

THE SEROLOGICAL AND IMMUNOLOGICAL RELATIONSHIP OF TYPE STRAINS A AND D OF *PASTEURELLA MULTOCIDA* TO FIELD ISOLATES FROM SHEEP

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ABSTRACT

CAMERON, C. M., ENGELBRECHT, MARIA M. & VERMEULEN, ALETTA S. M., 1978. The serological and immunological relationship of type Strains A and D of *Pasteurella multocida* to field isolates from sheep. *Onderstepoort Journal of Veterinary Research*, 45, 215-220 (1978).

Investigations with antisera prepared in rabbits and sheep and active immunity experiments in mice showed that strains of *Pasteurella multocida* which, according to the haemagglutination test, are serologically related, do not necessarily give rise to cross immunity. Conversely, it was found that serologically unrelated strains may exhibit an appreciable degree of cross protection.

The unreliability of the haemagglutination test as an indicator of immunological identity of strains was further confirmed in that peritoneal fluid from immunized mice showed passive protection properties despite the absence of haemagglutinating antibodies.

There was no consistent agreement between the results obtained with antisera prepared in different species and active immunity experiments in mice, and this leads to the conclusion that different species probably vary quantitatively and qualitatively in their immune response to immunization with *P. multocida*.

Attempts to determine whether combined bacterins afforded a better immunity than monovalent bacterins were inconclusive.

Résumé

RELATION SÉROLOGIQUE ET IMMUNOLOGIQUE ENTRE LES SOUCHES DE TYPE A ET D DE *PASTEURELLA MULTOCIDA* ET DES SOUCHES SAUVAGES PRÉLEVÉES CHEZ LE MOUTON

Des recherches effectuées avec des antisera préparés chez le lapin et le mouton ainsi que des expériences d'immunité active chez la souris ont montré que des souches de *Pasteurella multocida* qui sont sérologiquement apparentées d'après le test d'hémoagglutination ne suscitent pas nécessairement une immunité croisée. Inversement on a trouvé que des souches sans parenté sérologique peuvent montrer de la protection croisée à un degré appréciable.

La non-fiabilité du test d'hémoagglutination comme signe d'identité immunologique des souches a trouvé une autre confirmation dans l'observation que le liquide péritonéal de souris immunisées manifestait des propriétés de protection passive malgré l'absence d'anticorps hémoagglutinants.

L'accord entre les résultats fournis par les antisera venant de diverses espèces et les expériences d'immunité active effectuées chez la souris s'est avéré inconsistant. Ceci mène à la conclusion que différentes espèces varient sans doute quantitativement et qualitativement dans leur réponse immunitaire à l'immunisation avec *P. multocida*.

Des essais effectués en vue de déterminer si des vaccins combinés confèrent une meilleure immunité que des vaccins monovalents n'ont pas été concluants.

INTRODUCTION

The first documented outbreak of ovine pasteurellosis in southern Africa was published by Maybin (1931), and subsequently Henning & Brown (1936) studied an outbreak of the disease near Pretoria. The disease has steadily become so widespread in South Africa that a bacterin was required to control it. Cameron & Smit (1970) developed a polyvalent alum-precipitated bacterin which gave good immunity in both sheep and mice when they were challenged with homologous strains of *P. multocida*, but there have been reports of vaccine failures in the field.

Various schemes have been introduced for the classification of *P. multocida*. Little & Lyon (1943) devised one based on slide agglutination tests but their system was found to be unsatisfactory (Carter, 1958) and has fallen into disuse. Roberts (1947) used specific antisera in passive protection tests in mice to divide his isolates into 5 immunotypes. He noted a varying degree of 'aggressiveness' among his strains and also recorded a degree of cross protection among their respective antisera. Subsequently, Carter (1955) introduced a haemagglutination test based on the capsular polysaccharide whereby he was able to distinguish 4 serotypes. Originally, types A, B, C and D were recognized, but type C has since been rejected, while type E, which is associated with East African haemorrhagic septicaemia, has been added.

This classification was extended by Namioka & Murata (1961a; 1961b) who introduced a subtyping system based on the organisms' somatic (O) antigens.

