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IMMUNE RESPONSE OF RABBITS, MICE AND SHEEP TO POLYVALENT *PASTEURELLA* VACCINE

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ABSTRACT

CAMERON, C. M. & SMIT, GERTRUIDA. Immune response of sheep and mice to polyvalent *Pasteurella* vaccine. *Onderstepoort J. vet. Res.*, 37 (4), 217-224 (1970).

A polyvalent alum-precipitated vaccine containing *Pasteurella multocida* (Lehmann & Neuman, 1899) types A and D and four serotypes of *Pasteurella haemolytica* (Newsom & Cross, 1932) produced a sound immunity in mice and sheep to challenge with the homologous strains of *P. multocida*. The vaccine also stimulated a marked increase in the haemagglutination titres and passive protection values of the serum to the homologous strains of *P. multocida*.

The antibody response to the *P. haemolytica* strains was poorer but possibly sufficient to contribute to an increase in resistance to infection.

Introduction

Although there is a large volume of literature pertaining to the preparation of vaccines and immunization of animals against classical haemorrhagic septicaemia and fowl cholera, there is little information available on the immunization against pneumonic forms of pasteurellosis caused by *Pasteurella multocida* (Lehmann & Neuman, 1899) and *Pasteurella haemolytica* (Newsom & Cross, 1932).

In this respect most work has been done with polyvalent vaccines for the protection of animals against shipping fever. These vaccines contain myxovirus para-influenza-3 and strains of *P. multocida* and *P. haemolytica* isolated from cases of shipping fever (Gale, Hamdy & Trapp, 1963). In some studies poor results were obtained (Hamdy & Trapp, 1964) while in others they were very encouraging (Matsuoka, Gale, Ose & Berkman, 1966; Mayr, Wizigmann, Schels & Plank, 1969).

In South Africa pasteurellosis occurs primarily in sheep under stress conditions, particularly during severe droughts, and is characterized by a severe fatal pneumonia. *P. haemolytica* can usually be isolated from pneumonic lungs in pure culture but very frequently *P. multocida* is also present.

P. haemolytica is an important pathogen of sheep and is responsible for a variety of pathological conditions in this species (Smith, 1960; Carter, 1964a; Carter, 1967; Biberstein, Nisbet & Thompson, 1967). Biberstein, Gills & Knight (1960) divided *P. haemolytica* into 11 serotypes and found that Types 1, 2, 3, 4 and 10 were most commonly associated with disease. Biberstein (1965) has, however, also shown that serological cross reactions may occur between some strains.

As far as *P. multocida* is concerned, Types A and D are universally associated with pneumonia in sheep (Namioka & Bruner, 1962; Carter, 1967).

The object of this investigation was to examine the immunizing properties of a polyvalent vaccine containing *P. multocida* Types A and D as well as the most common serotypes of *P. haemolytica* encountered in South Africa.

Materials and Methods

Strains

The following strains of *P. haemolytica* for the preparation of typing antisera were kindly supplied by Dr E. L. Biberstein*: I 29 (Type 1), J 28 (Type 2), D 2 (Type 3), S (Type 4), G 132 (Type 5), A 30 (Type 6), HI (Type 7), H21Q (Type 8), B 1 (Type 9), JF 2 (Type 10), KC 282 (Type 11), and 5209 (Type 12). Strains 4367 (Type 4) and 14834 (Type 6) were isolated from sheep with pneumonia. *P. multocida* strains A14g (Type A) and DI (Type D) were obtained from Dr P Perreau**.

Serotyping of P. haemolytica

Antisera were prepared and indirect haemagglutination tests were done as described by Carter (1955), except that guinea pig erythrocytes were used instead of human Type O erythrocytes.

Preparation of vaccines

P. haemolytica strains I 29 (Type 1), 4367 (Type 4), 14834 (Type 6), HI (Type 7) and *P. multocida* strains A14g (Type A) and DI (Type D) were used for preparation of polyvalent vaccines.

