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Cross-Reactivity and Antigenic Heterogeneity among *Actinobacillus pleuropneumoniae* Strains of Serotypes 4 and 7

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Actinobacillus pleuropneumoniae **strains of serotypes 4 and 7 were studied for their antigenic properties by means of agglutination, coagglutination, indirect hemagglutination, immunodiffusion, and counterimmunoelectrophoresis tests. Strains of serotype 4 showed cross-reactivity with those of serotype 7 in various serological tests. Serotype 7 strains were antigenically heterogeneous and shared common antigens with several other serotypes. By using boiled whole-cell saline extract as the antigen in the immunodiffusion test, serotype 7 strains could be divided into four subgroups. Subgroup I strains did not have antigens in common with other serotypes, whereas subgroup II strains had antigens in common with serotype 4; subgroup III strains had antigens in common with serotype 10, and subgroup IV had antigens in common with serotypes 1, 9, and 11. The indirect hemagglutination test using unheated whole-cell saline extract as the antigen detected serotype-specific activity. Quantification of serotype-specific and group-specific antigens by coagglutination and immunodiffusion tests was found useful for identifying strains that belonged to serotype 4 or 7.**

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is one of the major problems in the modern swine industry all over the world. The organism may cause an acute respiratory infection, resulting in high morbidity and mortality, or a chronic persistent infection, resulting in severe economic losses due to loss in body weight of the pigs (21). Strains of *A. pleuropneumoniae* possess several antigens, some of which are shared by different serotypes. To date, 12 serotypes of *A. pleuropneumoniae* have been reported (15). Serological diagnosis and vaccination programs have been used in an attempt to control the disease. Both of these programs require an extensive knowledge of the different serotypes which exist in a particular region (16). More than 3,000 strains of *A. pleuropneumoniae* isolated from lung tissues of pigs that died because of acute pleuropneumonia or isolated from tonsils or nasal cavities of pigs that originated from chronically infected herds have been serotyped in our laboratory during the last 10 years. Strains isolated from acute infections were predominantly serotypes 1 and 5, with an average percentage of 68 and 23, respectively. Strains of serotypes 3, 6, 7, 8, 10, and 12 together accounted for about 9%. However, 16% of the strains isolated from the chronically infected herds belonged to serotype 7. To date, serotype 4 strains have not been isolated in Quebec. It is, therefore, important to keep an eye on the emergence of serotype 4 isolates in this region. Tests currently being used for serotyping are tube agglutination (3), slide agglutination (7), immunodiffusion (ID) (13), ring precipitation (7), indirect hemagglutination (IHA) (8), immunofluorescence (20), coagglutination (COA) (9), counterimmunoelectrophoresis (CIE) (18), and slide precipitation (4). Serotyping of *A. pleuropneumoniae* is based on identification and characterization of serotype-specific antigens. COA and ID tests are routinely used in our laboratory for serotyping *A. pleuropneumoniae* strains. Strong cross-reactions between strains of serotypes 4 and 7 as well as between strains of serotype 7 and those of other serotypes may cause a great deal of problems in both serotyping and serodiagnosis. It is not uncommon to isolate serotype 7 strains from pigs with clinical cases of porcine pleuropneumonia in Quebec; however, most of the clinical outbreaks in this region are caused by strains belonging to serotypes 1 and 5. Precise identification of serotype 7 thus becomes extremely important, especially in view of the recently observed cross-reactivity between some strains of serotypes 1 and 7 in ID tests. The ID test is used all over the world as a confirmatory test for serotyping *A. pleuropneumoniae* isolates (14). The objective in the present study was to evaluate different serological tests to identify *A. pleuropneumoniae* strains of serotypes 4 and 7 more precisely.

MATERIALS AND METHODS

A. pleuropneumoniae **strains.** The reference strains representing *A. pleuropneumoniae* serotypes 1 through 12 were S4074, S4226, S1421, M62, K17, Femo, WF83, 405, CVJ13261, D13039, 56153, and 8329. A total of 120 isolates of serotype 7, all of which originated from pulmonary tissues of pigs that had died of acute pleuropneumonia, were received from different diagnostic laboratories (104 strains from Quebec, Canada, 10 from the United States, 2 from Mexico, and 4 from Australia). Twenty-two serotype 7 strains were also isolated from the tonsils or nasal cavities of chronically infected pigs. Six strains of serotype 4 (one from Denmark and five from Mexico) were also included in the present study. The strains were identified as *A. pleuropneumoniae* if they were gram-negative, V-factor dependent but X-factor independent, and positive for CAMP and urease reactions (1). ID and IHA tests were used to classify the 12 reference serotypes.