By this method Namioka & Bruner (1962) were able to identify numerous subtypes within Carter's types.

An independent typing system employing a gel diffusion technique is used for *P. multocida* isolates from poultry (Heddlston, Gallagher & Rebers, 1972). A similar procedure has also been used by Penn & Nagy (1974) for isolates of mammalian origin.

Little has been published on the relation between the antigens which are identified by these serological tests and their actual role in inducing immunity. A correlation has, however, been established between haemagglutinating antibodies to capsular antigens and immunity in types B and E (Carter, 1964; Penn & Nagy, 1974). Conversely, Mukkur & Nilakantan (1969) contend that haemagglutinating antibodies play a limited role in protection. No such direct evidence has been reported with respect to types A and D.

Cameron & Smit (1970) found that there was a general association between the haemagglutination titres of sheep sera and their protective properties, but they found no direct proof that these antibodies are directly responsible for immunity. In fact, in the case of *P. haemolytica*, it appears that haemagglutinating antibodies are not responsible for immunity (Cameron, 1966; Knight, Biberstein & Allison, 1969).

Reports of vaccine failures in the field prompted these studies on the strain specificity of immunity and a serological investigation of field isolates. Bacterins and antisera were prepared from recognized type strains as well as from a number of field isolates of *P. multocida*. The cross immunity among the strains as well as their serological relationship was studied, using various experimental models.

MATERIALS AND METHODS

Bacterial strains

P. multocida strains A14g (Type A), B921 (Type B) and D1 (Type D) were obtained from Dr P. Perreau*, while strains 33191 (1), and 4009 were isolated from cases of fatal ovine pneumonic pasteurellosis in South Africa.

Experimental animals

Mice. Conventionally reared albino mice were obtained from the colony maintained at the Institute. For the active immunity experiments 6–8-week-old male mice were used while 8–10-week-old males were used in the passive immunity experiments. They were housed in plastic cages and fed a balanced pelleted ration. The number of animals used varied according to the requirements of each experiment.

Rabbits. New Zealand-White type rabbits were used for the production of antisera. They were housed in wire cages and fed a balanced pelleted ration.

Sheep. Antisera were prepared in 6-month-old Merino wethers.

Preparation of bacterins

Bacterins for the immunization of mice were prepared by growing the bacteria in a slightly modified Bain & Jones's medium (Bain & Jones, 1958; Cameron & Smit, 1970) for 18 h at 37 °C in shake flasks. The cultures were inactivated by the addition of 0,5% formalin and the density adjusted to contain 1,0% packed cells. The inactivated culture was precipitated by the addition of 10 ml of an 11% solution of potassium alum per 100 ml to give a concentration of 1,0%. Finally, the pH was adjusted to 6,5 with KOH.

For the preparation of combined bacterins, Strains A14g and D1 were grown and inactivated as above. Equal volumes of the bacterins were mixed and precipitated with potassium alum. A 0,2 ml dose of the combined bacterin therefore contained half as much antigen of each strain compared with the monovalent bacterins.

Acetone-dried bacteria were used for the production of antisera in rabbits and sheep. Bacteria were grown in Bain and Jones's medium as above, collected by centrifugation, washed twice with saline and thereafter 4 times with acetone. The acetone-washed cells were placed in a beaker and allowed to dry for 18 h at 37 °C.

Production of antisera

Rabbits. Groups of 6 rabbits were used for each strain. The desired mass of dried bacteria was suspended in 0,5 ml of saline and injected intravenously. Each rabbit was given 6 injections of 0,5; 1,0; 2,0; 3,0; 4,0 and 5,0 mg at 3- and 4-day intervals alternately, and bled 7 days after the last injection.

The haemagglutination titres and passive protection value of the sera were determined individually (*vide infra*). The best sera were pooled and stored at –20 °C for use in further experiments.

Sheep. Antisera were prepared by a procedure similar to that used in rabbits except that the dosages of antigen were doubled. Many sheep did not yield satisfactory antisera and the procedure was consequently repeated for some strains.

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Induction of ascites in mice

A modification of the procedure described by Sommerville (1967) was used.