The medium used for producing vaccines was prepared as described by Bain & Jones (1958) with minor modifications. The final composition was as follows:

Casein hydrolysate (Oxoid)	15 g
Yeast extract (Oxoid)	10 g
Tryptone (Oxoid)	5 g
Sucrose	2 g
$MgSO_4.7H_2O$	1 g
KH_2PO_4	2.72 g
$Na_2HPO_4.12\ H_2O$	10.745 g
Distilled water	1000 ml

1. *Formalin inactivated vaccine*

(a) *Whole bacteria:* Bacteria were grown in 200 ml volumes of B and J medium in Roux shake flasks for 24 hours at 37°C. The packed cell volume was determined, adjusted to 1.0 per cent with 0.85 per cent NaCl and 0.5 per cent formalin added. As soon as the cultures were found to be sterile, equal volumes of the six strains were mixed and 20 ml of an 11 per cent solution of potassium alum added to each 100 ml of vaccine to give a final concentration of approximately 2.0 per cent. The pH was adjusted to 4.5 with 1.2 N KOH in order to obtain optimal precipitation. The polyvalent vaccine then contained 0.16 per cent packed cells per strain. For one experiment vaccines containing 5.0, 1.0 or 0.2 per cent packed cells of strain A14 g alone were prepared in a similar way.

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For the preparation of oil adjuvant vaccines, equal volumes of bacterial suspensions and an oil adjuvant were mixed. The oil adjuvant was prepared by mixing a light mineral oil with appropriate emulsifiers.

(b) *Ultrasonically disrupted bacteria:* A 4.0 per cent packed cell suspension was prepared in 0.85 per cent NaCl and subjected to ultrasonic vibration in a Branson Sonifier Model S75 (Branson Instruments Inc., Danburg, Connecticut) at maximum output for 20 minutes and centrifuged at 10,000*g* for 20 minutes. The supernatant fluid was collected and the sediment resuspended in the original volume of 0.85 per cent NaCl and again subjected to ultrasonic vibration. The procedure was repeated another three times. The supernatant fluid from the first three cycles was pooled together with all the material remaining after the fourth cycle and 0.5 per cent formalin added. When the material was found to be sterile, dilutions were prepared and potassium alum added as for the whole cell vaccine.

2. *Phenol, merthiolate or heat inactivated vaccine*

In order to examine the effect of different inactivation procedures on the antigenicity of the vaccine, a batch of polyvalent vaccine was divided into four aliquots which were inactivated with either 0.5 per cent formalin, 0.5 per cent phenol, 1:5000 merthiolate or by autoclaving at 120°C for 15 minutes. The vaccines were mixed with an equal volume of oil adjuvant.

Immunization of rabbits: Two doses of 5 ml oil adjuvant vaccine were administered subcutaneously to groups of six rabbits with an interval of 4 weeks between the two injections. Each dose was divided into five 1 ml volumes and given at different sites. The animals were bled at fortnightly intervals and the haemagglutination titres against all six strains determined.

Assay of immunity in mice: Potency tests were done essentially as described by Ose & Muenster (1968). Six groups of 10 female albino mice aged 6 weeks, were used in all the experiments. Each animal received two injections of 0.2 ml monovalent, polyvalent or sonically disrupted vaccine subcutaneously, at an interval of 3 weeks, and was challenged 10 days after the second injection. For challenge, either strain A14g or DI was grown on blood tryptose agar at 37°C overnight. A suspension was prepared in 0.85 per cent NaCl and the concentration adjusted to give an optical density reading of 0.046 using an Eel colorimeter with a blue filter. Groups of ten experimental mice were challenged by intraperitoneal injection of 0.2 ml of 10^{-1} to 10^{-6} dilutions of the suspension. Groups of control mice were similarly challenged with 10^{-4} to 10^{-9} dilutions of the same suspension. Deaths were recorded daily for 3 days and the LD_{50} calculated by the method of Reed & Muench (1937).

The potency of a vaccine was expressed in terms of the logs protection obtained as compared with the controls.

