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Morphological Variations of *Haemophilus parasuis* Strains

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Haemophilus parasuis **strains isolated from the noses of apparently healthy animals and from animals with pathological conditions were examined for the presence of a capsule, for their ability to agglutinate in acriflavine or after boiling, and for their peptide profile after polyacrylamide gel electrophoresis (PAGE). The capsule was identified by precipitation against hexadecyl trimethylammonium bromide (Cetavlon), by demonstration of iridescence, and by means of a capsule-staining method. We found a group of capsulated strains showing a rather coccobacillary morphology compared with the morphology with polymorphism, varying from rod-like to filamentous, in strains without detectable capsules. The strains of the latter group were agglutinated by acriflavine or by boiling. Soluble antigens of capsulated strains reacting with Cetavlon were thermostable and resisted proteolytic enzymes, thus suggesting the presence of an acidic polysaccharide. A few of the capsulated strains did not precipitate with Cetavlon, which indicated that their chemical composition was different. Acriflavine-positive strains belonging to a definite PAGE pattern (type II) seemed to be associated with pathological conditions more frequently than were capsulated strains which were mostly isolated from nasal cavities of apparently healthy pigs. We put forward the hypothesis that the agglutinability in acriflavine, together with the PAGE profile type II, may be associated with particular structures responsible for virulence.**

It is generally recognized that *Haemophilus parasuis* is the causative agent of Glässer's syndrome (fibrinous polyserositis with polyarthritis and meningitis). It is also occasionally isolated from animals with acute septicemia and frequently associated with pneumonic lesions. At the same time, it is considered to be commonly present in the nasal cavities of young pigs (14).

H. parasuis infections cause economical problems in the modern pig industry, particularly in specified pathogen-free (SPF) farms, where a fulminant course of the disease with fatal cases can be observed. Therefore, a better understanding of the nature of this infectious agent and of its epidemiology seems to be essential.

The studies of Bakos and Thal in 1952 (1) and Bakos in 1955 (Ph.D. thesis, Royal Veterinary School, Stockholm, Sweden) did not show any substantial correlation between the morphology or serology and the pathogenicity of strains examined. A few experimental infections in pigs and laboratory animals clearly showed a difference in virulence among the different strains studied (1). In more recent investigations with partly capsulated strains, the high virulence of *H. parasuis* for SPF pigs has been demonstrated experimentally (6, 8, 10).

The main difficulty in characterizing *H. parasuis* lies in the fact that it is impossible to differentiate the strains by using biochemical and enzymatical tests (unpublished data) or simple serological methods. Two different polyacrylamide gel electrophoresis (PAGE) patterns (types I and II) (12, 13) could be identified by using a PAGE with sodium dodecyl sulfate (SDS)-solubilized whole cells. All strains from animals with Glässer's disease and some from respiratory tracts were found to belong to type II, suggesting a correlation between the peptide pattern and pathogenicity.

In accordance with this observation, it seemed important to us that the structure of pathogenic isolates should be defined exactly to allow the detection of possible virulence factors. For this purpose, we examined 10 strains isolated from the nasal cavities of healthy pigs and 11 strains isolated from animals with pathological conditions. In addition, strains from different culture collections as well as some other *Haemophilus* species isolated from pigs were included. The main objective was to determine the presence of a capsule and to specify the acidic polysaccharidic nature of the capsular material with hexadecyltrimethylammonium bromide (Cetavlon; Fluka, Buchs, Switzerland), as used by Ørskov (16) for *Escherichia coli*. We further tested the agglutinability in acriflavine and after boiling to observe the behavior of the superficial structures.

MATERIALS AND METHODS

Bacterial strains. We used 32 strains of *H. parasuis* (Table 1), 2 strains of *Haemophilus* sp. taxon C (CAPM 5111 and CAPM 5113), and *Haemophilus pleuropneumoniae* S1536 (11). Eighteen of the 32 *H. parasuis* strains were obtained from the stock culture collection, National Institute of Animal Health, Hokuriku Branch, Niigata, Japan. The remaining strains were derived from the stock culture collection of our institute.