Bacterin was prepared from *P. multocida* strains A14g, D1, 33191 (1) and 4009, as outlined above, but the density was adjusted to 2,0% packed cell volume (pcv) and emulsified with an equal volume of Freund's complete adjuvant (Difco).

The following immunization schedule was used.

Day 1: 0,25 ml of the bacterin in Freund's complete adjuvant intraperitoneally (ip).

Day 14: Repeat as for Day 1.

Day 28: Equal volumes of incomplete Freund's adjuvant (Difco) and physiological saline only. 0,2 ml ip per mouse.

Day 35: 0,25 ml of the following emulsion intraperitoneally.

Mycobacterium smegmatis was grown for 7 days on Löwenstein-Jensen agar and the yield obtained from 30 slants (20 ml round McCartney bottles) suspended in 30 ml physiological saline. The suspension was subsequently emulsified with 30 ml of a solution composed of Bayol 72* (25,5 ml) and Arlacel A** (4,5 ml) and containing 30 mg of dry *Mycobacterium avium*.

Ascites fluid was collected 2–3 weeks later.

Passive and active protection tests in mice

The protective value of both the antisera and peritoneal fluid was determined by the method of Ose & Muenster (1968) as modified by Cameron & Smit (1970). Groups of 40 mice were given 0,2 ml of the antiserum intravenously and challenged with the appropriate strain 24 h later, as outlined below.

For the active immunity experiments, groups of 40 mice were given either 1 or 2 subcutaneous injections of 0,2 ml alum-precipitated bacterin at intervals of 4 weeks, and 36 of them were challenged 14 days after the 2nd injection.

During the course of these studies it appeared that the type Strains A14g and D1 possess common antigens and it was postulated that a combined bacterin may afford a better protection than homologous monovalent bacterins. The possibility that combined bacterins prepared from the type strains would give an improved protection to the 2 isolates was therefore investigated. For the sake of comparison, one group of mice was given 2 doses of combined bacterin simultaneously at different sites (see Table 8).

Challenge of experimental mice

The strain or strains of *P. multocida* used for challenge were grown on blood tryptose agar for 18–24 h, suspended in tryptone water and the density nephelometrically adjusted to give a concentration of approximately $1-2 \times 10^9$ bacteria/ml. Tenfold serial dilutions of this suspension were made in tryptone water.

To obtain quantitative results, 6 mice were challenged by intraperitoneal injection of 0,2 ml of the various dilutions. Normally 6 dilutions were used and the range was varied depending on the virulence of a particular strain. Thus, for example, dilutions of

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** Atlas Chemical Industries, Inc., Wilmington, Delaware 19899, U.S.A.

10⁻⁴ to 10⁻⁹ were used for Strain A14g and 10⁻¹ to 10⁻⁶ for Strain D1. The remaining 4 mice were discarded.

Deaths which occurred within 4 days after challenge were recorded and used to calculate the log LD50 of each experimental group as well as that of the untreated or non-immunized groups (Reed & Muench, 1937). The degree of protection was expressed as the difference in the log LD50 of the experimental and control groups.

Serology

The haemagglutination titres of both rabbit and sheep sera as well as mouse peritoneal fluid were assessed according to the method originally introduced by Carter (1955) as modified by Cameron & Smit (1970).

RESULTS

Challenge procedure

Since consistently reproducible results were not obtained with either freeze-dried bacteria or bacteria frozen at -20 °C, fresh cultures were used to challenge the animals. Inherent variation in the standardized suspensions necessitated titration of these suspensions so that the results obtained in the different experiments could be compared.

An example of such a titration is shown in Table 1. This table also demonstrates the method of expressing the degree of protection in quantitative terms.

Passive protection

Rabbit antisera. The haemagglutination titres of 3 antisera are shown in Table 2. Although the titres for strains A14g and D1 are not high, they are sufficiently specific, and the field strains, 33191 (1) and 4009, could be identified as types D and A respectively.

The protective properties of these antisera are shown in Table 3. Antisera to Strains A14g and B921 gave a good homologous protection with minimal cross protection to the other strains. Antiserum to Strain D1 did not give a good homologous protection but it protected against Strain 33191 (1). Antiserum to Strain 33191 (1) gave very good homologous protection and also protected against Strain D1. Strain 33191 (1) would therefore be regarded as a type D strain and in rabbits it is apparently a better immunogen than Strain D1.