Assay of antibody response and immunity in sheep

Groups of six or eight Merino wethers were used in all the experiments. Formalin inactivated whole cell vaccine was given at various intervals depending on the requirement of the experiment. A dose of 5.0 ml was used throughout and always given subcutaneously. The immune response was studied by determining the indirect haemagglutination titres and the passive protection potency of serum samples obtained at 4 weekly intervals. Some sheep were challenged 2 weeks after the last dose of vaccine by intravenous injection of live bacteria.

Indirect haemagglutination tests were done using the method mentioned above. Serum dilutions starting at $\frac{1}{10}$ were made using Cooke microtiter equipment (Cooke Engineering Company, Alexandria, Virginia, U.S.A.).

Groups of ten mice were used to determine the protective potency of pooled sheep sera. Each mouse was given 0.2 ml of a $\frac{1}{5}$ dilution of serum intravenously. The mice were challenged 18 to 24 hours later by intraperitoneal injection of live bacteria as already described. Appropriate controls were challenged simultaneously. The potency of the sera was expressed in terms of the logs protection afforded.

Immunity of sheep to septicaemic infection was tested by the intravenous injection of 2.0 ml of a suspension of either *P. multocida* A14g (Type A.), *P. multocida* DI (Type D), *P. haemolytica* 129 (Type 1), *P. haemolytica* 4367 (Type 4), *P. haemolytica* 14834 (Type 6) or *P. haemolytica* HI (Type 7) with an optical density of 0.222 as measured with an Eel colorimeter with a blue filter. The sheep were observed for 14 days and rectal temperatures recorded daily.

Results

Prevalence of P. haemolytica *serotypes in sheep in South Africa*

Fifty five isolates of *P. haemolytica* obtained from cases of sheep pneumonia from all parts of the country were typed, using monospecific sera. The results are shown in Table 1. Serotypes 1, 4, 6 and 7 were the most prevalent at the onset of this study and were therefore incorporated into the polyvalent vaccine. During the course of the work Type 8 was isolated more frequently.

Table 1 *Serotypes of* P. haemolytica *isolated from sheep with pneumonia*

Type	Number of strains typed
1	7
2 (11)	1
3	0
4	8
5	5
6	12
7	4
8	6
9	4
10	1
12	1
Not typable	6
Total	55

Effect of method of inactivation and adjuvant on the immune response

The average haemagglutination titres obtained in rabbits that had received vaccines containing all six strains and which had been inactivated by different methods were followed for 6 weeks (Fig. 1).

Vaccine inactivated with 1:5000 merthiolate gave the best results but because severe reactions occurred at the injection sites, formalin was used for inactivation of vaccines in further experiments.

A comparison was made between the haemagglutination titres obtained with oil adjuvant vaccine and alum-precipitated vaccine in sheep (Fig. 2).

It is apparent that there is no significant difference between the two adjuvants, but because a 5 ml dose of

oil adjuvant vaccine frequently gave rise to suppuration at the injection site, alum-precipitated vaccine was used in all the other experiments.

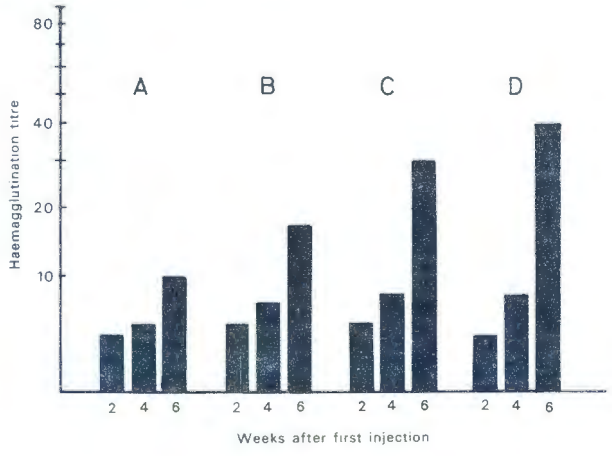

FIG. 1 Haemagglutination titres of rabbits which had received formalized, phenolized, merthiolate and heat inactivated vaccines.
A — Phenolized vaccine
B — Heat inactivated vaccine
C — Formalized vaccine
D = Merthiolate inactivated vaccine

