JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2590–2594
0095-1137/91/112590-05$02.00/0

Vol. 29, No. 11

Characterization of Six New Capsular Types (23 through 28) of *Streptococcus suis*

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Received 28 June 1991/Accepted 19 August 1991

Six new capsular types of *Streptococcus suis* (types 23 to 28) are described. All reference strains were isolated from diseased pigs and were morphologically and biochemically similar to previously described capsular types 1 to 22. Clear and specific reactions were obtained for each of the new capsular types with three different typing techniques; no cross-reactions were detected among them or with other *S. suis* capsular types. Their capsular material presented similar ultrastructural characteristics, as shown by electron microscopy, and fimbriae similar to those described for other capsular types of *S. suis* were observed. When untypeable field isolates were tested with antisera raised against the six new capsular types, capsular type 23 appeared to be the most prevalent, representing more than 50% of all these isolates. Most isolates were recovered from cases of pneumonia, septicemia, and meningitis. Presumptive biochemical identification described for *S. suis* capsular types 1 to 22 may also be used for capsular types 23 to 28.

Streptococcus suis is responsible for a variety of pathological conditions in pigs (11). It is also implicated in different types of infections in other animals such as ruminants and horses (4, 10). It is noteworthy that *S. suis* is now considered one of the most important microorganisms among zoonotic agents (1, 17).

Since *S. suis* was officially recognized as a defined species in 1987 (15), the number of capsular types has increased from 9 to 23 (6, 16). So far, all these capsular types have been found in North America (5, 11). Capsular type 2 still represents at least one-third of all isolates from diseased pigs, followed by capsular types 3, 1/2, and 8 (11).

A presumptive identification of *S. suis* isolates may be reached by the use of biochemical tests (3, 9); however, standardized capsular typing remains the only way to achieve a definitive identification. Despite the high number of known capsular types of *S. suis*, untypeable isolates are still recovered in pure culture or in significant numbers from diseased pigs (5, 8).

As long as *S. suis* untypeable isolates are associated with clinical diseases in swine, it will be very difficult to collect valuable epidemiological data and the development of control methods will be impaired. The aim of this study was the proposition and characterization of six new capsular types of *S. suis*.

MATERIALS AND METHODS

Bacterial strains and field isolates. Bacterial strains used in this study included reference strains of *S. suis* capsular types 1 to 22 and 1/2 (6, 16) and also six other strains representing proposed capsular types 23 to 28. A total of 46 field isolates, each belonging to one of these six new capsular types, were also included in this study. These isolates were recovered in pure culture or as the predominant organism from different tissues of diseased pigs after culture on 5% bovine blood agar plates (tryptic soy agar containing 5% bovine blood) incubated at 37°C with 5% CO_2. Presumptive identification with biochemical tests was carried out as previously described (9).

Production of antisera. Antisera against all reference strains were raised in rabbits. Antigens for immunization were prepared as previously described (9).

Capsular typing. Capsular typing was carried out by three different techniques: coagglutination, capsular reaction, and capillary precipitation tests. The coagglutination test was performed as described earlier (6), and positive results were recorded when a strong reaction was obtained within 1 min. The capsular reaction test was conducted as described earlier (9). The capillary precipitation test was carried out with antigens extracted with 0.1, 0.06, and 0.01 N hydrochloric acid. All reference strains and field isolates were also examined for the presence of Lancefield group D antigen with a commercial reagent (Phadebact; Pharmacia Diagnostic, Uppsala, Sweden), according to the manufacturer's recommendations.

Biochemical identification. Reference strains of capsular types 23 to 28 of *S. suis* as well as all field isolates were subjected to the following conventional biochemical tests: arginine hydrolysis; production of acetoin (Voges-Proskauer test); production of acid from inulin, salicin, trehalose, lactose, sucrose, sorbitol, mannitol, and glycerol; and production of amylase. All strains and field isolates were also tested for their ability to grow in the presence of 6.5% NaCl. Acid production in phenol red broth was tested, and reading was carried out after 48 h of incubation at 37°C under aerobic conditions. Amylase activity was tested on Mueller-Hinton agar supplemented with 1 g of soluble starch per liter. The strains and field isolates were spot inoculated, and the plates were flooded with iodine after 18 h of incubation at 37°C under aerobic conditions. Results were interpreted as previously described (3). Reference strains of *S. suis* capsular type 2 (strain 735), *Streptococcus bovis* (ATCC 9809), and *Enterococcus faecalis* (ATCC 29212) were used as controls. Finally, all strains and isolates were tested with the Rapid-Strep system (API system; Laboratory Products Ltd., Saint-Laurent, Quebec, Canada) according to the manufacturer's recommendations. Susceptibility to optochin was determined by inoculating a young culture on Mueller-Hinton