There is therefore general agreement between the protective properties of the antisera and their haemagglutination titres. Since the quantity of serum which is obtainable from rabbits is limited, sheep antisera were used for more extensive studies.

TABLE 1 Example of immunity assay in mice immunized with different bacterins and challenged with a single strain

Dilution of challenge strain 33191(1)	Vaccine strain																			
	A14g				B921				D1				33191(1)				Control			
	Deaths		Cum.		Deaths		Cum.		Deaths		Cum.		Deaths		Cum.		Deaths		Cum.	
	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L
10 ⁻¹	10	0		0	10	0		0	10	0		0	4	6	11	6	10	0		0
10 ⁻²	10	0		0	10	0		0	10	0		0	3	7	7	13	10	0		0
10 ⁻³	10	0		0	10	0		0	8	2		2	1	9	4	17	10	0		0
10 ⁻⁴	8	2		2	7	3		3	7	3		5	0	10	3		10	0		0
10 ⁻⁵	9	1		3	10	0		3	7	3	24	8	0	10	3		9	1		1
10 ⁻⁶	7	2	20	5	9	1	19	4	6	4	17	12	1	9	3		9	1	26	2
10 ⁻⁷	8	2	12	7	7	3	10	7	8	2	11	14	2	8	2		9	1	17	3
10 ⁻⁸	3	7	4	14	3	7	3	14	3	7	3	21	0	10	0		5	5	6	8
10 ⁻⁹	1	9	1	23	0	10	0	24	0	10	0	31	0	10	0		1	9	1	17
Log LD50					6,59				6,59				1,49				7,83			
Log ₁₀ protection					1,24				1,24				6,34							

Cum—Cumulative deaths
D—Died
L—Lived

TABLE 2 Reciprocal haemagglutination titres of rabbit antisera to various known and field strains

Antiserum	Antigen				
	A14g	B921	D1	33191(1)	4009
A14g	128	2	2	0	128
B921	0	2 048	0	0	0
D1	0	0	32	16	0

TABLE 3 Log₁₀ protection afforded by rabbit antisera to homologous and heterologous strains of *P. multocida*

Challenge strain	Antiserum			
	A14g	B921	D1	33191(1)
A14g	4,5	0	0	0
B921	0,1	>4	0,1	0,4
D1	0,2	0,3	0,8	3,0
33191(1)	0	0	1,3	>5

Sheep antisera

Many sheep did not respond well to hyperimmunization and only after repeated attempts was a set of usable sera obtained. The results of the haemagglutination assays given in Table 4 show that the titres were generally poor and numerous cross reactions are apparent. Type B antiserum gave good specific protection, however, and this serotype can be clearly distinguished from the others. As in the case with the rabbit antisera, Strain 4009 would be identified as type A but it also reacted with type D antiserum. Since the type D antiserum cross-reacted with type A, this serum would appear to be unsatisfactory.

TABLE 4 Reciprocal haemagglutination titres of sheep antisera

Antiserum	Antigen				
	A14g	B921	D1	33191(1)	4009
A14g.....	32	16	2	0	64
B921.....	2	512	0	0	2
D1.....	32	8	8	2	32
33191(1)....	2	2	2	0	2
4009.....	0	0	0	0	0

The protective properties of these sera were assayed in mice and the results are shown in Table 5. Antiserum to Strain A14g gave nearly 2 logs homologous protection and also protected against D1 and 33191 (1), although its haemagglutination titre to these strains was very low. Conversely, it did not give any protection to Strain 4009 to which it is serologically related.

TABLE 5 Log₁₀ protection afforded by sheep antisera in mice to challenge with homologous and heterologous strains of *P. multocida*

Challenge strain	Antiserum				
	A14g	B921	D1	33191(1)	4009
A14g.....	1,9	0	1,2	0	0
B921.....	0	3	0	0	—
D1.....	1,4	0,4	1,4	2,0	1,7
33191(1)....	1,2	0,5	1,5	3,0	3,0
4009.....	0,2	0	0	0	1,8

Type B antiserum was very specific in its activity and this serotype can be readily distinguished from all the other strains.