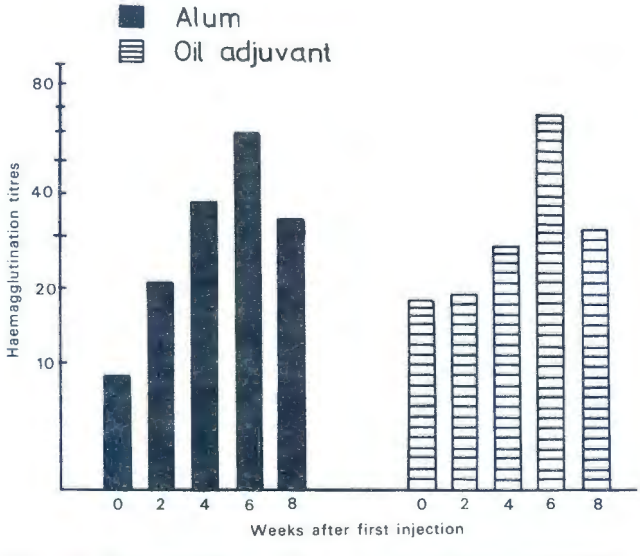

FIG. 2 Haemagglutination titres of groups of sheep given either alum-precipitated vaccine or oil adjuvant vaccine

Immune response of mice to P. multocida

The logs protection afforded to mice by monovalent vaccines containing 5.0 per cent, 1.0 per cent or 0.2 per cent packed cells of strain A14g, was determined (Fig. 3). In this particular experiment a final LD_{50} end point was not obtained in the control group. Consequently the exact degree of protection could not be calculated but was more than 4.5 logs throughout.

Vaccine containing only 0.2 per cent packed cells therefore still gives a very solid immunity. This concentration is slightly higher than in the polyvalent vaccine. The concentration of each strain in the polyvalent vaccine was approximately 0.16 per cent packed cells and this concentration was also quite adequate to give a solid immunity in mice (Table 2). Mice challenged with strain A14g showed a protection of 6.6 logs while those challenged with strain DI showed a protection of 4.7 logs.

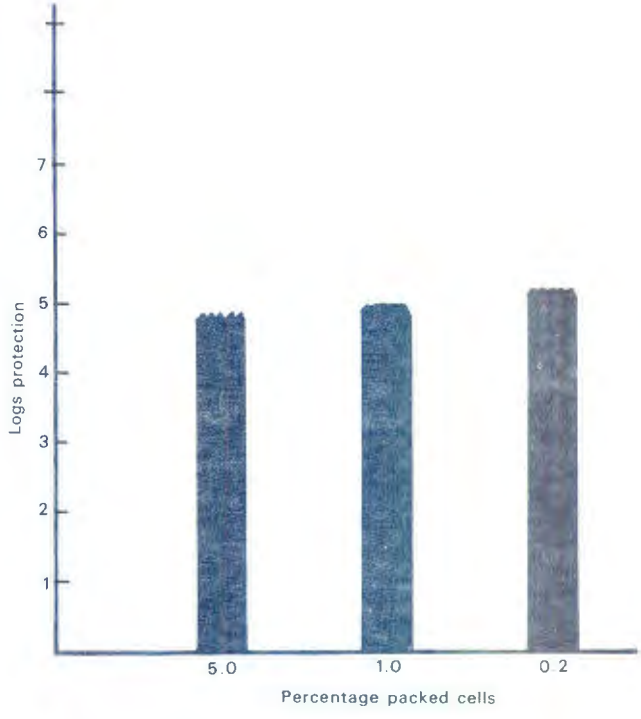

FIG. 3 Effect of concentration of vaccine on immunity of mice to *P. multocida* strain A14g