Media. The medium used was YCM agar (10), consisting of 50% (vol/vol) chicken meat infusion (50% [wt/vol]), 1% (wt/vol) soya peptone (Polypepton S; Daigo Co., Japan), 0.5% (wt/vol) NaCl, 5% (vol/vol) fresh dry yeast extract (25% [wt/vol]) and 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.). To observe the influence of the growth conditions (strains CCM 5751, no. 4, and Bakos B26) on the Cetavlon test, YCM agar was also prepared with other peptones such as Soytone (Difco), soya peptone (Merck) or neutralized soya peptone (Oxoid Ltd, London, England).

Preparation of antigens. A bacterial suspension (0.2 g/ml in physiological saline) from cultures, grown for 16 to 20 h at 37°C under a 5% CO_2 atmosphere, was heated at 60°C for 20 min in a water bath and centrifuged at 12,000 × *g* for 5 min. The supernatant was used as antigen for electrophoresis. Alternatively, some supernatants were autoclaved (121°C) for 2 h or treated with proteolytic enzyme (0.5% pronase

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TABLE 1. *Haemophilus* strains used in this study

Organism, organ of isolation, strain	Pathologic lesion	Source
Haemophilus parasuis		
Nasal cavity		
No. 4	—[a]	Japan
SW114	—	Japan
SW124	—	Japan
SW140	—	Japan
SW143	—	Japan
T128	—	Japan
T143	—	Japan
T147	—	Japan
T368	—	Japan
T678	—	Japan
Brain		
HA66	Meningitis	Japan
S1690	Septicemia	Switzerland
Pleura		
SW3	Pleuritis with high fever	Japan
SW35	Polyserositis	Japan
Lung		
Chuetsu	Pneumonia	Japan
Tako	Pneumonia with pleuritis	Japan
8Z14	Polyserositis, arthritis, and meningitis	Japan
Morioka	Septicemia with meningitis	Japan
Nagasaki	Septicemia with meningitis	Japan
Unknown		
6508	Glässer's disease	Denmark
4800	Glässer's disease	Denmark
NCTC 4557	?[b]	England
NCTC 6359	?	England
NCTC 7440	?	England
NCTC 7441	?	England
CCM 5747	?	Czechoslovakia
CCM 5751	?	Czechoslovakia
CIP 52203	?	France
Bakos A9	?	Sweden
Bakos B26	Glässer's disease	Sweden
Bakos C5	?	Sweden
Bakos D74	?	Sweden
Haemophilus sp. taxon C		
Lung		
CAPM 5111	?	England
CAPM 5113	?	England
Haemophilus pleuropneumoniae		
Lung		
S1536	Pleuropneumonia	Switzerland

[a] —, Strain obtained from an apparently healthy pig.
[b] ?, Unknown.

[Boehringer GmbH, Mannheim, Federal Republic of Germany], 0.5% trypsin [Serva, Heidelberg, Federal Republic of Germany]) at 37°C for 2 h.

Cetavlon electrophoresis. We basically used the method described by Ørskov (15). A Veronal buffer (pH 8.6) was used in the electrophoresis chamber. Each glass slide was coated with 4 ml of 1% agarose (Difco) prepared with Veronal buffer diluted with an equal volume of distilled water. The well was filled with 10 μl of antigen. An equal volume of 2% bovine albumin bromphenol blue (BPB) solution was used for the control of migration. The electrophoresis was carried out at a constant current (2.4 mA/cm) for 60 min. After this, each trough was filled with 120 μl of 0.2% Cetavlon solution. The gels were kept in a moist chamber at room temperature for 20 h.

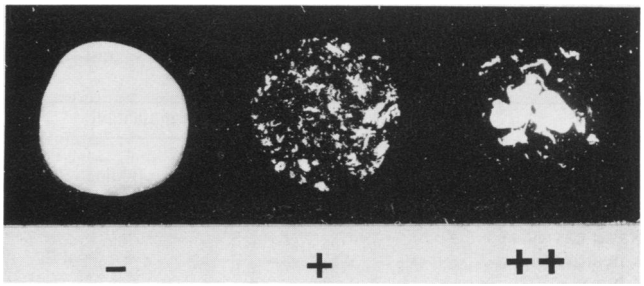

FIG. 1. Acriflavine test (agglutination on glass slide). Symbols: −, milky background; +, fine clumps; ++, large clumps with clear background.