TABLE 6 Log₁₀ protection afforded by *P. multocida* bacterins to homologous and heterologous challenge in mice

Challenge strain	Vaccine strain									
	A14g		B921		D1		33191(1)		4009	
	1 inj.	2 inj.	1 inj.	2 inj.	1 inj.	2 inj.	1 inj.	2 inj.	1 inj.	2 inj.
A14g.....	1,87	4,6	0,82	1,2	0,17	2,0	0,89	4,2	0,03	2,1
B921.....	0,0	n.d.	2,62	n.d.	0,26	n.d.	0,0	n.d.	0,0	n.d.
D1.....	0,0	2,1	0,97	1,1	1,04	3,6	0,44	1,0	0,96	0,6
33191(1)....	0,14	0,5	0,0	0,6	1,53	1,2	2,11	6,3	0,0	0,0
4009.....	0,34	0,0	1,14	0,0	0,32	0,0	0,0	0,0	4,63	3,5

n.d.—not done
inj.—injections

As in the case of the serological tests, antiserum to Type D1 gave appreciable cross protection to Type A and also protected against Strain 33191 (1). Antiserum to Strain 33191 (1) gave very good homologous protection and also protected against Type D. Strain 4009 antiserum cross-protected against Strains D1 and 33191 (1) while neither of these sera protected against challenge with Strain 4009. Strain 4009 did not protect against type A to which it is serologically related.

Active immunity

The degree of cross immunity which developed in mice after either 1 or 2 injections of alum-precipitated bacterin of the various strains is shown in Table 6.

Strain A14g gave near specific immunity but there was some cross protection to Strain D1 after 2 injections. Strain B921 gave specific immunity. Strain D1 gave the best homologous protection, but on the other hand it gave less specific immunity to Strains A14g and 33191 (1). Strain 33191 (1) gave essentially similar results as Strain D1. Strain 4009 gave a very specific homologous immunity after 1 injection, but after 2 injections it cross-protected to Strain A14g.

In Table 7, data on the active immunity provoked in mice are summarized for comparison with the passive protection induced by the peritoneal exudate fluid.

As in the previous experiment, Strain A14g gave a specific immunity, although there was some cross protection to Strain 4009 in the actively immunized animals. Strain D1 again gave good homologous immunity as well as extensive cross immunity. Similar results were given by Strain 33191 (1). Strain 4009 again gave a very good homologous protection as well as a lesser degree of immunity to Strain A14g in the active protection test.

Although there are some discrepancies, there is general agreement between the results obtained with direct challenge of immunized mice and the protection afforded by their peritoneal exudate fluid. Despite the protection given by the peritoneal exudate fluid, no antibodies could be detected in it by the haemagglutination test.

Immunogenic synergism and antagonism

The results of an experiment designed to investigate the possibility that combined bacterins prepared from the type strains would give an improved protection to the 2 isolated strains are shown in Table 8.

The data obtained were, however, inconclusive and confusing.

TABLE 7 Comparison of active and passive immunity (peritoneal fluid) in mice

Challenge strain	Vaccine strain							
	A14g		D1		33191(1)		4009	
	Act.	Pas.	Act.	Pas.	Act.	Pas.	Act.	Pas.
A14g.....	3,9	3,0	2,3	1,75	2,8	2,15	2,5	1,3
D1.....	0,8	0,14	2,3	2,59	0,0	1,02	0,0	1,02
33191(1).....	0,5	0	1,2	0	4,7	1,94	0,0	0,02
4009.....	1,9	0	2,1	0	2,4	0	>4	0,33

Act.—Active immunity
Pas.—Passive protection

TABLE 8 Log₁₀ Protection afforded by monovalent and combined bacterins to homologous and heterologous strains