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TABLE 1. Capsule thickness of agar-grown *S. suis* strains after stabilization with homologous antisera and staining with ruthenium red

Reference strain	Capsular type	Capsule thickness (nm)[a]
89-2479	23	110–130
88-5299A	24	150–170
89-3576-3	25	65–90
89-4109-1	26	85–95
89-5259	27	110–150
89-590	28	90–110

[a] Each value is based on 20 to 25 measurements per preparation.

agar, and commercial discs (Difco Laboratories, Detroit, Mich.) were used according to the manufacturer's recommendations.

Electron microscopy. Preparation for transmission electron microscopy was carried out as previously described (13). Capsular material was stabilized with homologous whole-cell antisera and stained with ruthenium red; normal rabbit serum was used as a negative control. Thin sections were examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV. Negative staining was also performed as described earlier (13). Briefly, a drop of each preparation was placed on a 200-mesh Formvar-coated electron microscope grid and blotted partially dry. A drop of 2% (wt/vol) phosphotungstate (pH 7.0) was then applied to the grids, which were examined with the electron microscope.

RESULTS

Six new capsular types of *S. suis* are described in this report. All reference strains showed colonial morphologies and biochemical patterns similar to those of previously described capsular types of *S. suis* and possessed the Lancefield group D antigen.

Clear and specific reactions were obtained for each of the new capsular types with all three typing techniques. No cross-reactions were detected among them or with other capsular types of *S. suis*. As demonstrated by the capillary precipitation test, the type antigen of capsular types 23, 24, and 26 could be successfully extracted with 0.06 N HCl; in contrast, 0.01 N HCl was needed to obtain a clear reaction for capsular type 25, and 0.1 N HCl was needed for capsular types 27 and 28.

Electron microscopy of thin sections of bacterial cells belonging to the new capsular types and stabilized with homologous antiserum showed the presence of a layer of capsular material (Table 1). The thickest capsular material layer (150 to 170 nm) was seen around cells from *S. suis* capsular type 24 (Fig. 1A), whereas the thinnest capsular material layer (65 to 90 nm) was seen around cells of *S. suis* capsular type 25 (Fig. 1B).

