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Some Antigenic Properties of *Haemophilus parasuis* and a Proposal for Serological Classification

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We propose a serological classification of *Haemophilus parasuis* into at least five serovars, using an agar-gel-precipitation test with extracts from autoclaved cells. Thirty-two strains were examined, and it was possible to classify 26 of them. The specific antigens were thermostable and soluble and were not affected by pronase treatment but could be extracted by phenol, suggesting a polysaccharide. This polysaccharide seemed to be identical with the capsular substance of serovars 1, 2, and 3. In the presumably uncapsulated strains of serovars 4 and 5, the specific antigen was probably located in the outer membrane. The diversity of the specific substance within the different serotypes was shown by the differences in their electrophoretic migration patterns. Other extraction procedures showed that the washing supernatant and extracts at 60 and 100°C were identical with the 121°C extracts for serovars 1, 2, and 3. In serovars 4 and 5, washing antigens, if present, were different from 100 and 121°C extracts. Other common antigens, presumably proteinaceous antigens, were detected after extraction at 60 and 100°C. The slide and tube agglutination tests allowed classification only for the capsulated strains of serovars 1, 2, and 3. The specific agglutinogens were very sensitive to incubation temperature, and the absorption test showed them to be identical with the 121°C precipitinogens.

Haemophilus parasuis, a species established by Biberstein and White in 1969 (2), is common in pigs, sometimes found in the nasal cavities of apparently healthy pigs and sometimes as an etiological agent of Glässer's syndrome (fibrinous polyserositis, arthritis, septicemia), or associated with lung infections (9).

The antigenic properties of this bacterium have not been thoroughly investigated. No more information has become available since the studies carried out by Bakos et al. in 1952 (1) and Bakos in 1955 (K. Bakos, Ph.D. thesis, University of Stockholm, 1955). Bakos differentiated at least four serotypes (A, B, C, and D) with a precipitation test using cell extracts at 37°C. It has become apparent that the serological classification proposed by Bakos is not sufficient for typing strains of epidemiological importance. However, it was observed that strains found in connection with lesions have a definite pattern of peptides after polyacrylamide gel electrophoresis (PAGE), suggesting a certain heterogeneity among the different strains of *H. parasuis* (7, 8).

Recent observations on the morphological and structural properties of *H. parasuis* (5) revealed the existence of a capsule in a certain group of strains. The capsular material of most of these strains precipitated with hexadecyl trimethylammonium bromide (Cetavlon), which suggested acidic polysaccharides. Other strains shown to be iridescent and to have capsules by negative staining were negative in the Cetavlon test. However, the majority of the pathogenic strains did not seem to be capsulated, and their ability to agglutinate in acriflavine or after boiling suggests the presence of particular superficial structures.

The presence of a capsule in the genus *Haemophilus* is a well-known feature and, as reported for *H. influenzae* (3, 11) and *H. pleuropneumoniae* (6), the capsular material appears to be very soluble and type-specific.

These findings led us to study the antigenic properties of 32 strains isolated from nasal cavities and from pathological material as well as of a selection of strains available in different national culture collections. The aim of our investigation was to offer a better classification of this widespread organism and to allow better comprehension of the antigenic structure of pathogenic strains.

MATERIALS AND METHODS

Bacterial strains and culture medium. The 32 strains identified as *H. parasuis* (Table 1) are the ones investigated in a previous study (5). Chicken meat medium (YCM agar) (4) was used to prepare the bacterial suspensions and extracts for immunization and serology.

Antigens. (i) Agglutination tests. For slide agglutination tests, cultures incubated in 5% CO_2 atmosphere at 37°C for 20 h were suspended in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% NaN_3 and adjusted to a concentration of about 0.1 g/ml. For tube agglutination tests, bacterial suspensions from 14-h cultures were adjusted by spectrophotometry (Hitachi, UV-Vis) to a density equivalent to 5×10^8 cells per ml.

(ii) AGPT. A bacterial suspension (about 0.1 g/ml in 0.15 M NaCl) from 20-h cultures served for preparing the following extract antigens for agar gel precipitation tests (AGPT).

Washing antigens: the untreated suspension was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was used as an antigen.

Heat-treated antigens: extracts were prepared by heating bacterial suspensions at 60°C for 20 min, boiling for 1 h at 100°C, or autoclaving for 2 h at 121°C. After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatants, designated 60°C, 100°C, and 121°C antigens, respectively, were used as extracts.