Challenge strain	Bacterin strains							
	A14g		D1		Combined bacterin			
	0,2 ml		0,2 ml		0,2 ml		2 × 0,2 ml*	
Dosage	1 inj.	2 inj.	1 inj.	2 inj.	1 inj.	2 inj.	1 inj.	2 inj.
A14g.....	2,31	1,01	2,05	0,00	2,11	0,01	3,5	1,47
D1.....	0,70	0,00	1,18	1,88	1,28	1,67	1,08	2,46
33191(1).....	0,07	0,81	1,62	1,22	0,65	0,08	0,55	1,33
4009.....	1,18	2,12	0,02	1,28	0,00	0,00	0,00	0,62

* The 2 injections were given simultaneously but at different sites
inj.—injections

DISCUSSION

In the first place it is evident that serological identity between strains as determined by the haemagglutination test is not necessarily a measure for immunogenic homogeneity and, secondly, there is a discrepancy in the passive protection provoked by rabbit and sheep antisera and the active immunity which developed in mice after treatment with bacterins.

Haemagglutination tests with both rabbit and sheep antisera showed a close similarity between Strain A14g and Strain 4009. This similarity, however, was not evident in passive protection tests with sheep antiserum but was shown when mouse peritoneal fluid was used. The immunological relationship between these 2 strains was clearer in the active mouse protection tests. Homologous protection was, however, better than heterologous protection. It can therefore be deduced that there is a partial relationship but not absolute identity between Strains A14g and 4009.

Similar results were obtained with Strains D1 and 33191 (1). They cross-protected against one another, but this was variable particularly when antisera were used. In the active immunity experiments homologous protection was usually better than cross protection. Both these strains also gave cross protection to Strains A14g and 4009 when sheep antisera were used and in the active mouse protection test system but not with the rabbit antiserum.

The serology and immunology of *P. multocida* is thus vastly more complicated than is generally appreciated. A simple serological test is clearly inadequate

as a basis of typing them since it does not accurately reflect the immunological relationship among strains. It is apparent that *P. multocida* organisms are endowed with a complex antigenic structure; some antigens are prominent in serological reactions, while others are involved in inducing immunity. The degree to which the latter antigens are involved also appears to be influenced by the test system employed as well as by the animal species used for immunological assays.

The inability of both type Strains A14g and D1 either alone or in combination to provide solid protection against Strain 4009 is particularly important. Such bacterins would be ineffective under field conditions, whereas an autogenous bacterin would probably be effective. The existence of such strains poses profound problems relating to the formulation of universally effective bacterins and stresses the necessity for further intensive studies on the antigenic structure of *P. multocida* and the immunizing characteristics of sub-cellular components.

Certain studies have been undertaken in this respect and a number of immunogenic substances have been isolated. Rebers, Heddleston & Rhoades (1966) and Ganfield, Rebers & Heddleston (1976) isolated and described a lipopolysaccharide antigen with immunizing and toxic properties. Srivastava, Foster, Dawe, Brown & Davis (1970) studied the immunizing properties of *P. multocida* cell walls and protoplasm, while Baba (1977) was able to isolate a highly immunogenic ribosomal fraction. The relationship between these components and the antigens that are detected by serological tests has, however, not yet been established.

A major problem jeopardizing studies on the antigenicity of *P. multocida* is the inconsistency of antigenic expression. Recently Rebers & Heddleston (1977) showed that host-passaged *P. multocida* was more antigenic especially with respect to cross immunity to heterologous strains. It has also been found that certain antigens afford protection in some species but not in others. Thus Yaw & Kakavas (1957) found that polysaccharide antigens would protect both chickens and mice, whereas somatic or intracellular antigens would protect chickens but not mice. Our results confirm these species-related differences as the results obtained with rabbit and sheep antisera often did not agree. It seems, therefore, that certain animal species respond better to some antigens while the reverse is true for other antigens. Experimental results obtained in one species are therefore not necessarily valid in and cannot injudiciously be extrapolated to another species.

The immunology and serology of *P. multocida* thus remains a perplexing and complex practical and theoretical problem which can only be resolved by in-depth studies on the antigenic mosaic of the organism and a thorough investigation of host response to them as initiated by Woolcock & Collins (1976) and Collins (1977). There is an urgent need for the establishment of a serological typing system which would be a reliable indication of the immunological relationship among strains.

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