Staining of nucleic acid arcs. After observation of the precipitation arcs, the gels were stained with ethidium bromide (1 μg/ml; Merck & Co., Darmstadt, Federal Republic of Germany) in the electrophoresis buffer for 20 min (2) and then examined under a UV lamp (Ultra Violet Inc., San Gabriel, Calif.).

Iridescence and staining. After 14 to 16 h of incubation in CO_2 at 37°C, colonies grown on YCM agar were examined for iridescence by obliquely transmitted light. Gram-stained organisms were tested for their cell morphology. For capsule detection (negative staining) we made a smear consisting of a drop of bacteria suspension and a drop of India ink solution (3 ml of Pelikan drawing ink, 1 ml of calf serum, 2 ml of distilled water, 4 drops of Formalin). Smears were prepared with cover glass, fixed with methanol, stained with 25% (vol/vol) saturated crystal violet (Merck)-alcohol solution and well rinsed with water. A capsule was observed in the form of an unstained halo around purple-colored cells.

Acriflavine test and heat test. Bacteria harvested from 2-h cultures on YCM agar were suspended in physiological saline (about 0.1 g/ml). This suspension of bacteria (about 20 μl) was mixed with an equal volume of 0.1% (wt/vol) acriflavine (3,6-diamino-10-methyl-acridinium chloride; Fluka) on a glass slide for 1 min. The degree of agglutination was read as shown in Fig. 1. After performance of the acriflavine test, the remaining bacterial suspensions were diluted five times with physiological saline and then boiled for 60 min. Heat test-positive strains showed a bacterial agglutination, and cells sedimented with a clear supernatant at 4°C overnight.

SDS-PAGE. Solubilization of cells in SDS and PAGE were performed as described by Nicolet et al. (13).

RESULTS

Some morphological features of *H. parasuis* are summarized in Table 2. Strains were placed in three groups: strains from nasal cavities of healthy pigs, strains from lesions (polyserositis, pneumonia, etc.), and strains from culture collections.

TABLE 2. Results of the Cetavlon test and some morphological or physiological tests in *H. parasuis* collection strains and reference strains of *Haemophilus* sp. taxon C and *H. pleuropneumoniae*[a]

Source, strain	Cetavlon test result	Iridescence	Presence of capsule	Agglutination: Acriflavine	Agglutination: Boiling	PAGE Pattern
Nasal cavity of healthy pig						
No. 4	+	+	+	−	−	II
SW114	−	+	+	−	−	I
SW124	−	−	−	+	+	I
SW140	+	+	+	−	−	II
SW143	−	−	−	+	+	II
T128	+	+	+	−	w	I
T143	+	+	+	−	w	I
T147	−	−	−	+	+	II
T368	−	+	+	−	−	I
T678	−	+	+	−	−	I
Pathological material						
HA66	+	+	+	−	−	II
SW3	−	−	−	+	+	II
SW35	+	+	+	−	−	II
Chuetsu	−	−	−	++	+	II
Tako	+	+	+	−	−	II
8Z14	−	−	−	+	+	II
Morioka	−	−	−	+	+	II
Nagasaki	−	−	−	+	+	II
S1690	−	−	−	+	+	II
6508	−	−	−	+	+	I
4800	−	−	−	+	+	II
Collection						
NCTC4557	−	−	−	+	w	II
NCTC6359	−	−	−	+	+	II
NCTC7440	w	−	w	−	w	I
NCTC7441	−	−	−	++	+	II
CCM5747	−	−	−	++	+	II
CCM5751	+	+	+	−	−	I
CIP52203	−	−	−	++	+	II
Bakos A9	+	+	+	−	−	I
Bakos B26	−	−	−	++	+	II
Bakos C5	−	−	−	+	+	I
Bakos D74	−	−	−	+	+	I
Haemophilus sp. taxon C						
CAPM5111	+	++	+	−	−	D
CAPM5113	+	++	+	−	−	D
Haemophilus pleuropneumoniae S1536	+	++	++	−	−	D

[a] Symbols: ++, strongly positive; +, positive; w, weak reaction; −, negative; D, different.