Immune sera. Antisera to nine strains (Table 1) were prepared by immunizing rabbits with 12 to 14-h cultures. The cells were washed once in PBS, pH 7.2, containing 0.3% Formalin and adjusted to a concentration of 10^{10} cells per ml. Then 5 ml of a bacterin, consisting of an equal volume of cell suspension and Freund complete adjuvant, was injected into the footpads and subcutaneously at four sites as described by Nicolet (6). Three weeks later, four intravenous injec-

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TABLE 1. Serological differentiation of *H. parasuis* and relationships between serovars, PAGE type, and sources of strains

Strain	Grouping by agglutination test		Serovar by AGPT with 121°C antigen	Capsule	Agglutination with acriflavine	PAGE type	Strain source
	Slide	Tube					
No. 4[a]	I	I	1	+	−	II	Healthy[b]
T143	NT[c]	NT	1p[d]	+	−	I	Healthy
HA66	I	I	1p	+	−	II	Meningitis
SW35	I	I	1	+	−	II	Polyserositis
NCTC 7440	I	I	1	+	−	I	Unknown
SW140[a]	II	II	2	+	−	II	Healthy
T128	II	II	2	+	−	I	Healthy
Tako	II	II	2	+	−	II	Pneumonia, pleuritis
Bakos A9	II	II	2	+	−	I	Unknown
CCM 5751	II	II	2	+	−	I	Unknown
SW114[a]	III	III	3	+	−	I	Healthy
T368	III	III	3	+	−	I	Healthy
T678	III	III	3	+	−	I	Healthy
SW124[a]	NT	NT	4	−	+	I	Healthy
6508[a]	NT	NT	4	−	+	I	Unknown
T147	NT	NT	4	−	+	II	Healthy
SW3	NT	NT	4	−	+	II	Pleuritis
Chuetsu	NT	NT	4	−	+	II	Pneumonia
NCTC 4557	NT	NT	4	−	+	II	Unknown
CCM 5747	NT	NT	4	−	+	II	Unknown
NCTC 7441	NT	NT	4	−	+	II	Unknown
CIP 52203	NT	NT	4	−	+	II	Unknown
Nagasaki[a]	NT	NT	5	−	+	II	Septicemia[e]
SW143[a]	NT	NT	5p	−	+	II	Healthy
Bakos B26	NT	NT	5p	−	+	II	Glässer
4800	NT	NT	5p	−	+	II	Glässer
S1690[a]	NT	NT	NT	−	+	II	Septicemia
Morioka[a]	NT	NT	NT	−	+	II	Septicemia
8Z14	NT	NT	NT	−	+	II	Septicemia
NCTC 6359	NT	NT	NT	−	+	II	Unknown
Bakos C5	NT	NT	NT	−	+	I	Unknown
Bakos D74	NT	NT	NT	−	+	I	Unknown

[a] Strains used for the production of antiserum.
[b] *H. parasuis* isolated from the nasal cavities of apparently healthy pigs.
[c] NT, Not typable.
[d] p, Partial identity.
[e] With meningitis, polyserositis, or polyarthritis.

tions (0.5 ml) with Formalin-treated antigens were performed at 2-day intervals, followed 1 week later by four intravenous injections (1.0 ml) of living antigens. One week after the final injection, the rabbits were exsanguinated, and the sera obtained were stored at −20°C.

Slide agglutination test. About 0.03 ml of antigen and undiluted antiserum were mixed on the agglutination plate. The agglutination plate was tilted backwards and forwards slowly for 2 min.

Tube agglutination test. A twofold dilution of antiserum was done with PBS-NaN_3 from 1:10 in 0.2-ml quantities. The same volume of antigen was added to each tube. The incubation was performed under different conditions to compare agglutinating properties as follows: at room temperature overnight, at 37°C for 2 h, or at 50°C for 4 h, followed by overnight storage in a refrigerator.

AGPT. A 1% solution of agarose (Difco Laboratories) in PBS-NaN_3 was melted and then poured (4 ml) onto slide glass (26 by 76 mm). Wells (5 mm in diameter and 3 mm apart from each other) were made by a puncher. The wells were filled with antisera or antigens (about 40 μl) and incubated in a moist chamber at room temperature. The precipitate lines were read after 24 h.

