THE AGGLUTINATION REACTIONS OF *HAEMOPHILUS PARAPHRAPHILUS* AND *H. PARAPHROHAEMOLYTICUS*, AND SOME OBSERVATIONS ON THE AGGLUTINATION OF *H. APHROPHILUS* AND *H. HAEMOGLOBINOPHILUS* (*H. CANIS*)

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HITHERTO the agglutination reactions of only a few species of the genus *Haemophilus* have been investigated. Non-capsulate *H. influenzae* strains were found to be heterogeneous (Park, Williams and Cooper, 1918; Valentine and Cooper, 1919; Kristensen, 1922; Allison, Gordon and Zinnemann, 1943), but different polysaccharide capsular antigens were demonstrated by Pittman (1931) and six serological types are recognised on this basis. Khairat (1940) noted that the species *H. aphrophilus* was serologically distinct from the X-dependent, V-independent species *H. haemoglobinophilus* and *H. influenzae-murium*; he could not determine whether his new species *H. aphrophilus* was homogeneous or not, because all his strains had been isolated from one subject. Alexander (1948) reported that *H. parainfluenzae* and *H. suis* could be divided into capsular and non-capsular types; she stated that five capsulate *H. suis* strains were immunologically identical and were not related to *H. influenzae* and that there appeared to be more than one capsular type of *H. parainfluenzae*. The present study was primarily undertaken to define the antigenic relationships of *H. paraphrophilus* and *H. paraphrohaemolyticus* within the species, and to other members of the genus.

METHODS

Strains. Strains used for the preparation of antisera were: *H. influenzae* (non-capsulate): no. NCTC8405; *H. haemolyticus*: no. NCTC10659; *H. parainfluenzae*: Barts and MB (laboratory strains); *H. paraphrophilus*: Funagli (no. NCTC10557) and Poole (laboratory strain); *H. paraphrohaemolyticus*: Corinthian (no. NCTC10672) and Lawton (no. NCTC10671); *H. haemoglobinophilus* (*H. canis*): no. NCTC8540; *H. aphrophilus*: Khairat (no. NCTC5906).

Suspensions of the following strains were prepared and used as antigens in agglutination tests; strains without NCTC numbers are our laboratory strains: non-capsulate *H. influenzae*: no. NCTC8405; *H. haemolyticus*: strain Turk (no. NCTC10659); *H. parainfluenzae*: strains MB, Barts and Roberts; *H. parahaemolyticus*: strains Meredith and H11; *H. paraphrophilus*: strains Funagli (no. NCTC10557), Poole, Jordan, Hayward (no. NCTC10558), Reece (no. NCTC10556), Plummer, Turner, and five strains from Atlanta, Georgia, USA; *H. paraphrohaemolyticus*: strains Corinthian (no. NCTC10672), Lawton (no. NCTC10671), Blower, Richards, 121, 119, 129, 154, 109, P1, P2, 24, 56 and 402; *H. haemoglobinophilus*: no. NCTC8540, and strains RV (no. NCTC10169), MG and 066; *H. aphrophilus*: strain Khairat (no. NCTC5906), no. NCTC5908, and strains Oxford and Cassidy.

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Raising of antisera. During week 1, New Zealand White rabbits weighing 5–6 kg received three intramuscular injections of 0.5 ml of 0.5% phenolised suspension of the test organism adjusted to an optical density standard of 2.5×10^8 *H. influenzae* organisms per ml. The organisms were harvested from an overnight growth on heated horse-blood agar incubated in air, with added 5–10% CO₂ if indicated by growth requirements. The only exception to this was during the preparation of antiserum Funagli-“air”, when the plates were incubated in air. During weeks 2–4, living suspensions of the same standard density were injected intravenously thrice weekly in amounts from 0.2 ml increasing by 0.1 ml to a maximum of 1.0 ml. Test bleedings were taken from the marginal ear vein at the end of the first month and, if necessary, the injections were continued until the titre of the serum reached 1280. The sera were preserved in 30-ml screw-capped bottles by the addition of 0.5% phenol or 0.01% Mersalyl, and stored at 4°C. All sera agglutinated to a titre of 1280 or even 2560 with the homologous strains except the antiserum to *H. arophilus* strain Khairat, the titre of which could not be increased beyond 640, and the antiserum to *H. parainfluenzae* strain MB, the titre of which remained at 80 despite a very prolonged course of injections.