TABLE 2 *Protection of mice with polyvalent vaccine against challenge with* P. multocida *strains A*14g *(Type A) and DI (Type D)*

Dilution of challenge suspension	Deaths of mice/10 challenge with strain A14g		Deaths of mice/10 challenge with strain DI	
	Immunized	Controls	Immunized	Controls
10^{-1}	6	—	7	—
10^{-2}	3	—	10	—
10^{-3}	2	—	1	—
10^{-4}	1	10	6	10
10^{-5}	0	10	2	10
10^{-6}	2	10	0	10
10^{-7}	—	10	—	5
10^{-8}	—	7	—	2
10^{-9}	—	1	—	0
LD 50	−1.7	−8.3	−2.7	−7.4
Logs protection	6.6		4.7	

An attempt was made to determine whether ultrasonic disruption of the bacteria would enhance their immunizing properties. There was no significant difference between whole cells and disintegrated cells at the concentration tested. Again the exact number of logs protection could not be calculated but was more than 5.0 throughout (Fig. 4).

Immune response of sheep to P. multocida

The haemagglutination titres and passive protection values of sera from two groups of sheep which had received either two or three initial doses of vaccine and a booster dose 3 months later were followed for 11 months (Fig. 5).

It is apparent that although three injections did not markedly influence the level of antibody production the levels obtained persisted for a slightly longer period. It is also evident that by and large the haemagglutination titres were parallel to the passive protection values,

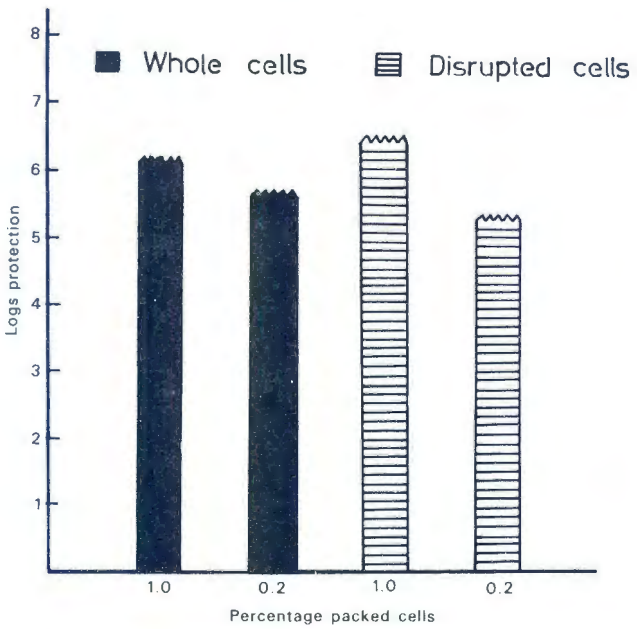

FIG. 4 Comparison of immunizing potency of whole cells with ultrasonically disrupted cells of *P. multocida* strain A14g in mice

which is in accordance with the findings of Carter (1964b).

The haemagglutination titres obtained to strain DI were also followed. As in the case of strain A14g, titres persisted for a longer period when three initial injections were used instead of only two (Fig. 6).

The duration of the immune response after three initial injections was approximately 20 weeks for both strains. A booster injection given at this stage elicited a response which persisted for a further 24 weeks.

In order to obtain some information which would relate actual resistance to infection to either the haemagglutination titre or the passive protection potency of the serum, six sheep were immunized with two doses of polyvalent vaccine given at an interval of 4 weeks. The haemagglutination titres of sera obtained 4 weeks after the second injection were determined and the sheep were challenged 1 week later with strain A14g. The pre-challenge sera from the sheep which subsequently survived the challenge and sera from control sheep that died were respectively pooled and the passive protection potency determined (Table 3).

Although the sheep had comparatively low haemagglutination titres, they were solidly immune to intravenous challenge, and the pooled sera protected mice to an exceptional degree, while pooled sera from the unimmunized sheep had no protective effect whatsoever.