After negative staining, the presence of a peritrichous

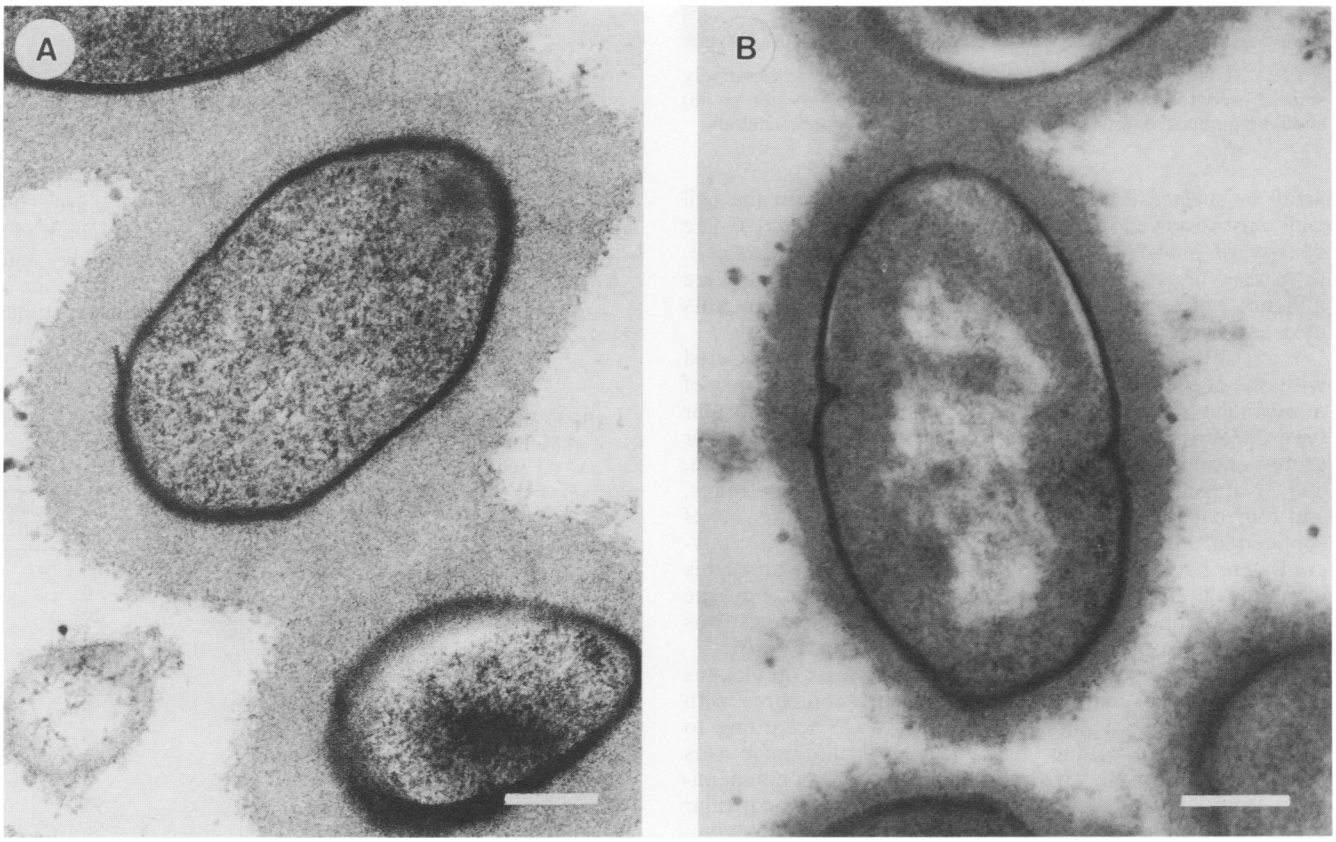

FIG. 1. Transmission electron micrographs of thin sections of *S. suis* grown on blood agar plates. Cells of strains 88-5299A, representing serotype 24 (A), and 89-3576-3, representing serotype 25 (B), were stabilized with whole-cell homologous antiserum and stained with ruthenium red. Bars = 200 nm.

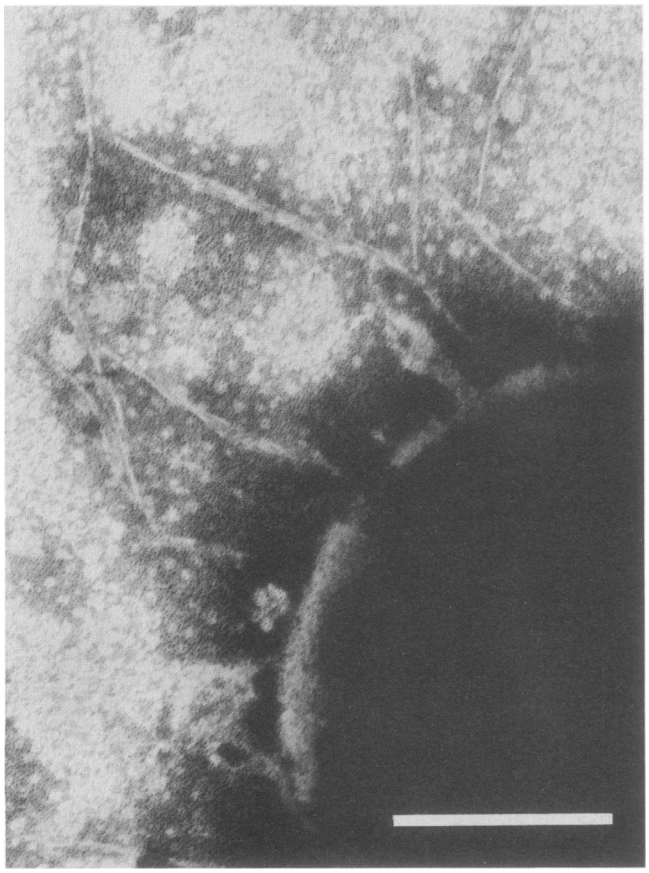

FIG. 2. Transmission electron micrograph of a negatively stained preparation of *S. suis* reference strain 89-590 (serotype 28) showing fimbriae extending from the cell surface. Bar = 200 nm.

array of surface fimbriae extending outward from the cell wall was observed in all six reference strains (Fig. 2). The density of fimbriae varied considerably from cell to cell, from very dense to sparse. In some strains, fimbriae were difficult to observe because of the presence of large amounts of capsular material.