Two separate antisera were raised to *H. paraphrophilus* strain Funagli because of the markedly differing colonial and microscopic appearance when this strain was incubated in air, or in air with added CO₂ (Zinnemann *et al.*, 1968).

Antigenic suspensions for agglutination tests were prepared as follows: Strains were subcultured on to heated blood agar containing 7% oxalated horse blood (Oxoid) and incubated overnight at 37°C in air, and CO₂ if so indicated by growth requirements. Single colonies were then inoculated into 5–6 ml Levinthal's broth (1918) which was incubated overnight at 37°C, again in air and CO₂ if so indicated. This liquid culture was used as the antigen suspension.

Haemophilus antigens free from horse-blood components were prepared by overnight growth in proteose peptone water with added growth factors (PPXV). This medium contained 2% Proteose Peptone (Difco) and 0.6% NaCl (Analar) together with 1 µg per ml. each of X-factor (haemin, equine type III, Sigma) and V-factor (DPN; NAD, Sigma) in sterile distilled water.

Most of the agglutination tests were done immediately after incubation, but results were not influenced if the overnight cultures were held at 4°C for up to a week.

Agglutination tests. The technique of Allison *et al.* (1943) was followed. Agglutination tubes were incubated at 37°C for 3–4 h and then stored overnight at 4°C. On the next day the tubes were allowed to reach room temperature before being shaken gently and read with a hand lens in oblique, transmitted light. Readings were checked again 1 h later. The highest dilution at which agglutination occurred was taken as the final titre. Results were recorded as positive (+) or negative (–). Agglutination reactions produced by our sera at dilutions of 1280 or 2560 were recorded as positive, and at 80 to 640 as moderately positive; reactions at dilutions of 40 or less were assigned to the negative category. All agglutination tests were repeated at least once. In case of a discrepancy of one dilution step between two tests, the lower figure was taken, but the test was repeated in duplicate if the difference was greater.

Bile-salt extracts and ultrasonically disintegrated cells were used as antigens (Omeland, 1964; May, 1968). Because the strains used to prepare these antigens were grown on heated horse-blood agar the presence of non-specific lines due to medium components in the antisera and the antigens were excluded as far as possible by using a “control” well filled with Levinthal broth.

RESULTS

Agglutination of Haemophilus paraphrophilus

Eight strains of *H. paraphrophilus* were agglutinated by the two antisera raised to the species with strains Funagli and Poole to titres of 640, 1280 or

TABLE I

Results of agglutination tests with 13 strains of Haemophilus paraphrophilus and two different antisera

Number or designation of strain of <i>H. paraphrophilus</i> used as antigen	Agglutination titre obtained with the stated antigen in tests with antiserum to	
	strain Funagli	strain Poole
Strains from England:		
Funagli	1280	2560
Poole	2560	2560
Jordan	2560	1280
Reece	2560	1280
Plummer	640	1280
Hayward	640	640
Turner	640	640
Fletcher	640	640
Strains from the USA:		
A8064	1280	ND
7776	640	ND
2937	1280	ND
2587	2560	ND
7432	640	ND

ND = not done.

2560. In addition, five strains from USA which were tested only with anti-serum Funagli agglutinated to similar titres (table I). Comparison of the two antisera, Funagli "air" and Funagli "CO₂" showed that they agglutinated preparations of three related strains and of the homologous strain at titres of 640–2560 (table II).

Agglutination of Haemophilus paraphrohaemolyticus

Two antisera were prepared to *H. paraphrohaemolyticus*, because the strains Corinthian and Lawton differed in their haemolytic ability when grown in air (Zinnemann *et al.*, 1971). After incubation for 24 h, strain Corinthian grew

TABLE II

Results of agglutination tests of Haemophilus paraphrophilus strains with antisera Funagli-"air" and Funagli-"CO₂"

Designation of strain used as antigen	Agglutination titre with antiserum to strain	
	Funagli-"air"	Funagli-"CO ₂ "
Funagli-"air"	2560	640
Funagli-"CO ₂ "	2560	1280
Poole	1280	2560
Hayward	2560	2560
Reece	2560	2560

as very small haemolytic colonies on fresh blood plates whilst strain Lawton produced only faint haemolysis without visible colonies. Both antisera reacted at a titre of 2560 with the homologous strain, but there was no cross-agglutination between these two strains of this species. Thus they belong to two different serotypes. Subsequently, all strains of *H. paraphrohaemolyticus* that became available were tested with each of the antisera prepared against strains Corinthian and Lawton.