Distinct Cetavlon precipitation arcs were observed in 10 of the 32 examined strains of *H. parasuis*, in both strains of *Haemophilus* sp. taxon C and in *H. pleuropneumoniae* (Table 2). These arcs, situated between the application well and the albumin BPB control, were not stained with ethidium bromide solution (Fig. 2). After treating the antigens by heat (121°C) or with proteolytic enzymes (pronase and trypsin), we observed similar precipitation arcs with the same migration. The precipitation arcs of antigens from the remaining strains (Cetavlon test negative) were differently situated, i.e., often close to the anodic end of troughs. It was not difficult to distinguish these precipitation arcs from nucleic acids, since the latter ones moved faster than the albumin BPB control, and their precipitation arcs were clearly stained with ethidium bromide solution.

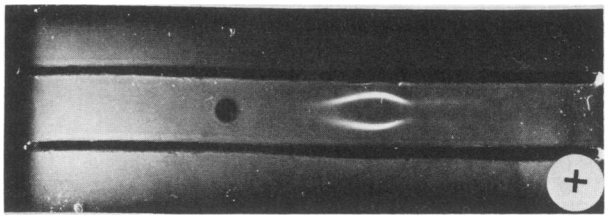

FIG. 2. Cetavlon precipitation arcs. Heat extract (strain no. 4) is in the well, and Cetavlon is in the troughs.

Further tests were performed to find out the effect of the age of the cultures as well as the effect of the peptone composition of the YCM medium on the formation of Cetavlon arcs. Antigens from 16- or 24-h cultures of strains CCM 5751 and no. 4 produced distinct precipitation arcs, independent of the kind of peptone tested, although these two strains showed more rapid and profuse growth on medium prepared from Polypepton S or Soytone. On the other hand, antigens from 48 cultures of Cetavlon-positive strains formed only very weak precipitation arcs or none at all.

It was remarkable that all Cetavlon-positive strains showed iridescent colonies on YCM agar. These strains, which had the morphology of capsulated coccobacilli or small rods, were not agglutinated by acriflavine solution and were mostly stable after 60 min of boiling. Cetavlon-negative strains were divided into at least two groups. Strains of the first group (SW114, T368, and T678) showed capsulated, polymorphous organisms and produced iridescent colonies. They were stable against acriflavine solution or heat treatment. The remaining strains of the second group showed noniridescent or bluish smooth colonies with noncapsular, filamentous, and pleomorphous rods. Bacteria of this group were agglutinated by acriflavine solution and boiling.

Six strains from the nasal cavities belonged to PAGE type I, and the remaining four strains belonged to PAGE type II. There was no evident correlation between Cetavlon-positive capsulated strains and PAGE type, although the three Cetavlon-negative capsulated strains belonged to PAGE type I. All but one of the isolates from pathological conditions belonged to PAGE type II; most of them (seven strains) were not capsulated and were agglutinated by acriflavine or boiling. Three strains were capsulated (Cetavlon positive).

Although most of the collection strains were not capsulated and belonged to PAGE type II (six strains) or to PAGE type I (Bakos C5 and D74), three capsulated strains of PAGE type I could be observed (CCM 5751, NCTC 7440, and Bakos A9).

DISCUSSION

The morphological and physiological study of a selection of *H. parasuis* strains isolated from different sites and of strains from international culture collections made it possible to distinguish between different kinds of structural properties.

The presence of a capsule was clearly demonstrated in 12 of 32 *H. parasuis* strains, in 1 *H. pleuropneumoniae* strain, and in 2 strains of *Haemophilus* sp. taxon C tested comparatively. The capsular material could easily be detected by electrophoresis of the heat-extracted capsular substance and by subsequent precipitation with Cetavlon, as had been done earlier with *E. coli* by Ørskov (15). This capsular material is thought to be an acidic polysaccharide, an assumption supported by the fact that our extracts migrated to the anode and that neither pronase and trypsin treatment nor autoclaving affected it.

Certain nucleic acids may contaminate the extracts and yield some arcs of precipitation. However, these arcs migrate much faster (beyond albumin stained with bromthymol blue as a position marker) and are easily recognized after staining with ethidium bromide. Such nucleic acid arcs were mostly seen in strains that did not show a precipitation with Cetavlon at the usual site. The intensity of Cetavlon precipitation arcs clearly decreased with the age of the culture (48-h incubation), indicating a loss of capsular material after prolonged incubation.

