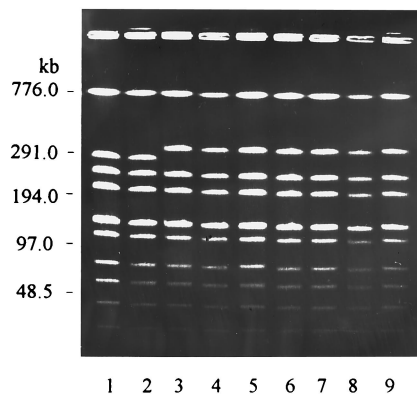

FIG. 2. Pulsed-field electrophoretic restriction patterns of chromosomal DNAs of nonmucoid (lanes 1, 2, and 8) and mucoid (lanes 3 to 7 and 9) *S. equi* subsp. *zooepidemicus* isolates from pigs (lanes 1 to 3 and 6 to 9) and monkeys (lanes 4 and 5) after the DNAs were digested with endonuclease SmaI.
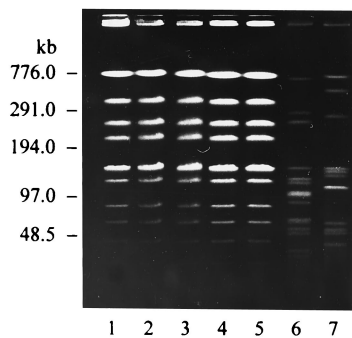
FIG. 3. Pulsed-field electrophoretic restriction patterns of chromosomal DNAs of mucoid (lanes 1, 4, and 5) and nonmucoid (lanes 2 and 3) *S. equi* subsp. *zooepidemicus* isolates from pigs after the DNAs were digested with endonuclease *Sma*I. The DNA restriction patterns of *S. equi* subsp. *zooepidemicus* 500 and W60, which were investigated for purposes of comparison, are shown in lanes 6 and 7.

ization and sequencing have led to important changes in streptococcal taxonomy. These techniques, which give information about the natural relationships of various streptococcal species, showed similarities between "*S. equi*" and "*S. zooepidemicus.*" According to a proposal of Farrow and Collins (8), "*S. zooepidemicus*" should be reclassified as *S. equi* subsp. *zooepidemicus.*

The *S. equi* subsp. *zooepidemicus* isolates of the present investigation displayed differences in growth properties on solid media and in liquid media and soft agar. Growth on solid media in large and mucoid colonies seemed to be related to a uniformly turbid growth of the bacteria in liquid media and to a diffuse colony morphology of the bacteria in soft-agar media. On the other hand, cultures with small, nonmucoid colonies grew as a sediment with a clear supernatant in liquid media and in compact colonies in soft agar. Comparable relationships in terms of microencapsulation, chain lengths, and the growth properties of the bacteria could be observed for various serotypes of the streptococci of serological group B (18, 25, 26). However, in contrast to those of group B streptococci which have neuraminic acid as a major part of the bacterial microcapsule (26), the encapsulation and growth properties of *S. equi* subsp. *zooepidemicus* seem to be influenced by hyaluronic acid capsular material. This influence could be demonstrated by the decapsulation test with a hyaluronidase-producing *S. aureus* strain. In addition, this type of encapsulation seems to influence the surface hydrophobicity of the isolates. This influence could be determined by the SAT. Comparable to the neuraminic acid of group B streptococci (26), the hydrophilic hyaluronic acid of *S. equi* subsp. *zooepidemicus* seems to mask hydrophobic surface structures.

The importance of group B streptococcal microencapsulation for pathogenicity could be demonstrated in adherence and phagocytosis experiments (18, 26). In addition, the hyaluronic acid capsule of group A streptococci is well known to be a major virulence factor (24). Further studies with encapsulated and unencapsulated *S. equi* subsp. *zooepidemicus* might elucidate the role of this encapsulation for bacterial pathogenicity.

To further characterize the epidemiological relationships of the bacteria, all 34 *S. equi* subsp. *zooepidemicus* strains isolated from pigs and monkeys from different islands of Indonesia were subjected to protein and DNA fingerprinting. The latter was performed by pulsed-field gel electrophoresis. Regardless of the origins of the fragments, whole-cell protein analysis and electrophoresis of large DNA fragments produced identical

profiles, indicating that a single virulent clone is the causative agent of the various pig and monkey infections on the island of Bali and the other islands of Indonesia. Further information about the trading connections among the islands and among the farmers and the monkey resorts might elucidate how these bacteria could spread in Indonesia.

However, the pulsed-field gel electrophoresis patterns of two pig isolates differed from the general outbreak pattern by two fragments. This difference was possibly caused by a deletion of DNA from the original DNA fragment. It was of interest that both strains were isolated from the same place but from two different pigs on the island of Bali. According to the criteria for interpreting the pulsed-field gel electrophoresis pattern described by Tenover et al. (22), this difference might be caused by a single genetic event, indicating that these two *S. equi* subsp. *zooepidemicus* strains are closely related to the other isolates. The two isolates belonged to the group of six unencapsulated strains, indicating that the degree of encapsulation does not seem to be directly related to the differences in the DNA fingerprints.

These nonmucoid colonies, which also included the three pig isolates from Sumatra, might possibly represent genetically identical phase variants of the mucoid strains. Encapsulated and unencapsulated phase variants of group B streptococci of various serotypes have been described previously (18, 19).

The occurrence of phenotypic variations among *S. equi* subsp. *zooepidemicus* isolates might support the adaptability of these bacteria to different environmental conditions and might help in the understanding of the process of infection with this microorganism.

As was seen for other isolates in the studies reported by Skjold et al. (20), fingerprinting of the chromosomal DNAs of the *S. equi* subsp. *zooepidemicus* isolates studied in the present investigation proved to be a useful and informative technique for further analysis of the epidemiological clusters of these bacterial isolates.

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