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Designation of 15 Serovars of *Haemophilus parasuis* on the Basis of Immunodiffusion Using Heat-Stable Antigen Extracts†

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Previous independent investigations of the serotyping of *Haemophilus parasuis* strains have led to the designation of serovars A to D, 1 to 7, Jena 6 to Jena 12, and ND1 to ND5. Heat-stable antigen preparations from the reference strains for these serovars were tested by immunodiffusion with rabbit hyperimmune antisera. The existence of 15 distinct serologic groups was apparent, for which we propose the designations serovars 1 to 15. Examination of 290 field isolates from swine in the former German Democratic Republic indicated a prevalence of serovars 4 and 5, which together accounted for 41% of the isolates examined. However, 26.2% of the isolates were nontypeable with this test procedure and available antisera. Intraperitoneal inoculation of specific-pathogen-free pigs with cells representing the 15 serovars indicated differences in virulence which may be serovar related. Cells of strains representing serovars 1, 5, 10, 12, 13, and 14 were the most virulent, causing death or moribundity in inoculated pigs. Cells of serovars 2, 4, 8, and 15 caused polyserositis, but not death, in inoculated pigs. However, inoculation of cells of strains representing serovars 3, 6, 7, 9, and 11 resulted in no clinical symptoms or lesions indicative of *H. parasuis* infection.

Haemophilus parasuis is the etiologic agent of porcine polyserositis and arthritis (Glässer's disease), which has historically been considered a sporadic, stress-associated disease of young pigs (17). In recent years, however, trends in swine production have resulted in large populations of swine maintained as isolated high health status or specific-pathogen-free (SPF) herds. Introduction of *H. parasuis* into such herds, if they represent a nonimmune population, may have a devastating effect: infection may spread as a contagious disease of high morbidity, affecting pigs of all ages without obvious associated stress factors (1, 20, 22). Recent outbreaks in Europe (5, 10) and North America (11, 12, 29) attest to the increasing importance of *H. parasuis* as a pathogen of economic significance in swine.

Considerable antigenic heterogeneity among *H. parasuis* isolates has been demonstrated by serotyping. In 1955, Bakos (2) described serovars A to D on the basis of examination of 120 isolates using a precipitation test. Schimmel et al. (27) serotyped 115 isolates from the former German Democratic Republic by using an agglutination test and defined three additional serovars. By using immunodiffusion with heat-stable antigen extracts, Morozumi and Nicolet (16) and Nicolet et al. (19) examined isolates from Japan and Switzerland and defined seven serovars, designated 1 to 7. Recently, Kielstein (4) and Kielstein et al. (8) examined 158 isolates from the former German Democratic Republic and reported the existence of seven new serovars provisionally designated Jena 6 to Jena 12. On the basis of examination of 243 isolates, primarily from North America, Rapp-Gabrielson and Gabrielson (24) reported the existence of five new serovars, which were designated ND1 to ND5.

The purpose of this investigation was to clarify the present knowledge of *H. parasuis* serologic diversity by examining the relationships among recently recognized serovars. A uniform scheme for designation of 15 *H. parasuis* serovars and reference strains is proposed. The prevalence of serovars among isolates from the former German Democratic Republic is also reported. Inasmuch as data from numerous investigations have indicated a possible association of the serovar of a strain with its pathogenic potential (2, 4, 8, 16, 21, 25, 26), we included a comparison of the host animal virulence of strains representing the 15 serovars.

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MATERIALS AND METHODS

Bacterial strains and field isolates. Reference strains for *H. parasuis* serovars A to D and 1 to 7 were provided by J. Nicolet, Bern, Switzerland. Strains representing serovars Jena 6 to Jena 12 and ND1 to ND5 were as previously reported (4, 8, 24). The 290 field isolates used were obtained from pigs from 20 farms in the former German Democratic Republic during a 5-year period. Duplicate isolates from the same farm were not eliminated from the study. Isolates were obtained from systemic organs of pigs with polyserositis, from lungs of pigs with pneumonia and/or pleuritis, and from pigs without obvious gross lesions. Field isolates were lyophilized at the second in vitro passage level. Results of preliminary serotyping of some of the field isolates have been previously reported (4). Growth, storage, and biochemical identification of all isolates were done as previously described (5, 24).

Serotyping procedures. Production of hyperimmune antisera in rabbits has already been described (4, 24). Briefly, rabbits were initially inoculated twice, subcutaneously, with formalin-inactivated cells in Freund's complete or incomplete adjuvant (Difco Laboratories, Detroit, Mich.) and then given a series of intravenous inoculations with inactivated

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TABLE 1. Proposed reference strains for 15 serovars of *H. parasuis*