Absorption test. Antisera for the tube agglutination test and AGPT were absorbed as follows. Portions of 0.1 ml of undiluted antiserum were mixed with 0.9 ml of bacterial suspension from a 16-h culture (0.1 g/ml in 0.15 M NaCl). The mixture was kept at room temperature for 2 h with constant slow rotation and then overnight at 4°C. The absorbed antiserum was obtained after centrifugation at 12,000 × *g* for 10 min at 4°C. Undiluted antiserum (0.5 ml) was also absorbed with an equal volume of 121°C extract under the same conditions. For tube agglutination, the absorbed sera were used at an initial dilution of 1:10; for AGPT they were concentrated five times with an Amicon Minicon D15.

Immunoelectrophoresis. Electrophoresis was carried out with 121°C extracts (concentrated twice with the Minicon) in agarose for 60 min at a constant current (2.4 mA/cm) with Veronal buffer, pH 8.6. After completion of the electrophoresis, the trough was filled with antiserum and the slides were kept in the moist chamber at room temperature for 20 h.

Pronase and phenol treatment of antigen extracts. The

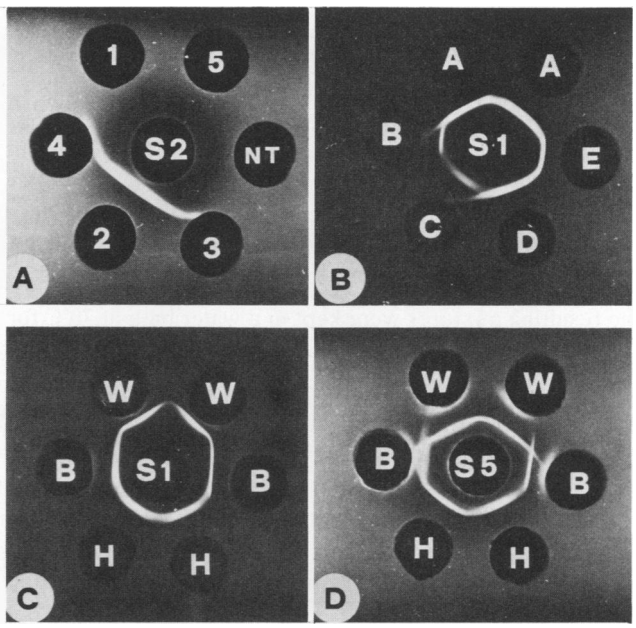

FIG. 1. Agar gel precipitation pattern obtained with 121°C extracts as antigen and its relationship with other extracts. (A) 121°C extracts in the outer wells. Serovar 1 (strain No. 4), serovar 2 (SW140), serovar 3 (SW114), serovar 4 (SW124), untypable (NT; strain S1690), and serovar 5 (Nagasaki). Center well S2, Antiserum to serovar 2 (SW140). (B) Identity of 121°C extracts of strains within the serovar 1 outer wells. Extract A (strain No. 4), B (T143), C (HA66), D (SW35), and E (NCTC 7440). Center well S1, Antiserum to strain No. 4. (C and D) Relationship with the different extracts. W, Washing extract; B, 100°C extract, H, 121°C extract. (C) Extracts of serovar 1 (strain No. 4) in outer wells. S1, Antiserum to serovar 1 (strain No. 4). (D) Extracts of serovar 5 (strain Nagasaki) in outer wells. S5, Antiserum to serovar 5 (Nagasaki).