However, 3 of the 12 capsulated strains (SW1114, T368, and T678) did not precipitate with Cetavlon, and this behavior suggested another polysaccharide structure. An early study on the chemical nature of the capsule of *H. parasuis* (18) reports a structure of repeating units of α-galactosyl-α-*N*-acetylglucosaminide which are polymerized through 4-4'-phosphodiester linkage. Our observations suggest that the capsular substance of *H. parasuis* may have different chemical compositions.

Moreover, the presence of a capsule is generally acknowledged to be an important virulence factor within the genus *Haemophilus*. By means of experimental infections (8, 10), attempts were made to maintain this hypothesis for *H. parasuis*. In one study, however, a noncapsulated strain was found to be much more virulent than a capsulated one (6). Among the strains examined in our study, capsules were mainly found in isolates from nasal cavities of apparently healthy pigs (7 of 10 strains) and were less frequently (3 of 11 strains) connected with isolates from pathological conditions. The number of strains is too low for any definite conclusions to be drawn, but the clarity of this trend is nevertheless of great interest.

All the noncapsulated strains tended to polymorphism with a distinct rod-like to filamentous morphology, unlike the coccobacillary form of capsulated strains. Moreover, the apparent absence of capsules was connected with the property of agglutination by acriflavine or by boiling. Such a phenomenon is generally interpreted as a sign of roughness, with a loss of the O-polysaccharides and a consequent loss of virulence. Such degenerated strains appeared after several subcultures undergoing the mucoid-smooth-rough (M-S-R) variation, a feature well known from *H. influenzae* (4, 16).

It is noticeable that most of the strains isolated from animals with pathological conditions, i.e., Glässer's syndrome (8 of 11 strains), were classified in this group. Accordingly, such strains may be more susceptible to the S-R variation. However, we do not have any evidence of such a variation, since the colonies were remarkably smooth, even in primary culture, and most of our strains had been lyophilized after a few subcultures.

The agglutinability of *H. parasuis* by acriflavine and possibly after boiling seems rather to be due to the chemical nature of superficially exposed structures and is not to be considered as an expression of roughness. This phenomenon was observed earlier with the Vi antigen of *Salmonella typhi* (5), the type D strains of *Pasteurella multocida* (3) and the virulent strains of *Yersinia* sp. (7).

Although some capsulated strains may be pathogenic after experimental infections (strain no. 4 in Table 2) (9), our observations confirmed that the strains which induce an acute course of the disease in field cases, e.g., septicemia, are acriflavine positive and belong to the PAGE type II. This viewpoint was also expressed in the reports on the increased virulence of an uncapsulated strain after experimental infection (6) and on the immunogenicity shown by vaccine strains (strain 4800 in Table 2) (17).

The morphological variation in *H. parasuis* strains was confirmed in a recent field study comprising 197 strains from nasal cavities of apparently healthy pigs and 39 strains from necropsy material (I. Bloch, D.V.M. thesis, University of Berne, Bern, Switzerland, 1985). A correlation was found between agglutinability in acriflavine and PAGE type II in 77% of the strains isolated from necropsy material, including all septicemia isolates, whereas only 26% of the strains from nasal cavities showed these properties.

These findings give rise to the hypothesis that the pathogenicity of *H. parasuis* strains is correlated with their property of agglutination in acriflavine or after boiling as well as with their peptide pattern (PAGE type II) after PAGE, as was stated in earlier reports (12, 13). Unquestionably, there are two different structural variations, since PAGE type II can also be found in acriflavine-negative strains and PAGE type I can be found in acriflavine-positive strains (Table 2).

The examination of culture collection strains allowed us to characterize them in accordance with testing criteria. Due to the lack of information concerning the exact conditions of isolation, it was not possible to draw conclusions on the pathogenic properties of these strains.

This study should stimulate further investigations on the nature of the virulence in *H. parasuis*, particularly on the role of the capsule and on the structures which are revealed by acriflavine and the peptide pattern.

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