Morphological Variations of *Haemophilus parasuis* Strains

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Received 29 July 1985/Accepted 4 October 1985

*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 cocccobacillary 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 (fibrosus polyserositis with polyarthritis and meningitis). It is also occasionally isolated from animals with acute septicemia and frequently associated with pneumatic 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 fulminating 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 enzymatic 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 polysaccharide nature of the capsular material with hexadecyltrimethylammonium bromide (Cetavlon; Fluka, Buchs, Switzerland), as used by Orskov (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% CO₂ 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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Haeomophilus

Haemophilius parasuis

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Organism, organ of isolation, strain | Pathologic lesion | Source
--- | --- | ---
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
6506 | 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
S1356 | Pleuropneumonia | Switzerland

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*a* — Strain obtained from an apparently healthy pig.

*b* ? — Unknown.

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.

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TABLE 1. *Haemophilus* strains used in this study

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

<table>
<thead>
<tr>
<th>Source, strain</th>
<th>Cetavlon test result</th>
<th>Iridescence</th>
<th>Presence of capsule</th>
<th>Agglutination</th>
<th>Boiling</th>
<th>PAGE Pattern</th>
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<td>+</td>
<td>+</td>
<td>D</td>
</tr>
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</table>

* 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 anti-
gens 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.

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 Soyotone. 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 cocccobacilli 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 pleomorphic 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 Orskov (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.

The 9 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 uncapsulated 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 uncapsulated 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 PACE 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 PACE type II in 77% of the strains isolated from necropsy material, including all septicaemia 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 after boiling as well as with their peptide pattern (PACE type II) after PAGE, as was stated in earlier reports (12, 13). Unquestionably, there are two different structural variations, since PACE type II can also be found in acriflavine-negative strains and PACE 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.

LITERATURE CITED