Serovar	Reference strain	Country or origin	Diagnosis/isolation site	Previous serovar designation(s)	Reference(s)
1	No. 4	Japan	Healthy/nose	1	16
2	SW140	Japan	Healthy/nose	2, A	16, 2
3	SW114	Japan	Healthy/nose	3	16
4	SW124	Japan	Healthy/nose	4	16
5	Nagasaki	Japan	Septicemia/meninges	5, B	16, 2
6	131	Switzerland	Healthy/nose	6	3, 19
7	174	Switzerland	Healthy/nose	7	3, 19
8	C5	Sweden	Unknown	C	2
9	D74	Sweden	Unknown	D, Jena 12	2, 4
10	H555	Germany	Healthy/nose	Jena 10	4
11	H465	Germany	Pneumonia/trachea	Jena 11, ND2	4, 24
12	H425	Germany	Polyserositis/lung	Jena 6, ND5	4, 24
13	84-17975	United States	Unknown/lung	ND4	24
14	84-22113	United States	Unknown/joint	ND3	24
15	84-15995	United States	Pneumonia/lung	ND1	24

and/or live cells. Isolates were serotyped by using the agar gel precipitation test originally described by Morozumi and Nicolet (16). The serotyping procedures used, including production of heat-stable antigen extracts by heating at 121°C for 2 h, have been described in detail elsewhere (4, 16, 24).

Virulence evaluation. The procedures used to evaluate the virulence of strains representing the 15 serovars, as well as the results of inoculation of pigs with some of the strains, have been described elsewhere (4, 8, 26). Briefly, primary SPF pigs, weighing approximately 6 kg, were inoculated intraperitoneally with an 18-h broth culture containing 5×10^8 CFU per dose. Clinical symptoms were monitored daily for 4 days postinoculation. Pigs were necropsied as soon as possible after death or moribundity, and gross lesions were recorded. Surviving pigs were euthanized and necropsied 4 days following inoculation. Criteria used to score clinical symptoms and gross lesions and estimate virulence were as previously described (4). For some serovars, multiple strains were tested for virulence. Except for serovars 9 and 11, of which only field isolates were examined, the reference strains for the serovars were included in the evaluation.

RESULTS AND DISCUSSION

Serotyping. Fifteen distinct serovars of *H. parasuis* can be distinguished on the basis of immunodiffusion using heat-stable antigens. As indicated in Table 1, we propose a scheme which recognizes previously proposed serovars 1 to 7 (3, 16, 19) and designates previously proposed serovars C and D (2) as serovars 8 and 9, respectively. Serovars 10 to 15 are represented by serovars which have recently been described in independent investigations in our laboratories (4, 8, 24).

Previous investigations have demonstrated the partial serological identity of serovars 2 and A and of serovars 5 and B (7, 16, 24). Testing of antisera and strains representing provisional serovars Jena 6 to Jena 12 and ND1 to ND5 was conducted independently by our laboratories. Cross-testing of antisera and antigen preparations indicated that serovar ND2 (strain 84-13676) was serologically indistinguishable from serovar Jena 11 (strain H465). Similarly, serovars Jena 6 and ND5 (strains H425 and MVP 3218, respectively) were serologically indistinguishable. Serovars 10 (strain H555), ND4 (strain 84-17975), ND3 (strain 84-22113), and ND1 (strain 84-15995) were serologically distinct from the other proposed reference strains. Serovar Jena 12 was originally proposed to be represented by strain H553 (4); however, subsequent testing with antisera and antigen preparations from strain D74, as well as field isolates representing serovars D and Jena 12, indicated serologic cross-reactions among these strains. The reactions observed were of partial identity, similar to those previously reported for serovar 7 strains (24).

On the basis of biochemical activity profiles, the indole-positive "*H. parasuis*-like" strains representing previously proposed serovars Jena 7, Jena 8, and Jena 9 (4) should be assigned to the recently proposed taxon F (14), a taxon distinct from *H. parasuis*.

Our designation of reference strains is based on the strains used to produce antisera in the initial reports (2, 4, 16, 19, 24). However, difficulty in producing serovar-specific antisera to some of the strains has been noted, particularly for strains 174 and D74, representing serovars 7 and 9, respectively (6, 24). This may reflect lack of expression of specific antigens, perhaps because of in vitro storage. Data also indicated the existence of antigenic heterogeneity among strains within serological groups, particularly serovars 7 (24) and, as indicated in this study, 9.

Recently, a lack of correlation between a tube agglutination procedure and the immunodiffusion test for serotyping of *H. parasuis* has been reported (7). Because of spontaneous agglutination of a high percentage of isolates (7, 27) and lack of specificity of antigen preparations made from washed cells or by heating at less than 121°C (16), immunodiffusion using heat-stable antigen extracts appears to be the test of choice for serotyping of *H. parasuis*. The thermostable, soluble antigen(s) appears to be a polysaccharide associated with the cell capsule or outer membrane (8, 16, 26).