Each of the 46 field isolates included in this study reacted with one of the sera raised against the new capsular types of *S. suis* (Table 2). Twenty-five of them belonged to capsular type 23 and five belonged to capsular type 28, and four groups of four isolates reacted with capsular types 24, 25, 26, and 27 antisera. Most isolates were recovered from lungs, and four capsular type 23 isolates originated from cases of septicemia. Pigs from which all these isolates were recovered were between 3 and 10 weeks of age; all field isolates exhibited alpha-hemolysis on bovine blood agar, and the colonial morphology of the different capsular types was similar to that of previously described types of *S. suis*.

Results of the conventional biochemical tests on isolates belonging to capsular types 23 to 28 were compared with expected results for capsular types 1 to 22 according to current literature (Table 3). No marked difference was observed between the two groups; moreover, no biochemical pattern was associated with any particular new capsular type. One isolate, belonging to capsular type 24, was glycerol positive; most isolates were arginine negative, and they were all mannitol negative. All isolates presented a characteristic narrow reaction zone on starch-containing plates.

TABLE 2. Distribution of 46 isolates of *S. suis*

Origin of isolates	No. of isolates of capsular type:					
	23	24	25	26	27	28
Lungs	15	1	2	2	0	3
Brain and/or meninges	1	0	0	0	2	0
Multiple tissues[a]	4	2	1	0	0	0
Heart	1	0	0	1	2	0
Other	4	1	1	1	0	2
Total	25	4	4	4	4	5

[a] Probably septicemia; isolates were found in three or more of the following tissues: lungs, brain and/or meninges, heart, joints, spleen, liver, and mesenteric lymph nodes.

Results of the biochemical tests carried out with the API system were compared with those obtained with previously described capsular types (Table 4). Three isolates were hippurate positive; more than half of the isolates were pyrrolidonylarylamidase positive, and two were amylase negative. Only one isolate was β-galactosidase positive; this test appeared to be variable for all other capsular types. Nine isolates were β-glucuronidase negative; they belonged to capsular types 24 (three isolates), 26 (two isolates), and 28 (four isolates). Only 30 isolates (65%) could be identified by that system as *S. suis*; the β-glucuronidase-negative isolates were presumptively identified as *Streptococcus pneumoniae*, but they were resistant to optochin. The Lancefield group D antigen could not be detected with a coagglutination reagent in 13% of isolates.

DISCUSSION

After the original description of de Moor's group R, S, and T streptococci (2), 23 capsular types of *S. suis* have been described during the following 26 years (6, 16). The characterization of six new capsular types of *S. suis* presented in this study is intended to add another step towards the establishment of a complete serotyping system, which is a prerequisite for further studies on the epidemiology and control of *S. suis* infections.

Antisera raised against reference strains of capsular types 23 to 28 showed homologous and specific reactions by three

TABLE 3. Results obtained with conventional biochemical tests of 46 isolates of *S. suis* capsular types 23 to 28 in comparison with existing data for capsular types 1 to 22

Biochemical test	Results for type 1–22 isolates[a]	% of type 23–28 isolates with positive result
Amylase	+	100
Acetoin (VP[b])	−	0
Arginine	V	20
Glycerol	−	2
Inulin	+	89
Lactose	+	100
Mannitol	(−)	0
6.5% NaCl	−	0
Salicin	+	96
Sorbitol	−	0
Sucrose	+	98
Trehalose	+	96

[a] Expected results (5, 9, 11, 12); V, variable results; +, positive; −, negative; (−), some exceptions (5).
[b] VP, Voges-Proskauer.