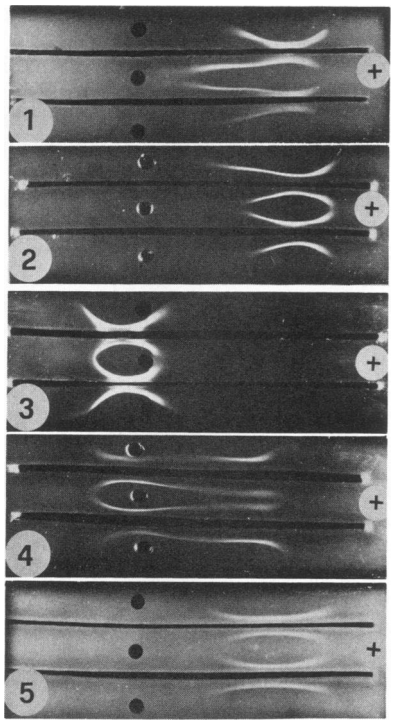

FIG. 2. Immunoelectropherogram of 121°C extracts from different serovars. Panels 1 through 5 correspond to serovars 1 through 5. The extracts in the wells and the antiserum in the troughs in each panel are identified from top to bottom. Panel 1: Wells, strains No. 4, T143, and HA66; troughs, antiserum to strain No. 4. Panel 2: Wells, strains CCM5751, SW140, and Bakos 9; troughs, antiserum to SW140. Panel 3: Wells, strains T678, SW114, and T368; troughs, antiserum to SW114. Panel 4: Wells, strains NCTC 7441, 6508 and CIP 52203; troughs, antiserum to 6508. Panel 5: Wells, strains Nagasaki, B26, and Nagasaki; troughs, antiserum to Nagasaki.

121°C antigens (concentrated twice) and 100°C antigens were mixed with an equal volume of 1% pronase (Boehringer Mannheim) solution and incubated at 37°C for 2 h. Thereafter phenol was added to a concentration of 45% (vol/vol). The resulting mixtures were kept in a water bath at 70°C for 30 min. After centrifugation at 12,000 × *g* for 10 min at 4°C, the aqueous phase was separated from the phenol phase, dialyzed against 0.15 M NaCl for 24 h, and then concentrated twice (Centricon 10) to the original antigen volume. The treated antigens were subjected to immunoelectrophoresis and AGPT.

RESULTS

Agglutination tests. The slide and agglutination tests allowed us to classify 13 strains into three groups, designated groups I, II, and III (Table 1). The remaining strains showed either weak or loose agglutination with nearly all antisera tested.

Apart from some minor cross-reactions which did not interfere with interpretation of the results, the slide agglutination gave clear results. The only exception was strain T143, which we tentatively classified into group I on the basis of its strong reaction.

For the tube agglutination we used untreated bacterial cell suspensions, since heat treatment (100 or 121°C) did not give more consistent results. Different incubation procedures (see Material and Methods) were tested. Incubation at room temperature overnight made it possible to detect specific reactions (disk-shaped agglutination) more accurately, while incubation at high temperatures revealed additional cross-reactions. The specificity of the reactions was confirmed by absorption tests with living cells or the supernatant of autoclaved cells (121°C extract). Titers at a serum dilution of 1:160 remained unchanged after heterologous absorption, whereas no agglutination could be detected after homologous absorption.

AGPT. To classify the strains by specific antigens and capsular substance, the AGPT was carried out with different methods for extracting soluble antigens.

For obtaining dense specific precipitation lines, the supernatant of cells autoclaved at 121°C proved to be the most suitable for all strains when tested against their homologous antisera (Fig. 1A), except for the untypable strains (Morioka and S1690), which showed only a faint reaction. The results allowed a tentative classification into five serovars based on precipitation with the 121°C extract (Table 1).

Serovars 1, 2, and 3 corresponded to agglutination groups I, II, and III. Only six isolates could not be included in this serological scheme. Some strains, particularly within serovars 1 and 5, seemed to be only partially identical with the respective antisera (Fig. 1B). They were included in the corresponding serovar, since other specific antigens could not be detected.

Cross-absorption tests with living antigens and 121°C extracts confirmed the specificity of the reactions. Since the specific 121°C antigen of all strains was not affected by pronase but could be extracted by phenol, this thermostable and soluble substance has the properties of a polysaccharide.

Other extraction procedures revealed various precipitation lines with different specificity. With untreated washing antigens, a specific precipitation line was formed within strains of serovars 1, 2, and 3 and proved to be identical with the 121°C antigen (Fig. 1C). From serovars 4 and 5, precipitation lines were inconsistently observed with homologous and partly heterologous antisera which are not related to the 121°C extract (Fig. 1D) and were destroyed by boiling and autoclaving.

Antigens obtained after heating at 60°C also yielded specific precipitation lines within strains of serovars 1, 2, and 3; but with many common lines. Special notice should be taken of common lines near the antigen well between the strains of serovars 1 and 3. From other strains common precipitation lines were observed currently, but type-specific lines were not detected. Moreover, they were partially eliminated after boiling or completely eliminated by autoclaving.

Antigens extracted after treatment at 100°C also allowed us to detect specific lines, not only for serovars 1, 2, and 3, but also for serovars 4 and 5. These lines were identical with those of the 121°C extracts (Fig. 1D). Other common lines may be distinguished; particularly noteworthy are distinct common precipitation lines near the antigen well between serovars 1 and 3 and between serovars 2, 4, 5, and an untypable strain (S1690). These common lines disappeared after autoclaving (Fig. 1D).