Preparation of hyperimmune sera in rabbits. Hyperimmune sera were prepared in rabbits by repeated inoculations of formalinized whole-cell suspensions of the reference strains of *A. pleuropneumoniae* of serotypes 1 through 12. The details of the procedure have been given earlier (7).

Preparation of antigens treated at different temperatures for various serological tests. Reference and field strains of *A. pleuropneumoniae* representing serotypes 4 and 7 were

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TABLE 1. Serological cross-reactivity of *A. pleuropneumoniae* reference strains of serotypes 4 and 7 with other serotypes

Strain (serotype)	Test	Antigen	Cross-reactivity of rabbit hyperimmune serum with serotype[a]:											
			1	2	3	4	5	6	7	8	9	10	11	12
M62 (4)	COA	WC	−	−	−	++++	−	−	+++	−	−	−	−	−
		BC	−	−	−	++++	−	−	+++	−	−	−	−	−
	ID	WC-SE	++	++	+	+++	+	++	++	++	++	++	++	++
		BC-SE	−	−	−	++	−	−	+	−	−	−	−	−
	IHA	WC-SE	−	−	−	≥1,280	−	−	−	−	−	−	−	−
		BC-SE	−	−	−	≥1,280	−	−	80	−	−	−	−	−
	CIE	WC-SE	++	+	+++	+++	+	++	++	++	++	++	++	++
		BC-SE	+	−	−	+	−	−	+	−	−	−	−	−
WF83 (7)	COA	WC	−	−	−	−	−	−	++++	−	−	−	−	−
		BC	−	−	−	−	−	−	++++	−	−	−	−	−
	ID	WC-SE	++	++	+	++	+	++	+++	++	+	++	++	++
		BC-SE	−	−	−	−	−	−	++	−	−	−	−	−
	IHA	WC-SE	−	−	−	−	−	−	≥1,280	−	−	−	−	−
		BC-SE	−	−	−	−	−	−	≥1,280	−	−	−	−	−
	CIE	WC-SE	++	+	+++	+++	+	++	+++	++	++	++	++	++
		BC-SE	+	−	−	−	−	−	+	−	−	−	−	−

[a] + to ++++ indicates the intensity of the reaction in results of the COA test and indicates the number of precipitation lines in the ID and CIE tests. − indicates negative reactivity. Results of the IHA test are expressed as the reciprocals of the highest dilution of antiserum giving a positive reaction.

grown on enriched pleuropneumonialike organism agar medium overnight at 37°C. Each plate was washed off gently in 3 ml of phosphate-buffered saline solution (pH 7.0) containing 0.5% formalin and then was pooled. The pooled whole-cell suspension of each strain was divided into two portions of 3 ml each. One portion was kept at room temperature, and the second portion was treated at 100°C in a water bath for 1 h. The two antigen preparations were referred to as WC and BC, respectively. Whole bacterial suspensions were used in COA, quantitative coagglutination (QCOA), and 2-mercaptoethanol tube agglutination (2ME-TA) tests. Soluble antigens for IHA, ID, quantitative immunodiffusion (QID), and CIE tests were prepared after centrifugation of WC and BC at 800 × *g* for 30 min and were referred to as WC-SE and BC-SE, respectively.

Serological tests. The details of the preparation of COA reagents and the procedure of this test have been described earlier (9). The 2ME-TA test was done as described earlier (12). The IHA test was performed with a Microtiter System (Dynatech Lab. Inc.) as described previously (8). The procedure used for the ID test was the same as that described earlier (10). Quantitation of serotype-specific and group-specific antigens was done by QCOA and QID tests as described earlier (11). The CIE test was carried out as described by Piffer et al. (18).

Absorption of rabbit antisera. Rabbit hyperimmune sera against the WC antigens of reference strains of serotypes 4 and 7 were absorbed with an equal volume of a 10% suspension of WC antigens of homologous and heterologous serotypes in phosphate-buffered saline solution containing 0.5% formalin. The mixtures were kept at 37°C for 2 h and then centrifuged at 800 × *g* for 30 min. The second absorption was done in the same way. The sera were tested before and after absorption for antibodies against homologous and heterologous serotypes.