TABLE 4. Results for 46 isolates of *S. suis* capsular types 23 to 28 obtained with the API system, in comparison with existing data for capsular types 1 to 22

Biochemical test	% of isolates with positive results in types:		
	1–8[a]	9–22[b]	23–28
VP[c]	0	0	0
Hippurate	0	4	7
Esculin	73	71	100
Pyrrolidonylarylamidase	6	49	54
α-Galactosidase	92	94	100
β-Glucuronidase	96	65	80
β-Galactosidase	68	45	2
Alkaline phosphatase	0	0	0
Leucine arylamidase	100	96	100
Arginine dihydrolase	84	81	65
Ribose	0	2	0
L-arabinose	0	0	0
Mannitol	2	38	0
Sorbitol	0	4	0
Lactose	100	95	100
Trehalose	100	96	96
Inulin	79	75	89
Raffinose	87	88	98
Starch	100	87	96
Glycogen	100	82	96

[a] Expected results according to the work of Hommez et al. (12).
[b] Expected results according to the work of Gottschalk et al. (5).
[c] VP, Voges-Proskauer.

capsular typing techniques when tested with reference strains of all other capsular types. It is noteworthy that when capsular types 9 to 22 were described, several nonspecific cross-reactions were detected with the coagglutination test (6). By using the standard concentration of hydrochloric acid (0.06 N) for the extraction of capsular material during the capillary precipitation test (6, 16), clear results were obtained with capsular types 23, 24, and 26; however, this concentration was not suitable for capsular types 25, 27, and 28. As it would be complicated to perform the extraction of capsular antigens with three different concentrations of HCl, the capsular reaction test remains the most reliable technique for the serotyping of *S. suis* isolates (9, 12).

The capsular material was stabilized during processing for electron microscopy when cells were treated with whole-cell homologous antisera. The presence of a capsule containing the type-specific antigen (polysaccharide) was demonstrated on each of the reference strains of *S. suis* capsular types 23 to 28 after immunostabilization and staining with ruthenium red. General ultrastructural characteristics were similar to those of previously described capsular types 1 to 8 and 1/2 (13), and the thickness of the capsular layer varied with each capsular type. After negative staining, fimbriae similar to those described for other capsular types of *S. suis* (7, 13) were observed. As is the case for other *S. suis* capsular types, the functions and properties of these surface appendages are unknown.

Capsular type 23 seems to be the most prevalent capsular type, since its antiserum reacted with 50% of the 46 field isolates tested. As is the case for previous types (8, 11), the majority (60%) of isolates belonging to this particular type were recovered from lungs; however, some isolates originated from cases of septicemia.

By using the conventional system for biochemical identification, no significant differences from previously described capsular types (5, 12) were observed. One capsular type 23 isolate fermented the glycerol; this test has consistently been negative for all other capsular types (5, 9). The presumptive identification based on only four biochemical tests (Voges-Proskauer, salicin, trehalose, and 6.5% NaCl) (9) can be successfully used for all new capsular types of *S. suis*, even though four isolates which were trehalose or salicin negative were identified as *S. suis* by capsular typing. As recently suggested by Devriese et al. (3), the amylase test appears to be very helpful for the identification of *S. suis* isolates. It is important to mention that each significant *S. suis* isolate recovered from a diseased pig should be serotyped even if it does not fit perfectly with the presumptive biochemical identification. As demonstrated with all other capsular types, the detection of the Lancefield group D antigen is not reliable for the identification of field isolates (5, 12, 15).

The Rapid-Strep system could not identify all field isolates as *S. suis*. Some features were similar to those presented by capsular types 9 to 22 (5); for instance, 20% of isolates, which could not be identified by the system as *S. suis*, but could be identified as *S. pneumoniae*, were β-glucuronidase negative. The most remarkable difference between capsular type 23 to 28 isolates and those belonging to all other capsular types was the almost complete lack of β-galactosidase activity. The API system was more sensitive than the conventional system for detecting the hydrolysis of arginine, but it was less sensitive for detecting amylase-positive isolates.

As protection against *S. suis* infection is probably type-specific (14), more efforts should be directed towards the characterization of all important capsular types of *S. suis*.

ACKNOWLEDGMENTS

We acknowledge the invaluable technical assistance of B. Foiry, S. Lacasse, M. Gaumond, N. Morier, and M.-A. Lussier.

This work was supported by a grant from Fonds pour la formation de chercheurs et l'aide à la recherche (89-EQ-4101), by Langford, Inc., and by the Ministère de l'Enseignement Supérieur et de la Science.

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