The immunoelectrophoresis with 121°C extracts of different serovars is shown in Fig. 2. A diversity of migration patterns of the specific substance is obvious. Extracts of serovars 1, 2, and 5 migrated towards the anode, those of serovar 3 towards the cathode, and those of serovar 4 seemed to show components on both sides.

Treatment of the 121°C extracts of representative strains of each serovar with pronase and phenol did not affect the specific arcs of precipitation.

DISCUSSION

In our attempt to classify *H. parasuis* strains serologically, we found that the agglutination tests were practicable only for a group of strains which are believed to be capsulated (5). However, the majority of the strains, mostly isolated from pathological material, were not identified by these tests, the more so as many of those strains show a tendency to autoagglutination.

The tube agglutination test performed at different incubation temperatures suggests that the specific substance is located on the surface and solubilized by high temperatures. A similar phenomenon has been reported for the capsular substance of *H. influenzae* (11) and of *H. pleuropneumoniae* (6). The AGPT was found to be more suitable for classification, since 26 of the 32 strains examined could be typed by it (Table 1). Of the different extraction methods tested, the supernatant of autoclaved cells (121°C extract) proved to yield the most reliable results. The supernatant of suspended cells (washing extract) may also show the same specificity relationship, but only in strains of serovars 1, 2, and 3.

Extracts obtained by heating the cells at 60°C and by boiling (100°C extract) were found to be less suitable, since besides the specific antigen, several other common antigens were detected. However, these extracts may reveal, near the antigen well, some important common antigens which are of help in the typing procedure. Since none of these common antigens were detected after treatment with pronase and phenol, their composition is probably proteinaceous.

Comparing the different extracts (washing, 100°C, and 121°C), full identity was found not only among the specific precipitinogens of serovars 1, 2, and 3 (Fig. 1C) but also, by means of the cross-absorption test, with the antigens reponsible for specific agglutination. This suggests that the specificity is given from the capsular substance. On the other hand, the washing antigen, if present in strains of serovars 4 and 5, was not identical with the specific antigen obtained from the 121°C extract (Fig. 1D). It may be assumed that the specificity of these probably uncapsulated strains is given by the cell envelope. The most constant specific antigen was obtained from the 121°C extract. Since this antigen was not affected by pronase and phenol treatment, it also seems to be a polysaccharide.

The diverse chemical nature of the specific antigens was further demonstrated by their migration patterns in immunoelectrophoresis (Fig. 2). These observations are in accordance with the property of the capsular substance of some strains to precipitate with hexadecyl trimethylammonium bromide (Cetavlon) (5).

According to a previous morphological study (5) and as shown in Table 1, all strains now classified as serovar 1, 2, or 3 were found to be capsulated. However, the strains of serovar 3 formed a group of capsulated strains which did not react to Cetavlon. Furthermore, all strains of serovars 4 and 5 as well as untypable strains without demonstrable capsule had the ability to agglutinate in acriflavine.

Due to the limited number of strains tested, the correlation between the PAGE pattern (7) and the serovars is not obvious, although PAGE type I was shown in all the strains of serovar 3 and PAGE type II in all the strains of serovar 5. Moreover, both types were present in serovars 1, 2, and 4 and in untyped strains (Table 1). On the other hand, PAGE type II was found to be associated with isolates from pathologic lesions, confirming the correlation between such isolates and pathologic conditions (8). Strains that cause Glässer's syndrome (including septicemia and meningitis) were found in serovar 1 and mainly in serovar 5 and nontypable strains, while strains that cause pneumonia or pleuritis belonged to serovars 2 and 4. In view of the fact that the number of strains tested was small, these results should be the subject of further investigations. Still, our findings indicate that pathogenic strains may be found in all the serovars (except serovar 3), provided that they belong to PAGE type II.

The present proposal of a serological classification for *H. parasuis* offers a more comprehensive analysis of the antigenic structure and a simple technique for performance. Two strains of Bakos and co-worker (1), classified in his serogroups A and B, belonged to serovar 2 and 5, respectively. The two remaining strains (C and D) as well as other untypable strains cannot be integrated until related field strains have been isolated. This group of untypable strains indicates that the species *H. parasuis* probably consists of more than five serovars.

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