RESULTS

Serological cross-reactions between reference strains of serotypes 4 and 7. Extensive cross-reactions were observed for both serotypes 4 and 7 with unheated antigen in the ID and CIE tests. Heat-treated bacterial cell suspensions or their saline extracts of the reference strain of serotype 4 showed cross-reactions in rabbit antiserum against serotype 7 in COA, ID, IHA, and CIE tests. However, heat-treated antigens of the reference strain of serotype 7 shared cross-reactivity with serotype 1 in the CIE test only (Table 1).

Effect of absorption of rabbit antisera against reference strains of serotypes 4 and 7 with homologous and heterologous whole-cell antigens. Antibodies reactive with serotype 4 and 7 antigens in rabbit hyperimmune sera in 2ME-TA, COA, and IHA tests were removed on absorption with homologous antigen only (Table 2).

Antigenic heterogeneity among field strains of serotype 7 as detected by ID test. Field strains of serotype 7 could be divided into four subgroups. Strains in subgroup I (24%) did not share common heat-stable antigens with other serotypes; strains in subgroups II (34%) and III (26%) showed cross-reactivity with strains of serotypes 4 and 10, respectively, whereas strains in subgroup IV (16%) showed cross-reactivity with strains of serotypes 1, 9, and 11 (Table 3). Some strains belonging to serotypes 1 and 10 showed cross-reactivity with antiserum against serotype 7 in the ID test which disappeared when antigen preparations were diluted for determination of antigen titers by QCOA and QID tests (data not shown).

Quantitation of serotype- and group-specific antigens in strains of serotypes 4 and 7 by COA and ID tests. Both reference and field strains of serotype 4 showed cross-reactivity with serotype 7 in COA and ID tests. The antigen titers for serotype 4 or 7 were manifold higher in QCOA and QID tests in homologous antisera than in heterologous antisera (Table 3).

DISCUSSION

Results shown in Table 1 indicate that strains of serotypes 4 and 7 shared extensive cross-reactive epitopes with strains of other serotypes. Cell-free soluble antigens of either serotype 4 or 7 did not show any cross-reactivity with each other in the IHA test when WC-SE was used as the sensitizing antigen. Superficially located antigens involved in the IHA test are mainly of capsular origin, as suggested by Keogh et al. (6) (with respect to antigens of *Haemophilus influenzae*),

TABLE 2. Effect of absorption of rabbit antisera against WC antigens of reference strains of serotypes 4 and 7 with homologous and heterologous antigens

Antigen from strain (serotype)	Serum absorbed with:	Activity in rabbit antiserum against strain (serotype)[a]:					
		M62 (4)			WF83 (7)		
		2ME-TA	COA	IHA	2ME-TA	COA	IHA
M62 (4)	Nil	640	++++	≥1,280	40	+++	0
	M62	0	−	20	0	−	0
	WF83	640	++++	≥1,280	0	−	0
WF83 (7)	Nil	0	−	0	640	++++	≥1,280
	M62	0	−	0	320	++++	≥1,280
	WF83	0	−	0	0	−	0

[a] Results in 2ME-TA and IHA tests are expressed as the reciprocal of the highest dilution of antiserum giving a positive reaction. Antigens used were WC for the 2ME-TA and COA tests and WC-SE for the IHA test.

and by Nielsen (15) (with respect to antigens of *A. pleuropneumoniae*). On the other hand, 2ME-TA, COA, ID, and CIE tests would presumably detect antigens of both somatic and capsular origins. Capsular polysaccharide antigens of *A. pleuropneumoniae* of serotypes 1 to 12 are type specific, and that serological cross-reactivity between different serotypes may be attributed to somatic antigens (17). The IHA test using unheated cell-saline extract as the antigen detects type-specific capsular antibodies (8).