Prevalence of serovars. As indicated in Fig. 1, examination of 290 isolates from the former German Democratic Republic indicated a high prevalence of serovars 5 and 4, representing 23.8 and 17.2% of the isolates, respectively. These frequencies are similar to the values reported for isolates from the United States and Canada, where serovars 5 and 4 represented 24.3 and 16.1%, respectively, of the isolates examined (24). Thus, data from both North America and Europe indicate serovars 4 and 5 as important isolates from pathologic material. The remaining serovars were detected at low frequencies, ranging from 0 to 5.5%. More than one-fourth of the isolates examined were nontypeable with available anti-

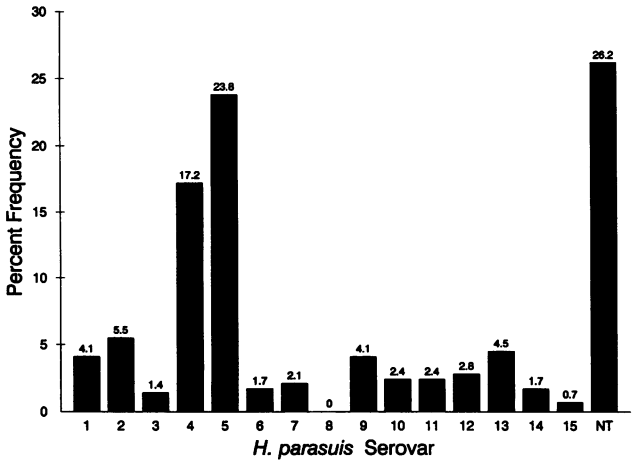

FIG. 1. Prevalence of serovars among 290 *H. parasuis* isolates from the former German Democratic Republic. NT, nontypeable.

sera, indicating the possible existence of additional serovars or the possibility that some strains have lost or do not consistently express a type-specific antigen(s). Examination of seven isolates from a single farm indicated the presence of multiple serovars. These seven isolates, originating from nasal secretions, lungs, or brain tissue specimens, represented four different serovars and three nontypeable strains. These data are in concordance with previous reports of the existence of multiple strains or serovars on a farm (4, 8, 24, 28).

Virulence in SPF pigs. As summarized in Table 2, inoculation of SPF pigs with cells from strains representing serovars 1, 5, 10, 12, 13, and 14 caused death or moribundity within the 4-day observation period. Inoculation of cells of serovar 2, 4, and 15 strains caused polyserositis, but not death, within the 4-day observation period, while inoculation with serovar 8 cells resulted in only mild symptoms and lesions. Cells of the remaining serovars (3, 6, 7, 9, and 11) produced no clinical symptoms or gross lesions in inoculated pigs. In general, results were similar when multiple strains representing a single serovar were tested. Strains Bakos A9 (serovar 2), SW 124 (serovar 4), and Nagasaki (serovar 5) were slightly less virulent than field isolates representing these respective serovars. It has been demonstrated that capsule expression may be influenced by in vitro and in vivo passage of *H. parasuis* (25). Extended in vitro storage or passage of the reference strains studied (strain Bakos A9 was originally isolated 40 years ago), but not the field isolates, may contribute to the apparent differences in virulence.

These data are in agreement with previous reports indicating differences in pathogenicity among strains which were not serologically characterized (9, 13) and among strains representing different serovars (4, 8, 21, 25). Previous reports have also demonstrated an association of phenotypic characteristics, such as encapsulation (4, 5, 15), whole-cell protein profile (3, 4, 16, 18, 26), and outer membrane protein profile (23), with isolation from systemic tissue sites in pigs. The reference and field strains examined differed not only in serotype specificity but also in these other phenotypic characteristics (4, 23, 26). Thus, it is possible that phenotypic differences, in addition to serovar differences, were associated with the apparent differences in pathogenicity which were observed following inoculation of these strains into SPF pigs.

TABLE 2. Virulence of strains representing *H. parasuis* serovars 1 to 15 in primary SPF swine

Serovar	No. of strains tested		No. of pigs inoculated[a]	Virulence[b]
	Reference	Field		
1	1	1	5	++
2	1	5	15	+[c]
3	1	1	6	0
4	1	2	7	+[d]
5	1	1	18	++[e]
6	1	0	3	0
7	1	0	3	0
8	1	0	3	±
9	0	2	6	0
10	1	1	6	++
11	0	2	6	0
12	1	1	6	++
13	1	0	3	++
14	1	0	3	++
15	1	0	3	+

[a] Pigs were inoculated intraperitoneally with an 18-h broth culture containing approximately 5×10^8 CFU.

[b] Virulence was scored as follows: ++, death of pigs within 96 h postinoculation; +, clinical symptoms and systemic gross lesions of polyserositis and arthritis at necropsy; ±, mild clinical symptoms or gross lesions at necropsy; 0, no clinical symptoms or gross lesions at necropsy.

[c] Five of the six strains tested were virulent, and one (Bakos strain A9) was nonvirulent.

[d] Reference strain SW124 was mildly virulent (±).

[e] Reference strain Nagasaki was moderately virulent (+).

The immune status of the host is a determinant in the pathogenic potential of *H. parasuis* infection (22). However, the data generated in this investigation corroborate those of previous reports indicating an association of phenotypic characteristics with virulence differences among strains. It is possible that strains with the ability to cause systemic disease in pigs represent a subpopulation, which can be distinguished by expression of specific phenotypic traits, of the strains which colonize the upper respiratory tract.

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