Four more strains agglutinated with antiserum Corinthian at a high titre; one other strain reacted with antiserum Lawton at high titre, and seven strains reacted with either antiserum at low titre. It appears, therefore, that there are at least three serotypes of *H. paraphrohaemolyticus* (see table III).

Studies with H. haemoglobinophilus

Antiserum raised against *H. haemoglobinophilus* strain no. NCTC8540 agglutinated the four available strains of *H. haemoglobinophilus* to titres of 640 or more (table IV).

Studies with H. aphrophilus

Antiserum raised against *H. aphrophilus* strain no. NCTC5906 agglutinated the homologous antigen at a titre of 640 and *H. aphrophilus* strain no. NCTC5908 at a titre of 1280, but the serum agglutinated strains Cassidy and Oxford at low titres of 20 and 10 respectively (table V).

Floccular cross-agglutinations giving titres of 160 to 640 occurred with antisera to *H. aphrophilus* strain Khairat and to *H. haemoglobinophilus* strain no. NCTC8540 when tested against *H. paraphrophilus* strains Funagli, Reece

TABLE III
Results of agglutination tests with 14 Haemophilus paraphrohaemolyticus strains and two different antisera

Number or designation of strain used as antigen	Agglutination titre with antiserum to strain	
	Corinthian	Lawton
Corinthian	2560	10
121	2560	40
119	1280	10
129	2560	20
154	640	20
Lawton	10	2560
Blower	20	2560
109	20	80
P1	20	10
P2	20	10
24	40	20
56	20	40
402	20	20
Richards	20	10

and Hayward as antigens on the one hand, and antiserum to *H. paraphrohaemolyticus* strain Corinthian and some strains of *H. parainfluenzae* and *H. parahaemolyticus* as antigens on the other. It was thought that these cross-agglutinations might have been due to the presence of a non-specific precipitin in the antisera resulting from the use of medium containing a horse-blood component for culturing cells used to raise the antiserum. It seemed that by growing the antigens destined for agglutination tests in a liquid medium free from blood components (PPXV), non-specific precipitation might be avoided. In tests with such antigens of *H. paraphrophilus*, the antisera against *H. aphrophilus* strain Khairat and *H. haemoglobinophilus* strain no. NCTC8540 gave titres of 40 or less. However, no such drop in titre was obtained with antiserum *H. paraphrohaemolyticus* Corinthian tested with antigen preparations of three strains of *H. parainfluenzae* and two strains of *H. parahaemolyticus* that were free from horse-blood components. No other cross-reactions occurred with the sera of either *H. paraphrophilus* or *H. paraphrohaemolyticus* to any other of the few tested representatives of *Haemophilus* species.

Agar-gel-diffusion studies

In these tests with *H. paraphrophilus* and *H. paraphrohaemolyticus*, reactions corresponded to the agglutination results. Two lines of precipitation that appeared to be respectively genus- and species-specific were observed with all strains of *H. paraphrophilus*, and three that appeared to be respectively

TABLE IV

Results of agglutination tests with strains of Haemophilus haemoglobinophilus

Number or designation of strain used as antigen	Agglutination titre with antiserum to strain no. NCTC8540
NCTC8540	2560
RV	640
MG	2560
066	2560

TABLE V

Results of agglutination tests with strains of Haemophilus aphrophilus

Number or designation of strain used as antigen	Agglutination titre with antiserum to strain Khairat
Khairat	640
NCTC5908	1280
Cassidy	20
Oxford	10

genus-, species- and strain-specific with *H. paraphrohaemolyticus*, were obtained in all tests. The three strains of *H. paraphrophilus* showed only slight differences in place or outline of lines in agar-gel-diffusion tests against heterogeneous species. However the three serological types of *H. paraphrohaemolyticus* strains evident in agglutination reactions could not be distinguished by the agar-gel-diffusion method. It was noted that V-dependent (X-independent) species showed some common antigens, and also that haemolysins might be responsible for one or more additional precipitation lines.