Absorption studies give clear evidence to the fact that strains of serotypes 4 and 7 belong to two distinct serogroups (Table 2). Results shown in Table 3 provide strong evidence for the presence of antigenic heterogeneity among strains of serotype 7. Rapp et al. (19) reported significant two-way cross-reactions between serotypes 4 and 7 in an immunofluorescent antibody test. They also reported occurrence of antigenic heterogeneity among strains of serotype 7. Two of 11 serotype 7 strains reacted with antisera to both serotypes 4 and 7, whereas the remaining 9 reacted only with antiserum to serotype 7 in the slide agglutination test. However, all 11

TABLE 3. Cross-reactivity and antigenic heterogeneity among strains of serotypes 4 and 7 as detected by ID, QCOA, and QID tests and demonstration of serotype-specific antigens by the IHA test[a]

Serotype and subgroup	Test	Reactivity of rabbit antiserum against serotype:											
		1	2	3	4	5	6	7	8	9	10	11	12
4													
	ID	−	−	−	++	−	−	+	−	−	−	−	−
	QCOA	0	0	0	256–1,024	0	0	1–16	0	0	0	0	0
	QID	0	0	0	8–32	0	0	1–2	0	0	0	0	0
	IHA	0	0	0	≥1,280	0	0	0	0	0	0	0	0
7													
I													
	ID	−	−	−	−	−	−	+ to ++	−	−	−	−	−
	QCOA	0	0	0	0	0	0	16–1,024	0	0	0	0	0
	QID	0	0	0	0	0	0	16–32	0	0	0	0	0
	IHA	0	0	0	0	0	0	≥1,280	0	0	0	0	0
II													
	ID	−	−	−	+	−	−	+ to ++	−	−	−	−	−
	QCOA	0	0	0	1–2	0	0	16–512	0	0	0	0	0
	QID	0	0	0	1–2	0	0	4–32	0	0	0	0	0
	IHA	0	0	0	0	0	0	≥1,280	0	0	0	0	0
III													
	ID	−	−	−	−	−	−	+ to ++	−	−	+	−	−
	QCOA	0	0	0	0	0	0	128–1,024	0	0	1–2	0	0
	QID	0	0	0	0	0	0	16–32	0	0	1–2	0	0
	IHA	0	0	0	0	0	0	≥1,280	0	0	0	0	0
IV													
	ID	+	−	−	−	−	−	+ to ++	−	− to +	−	+	−
	QCOA	1–8	0	0	0	0	0	128–1,024	0	0–1	0	1–4	0
	QID	1–2	0	0	0	0	0	8–32	0	0–1	0	1–2	0
	IHA	0	0	0	0	0	0	≥1,280	0	0	0	0	0

[a] + to ++ in ID test indicate the number of precipitation lines. − indicates a negative reaction. Antigens used for QCOA, QID, and IHA tests were WC, BC-SE, and WC-SE, respectively. Results for the QCOA and QID tests are expressed as the reciprocal of the highest dilution of antigen giving a positive reaction, and results for the IHA test were expressed as the reciprocal of the highest dilution of antiserum giving a positive reaction.

isolates reacted strongly with antisera to both serotypes 4 and 7 in the immunofluorescent antibody test.

Cordery et al. (2) studied the cross-reactivity among *A. pleuropneumoniae* strains of serotypes 1 to 6 by the flow microfluorimetry method. Strain M62 of serotype 4 reacted to a considerable degree with antisera to serotypes 1 and 3. These cross-reactions were observed because the flow microfluorimetry method is a highly sensitive technique. Cross-reactivity of serotype 4 with serotypes 1 and 3 is not generally encountered in laboratories which use routine serotyping methods such as agglutination, COA, and ID, which are less sensitive. Kamp et al. (5) reported a one-way cross-reaction between a phenol extract of the serotype 4 strain M62 and antiserum against the serotype 7 strain WF83 in the ID test but not in the rapid slide agglutination test. Perry et al. (17) studied the detailed structures of the specific capsular polysaccharides and cellular lipopolysaccharides of the 12 known serotypes of *A. pleuropneumoniae*. They reported that serological cross-reactions between *A. pleuropneumoniae* strains of serotypes 4 and 7 may be due to the presence of antibodies to their lipopolysaccharide components, since their associated lipopolysaccharide O chains are structurally similar, being composed of repeating tetrasaccharide units with identical trisaccharide residues which form a common backbone. One- or two-way cross-reactions between serotypes 4 and 7 and those between serotype 7 and serotype 1, 9, 10, or 11 may affect the reliability of serotyping and serodiagnostic results, although in most cases it is not difficult to identify the serotype-specific antigens, because of their stronger antigenic activity compared with cross-reacting common antigens, which are only minor in nature. A combination of two or three serological tests is suggested to identify the serotype-specific antigens in strains showing strong cross-reactivity. The IHA test which used WC-SE as the antigen clearly distinguished strains which belong to either serotype 4 or serotype 7 and should therefore be used for final confirmation.

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