DISCUSSION

The results of this investigation suggest that the species *H. paraphrophilus* is homogeneous, because agglutinations to high titre (640 or above) were obtained when eight strains were tested against the two antisera raised to this species, and when five additional strains were tested against one of the two antisera. The strains investigated were obtained from three different geographical areas—Leeds and Birmingham, England; and Atlanta, Georgia, USA—and their similar behaviour supports the view that the agglutinin of *H. paraphrophilus* is species-specific. Two of the strains received from Birmingham (Turner and Hayward) differed colonially from the holotype, strain Funagli, in that they produced sticky, cohesive growth on heated horse-blood agar. Despite this apparent colonial difference, these strains produced smooth turbid growth in overnight suspensions of Levinthal broth, and titres of 2560 were obtained with both antisera.

H. paraphrohaemolyticus strains Corinthian and Lawton were selected for the raising of antisera because of their different haemolytic ability and growth characteristics when grown in air on fresh blood agar. Subsequent agglutination tests showed that the two strains were also different antigenically.

At least three different serotypes of *H. paraphrohaemolyticus* have emerged from the results of agglutination tests with 14 strains. Four strains belonged to the same serological type as strain Corinthian, one to the same serotype as strain Lawton, and nine other isolates did not agglutinate significantly with antisera prepared against either strain Corinthian or Lawton. The serotypes Corinthian and Lawton were quite distinct because they agglutinated with one or other of the antisera at high titre (640 or greater), but never with both antisera. It appears, therefore, that *H. paraphrohaemolyticus* strains are not uniform serologically. The five non-agglutinating strains may well belong to one or more additional serotypes.

H. paraphrophilus strain Funagli was used to determine whether two different colonial forms of the same strain would raise different agglutinins. Each of the Funagli "air" and "CO₂" sera agglutinated both variants, and in addition, three other strains of the species *H. paraphrophilus* to titres of 640 or higher.

As far as these tests go, it appears that the species *H. haemoglobinophilus* is homogeneous and that the species *H. aphrophilus* is not.

When we prepared the antisera we were unaware of a possible antigenic effect of a component of horse-blood medium. Later, Branefors-Helander

(1972) drew attention to the antigenicity of contaminating substances of high molecular weight derived from horse blood in the medium. The appearance of such a non-specific component seems to be erratic since its presence could be shown only in antisera to *H. aphrophilus* strain Khairat and *H. haemoglobinophilus* strain no. NCTC8540. We therefore concluded that the cross-agglutinations with these two antisera were probably due to this component. Antisera against all the other *Haemophilus* species under investigation appeared to be free of this non-specific anti-horse-blood component and therefore the cross-agglutinations of antiserum *H. paraphrohaemolyticus* strain Corinthian with antigens derived from two other V-dependent species, *H. parainfluenzae* and *H. paraphrohaemolyticus*, were probably due to a shared minor antigen.

SUMMARY

Agglutination tests were used to study the surface antigens of two recently described species of the genus *Haemophilus*, *H. paraphrophilus* and *H. paraphrohaemolyticus*, and their antigenic relationship to other members of the genus. The results obtained with a few strains of *H. haemoglobinophilus* and *H. aphrophilus* are also reported. The species *H. paraphrophilus* appears to be homogeneous; no major cross-reactions were observed. The species *H. paraphrohaemolyticus* contains at least three serotypes, of which two have been defined in terms of agglutination reactions. Cross-agglutinations occurred between one strain of *H. paraphrohaemolyticus* and strains of the other V-dependent species, *H. parainfluenzae* and *H. parahaemolyticus*. Of the X-dependent species, *H. haemoglobinophilus* seems to be homogeneous, and the species *H. aphrophilus* is not.

A non-specific antibody against horse blood in the medium occurred erratically and was present in only two antisera, those raised to *H. aphrophilus* strain Khairat and *H. haemoglobinophilus* strain no. NCTC8540.

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