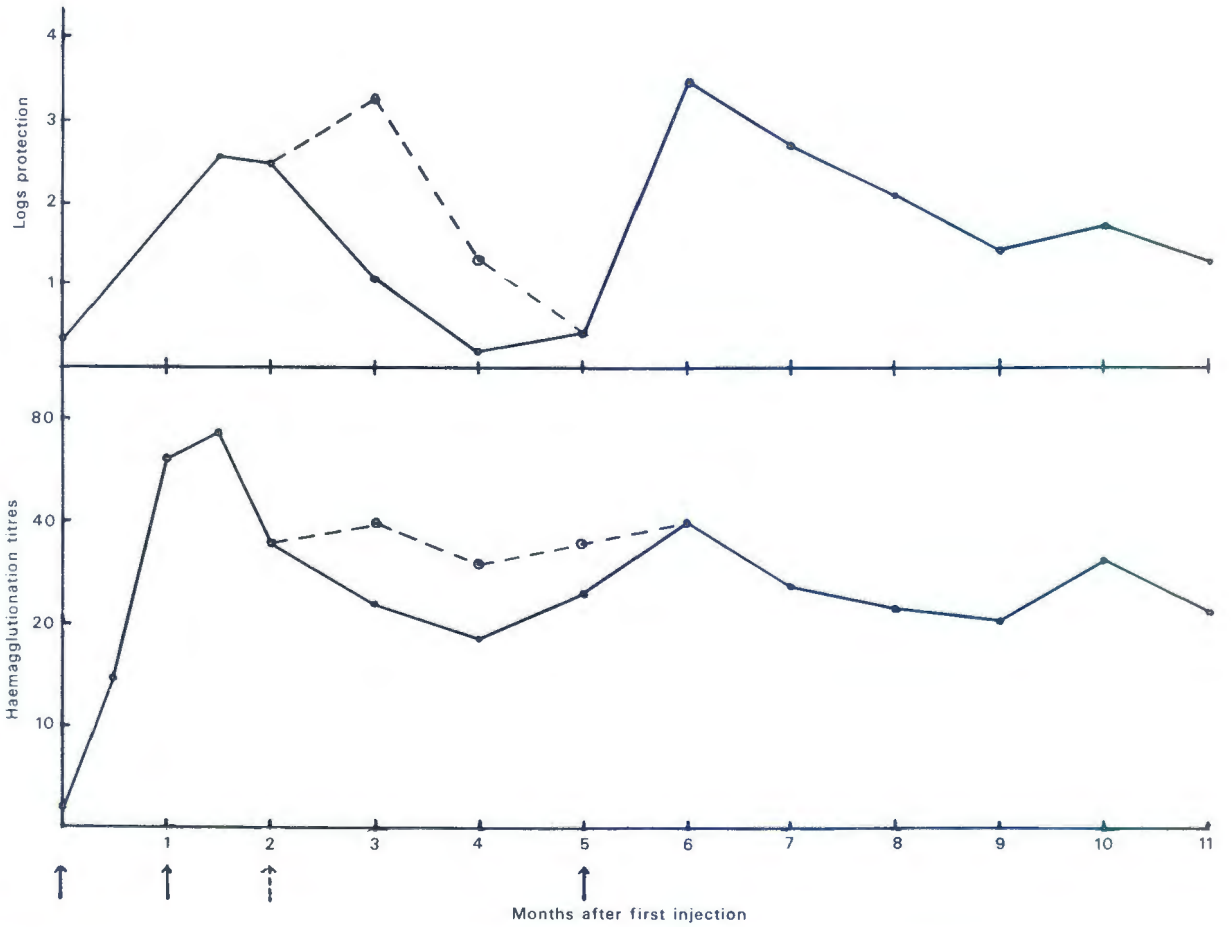

FIG. 5 Mean haemagglutination titres and passive protection values of sheep sera to *P. multocida* strain A14g (Type A) after administration of polyvalent vaccine. (Arrows indicate administration of vaccine and broken line indicates response of sheep which received three initial injections).

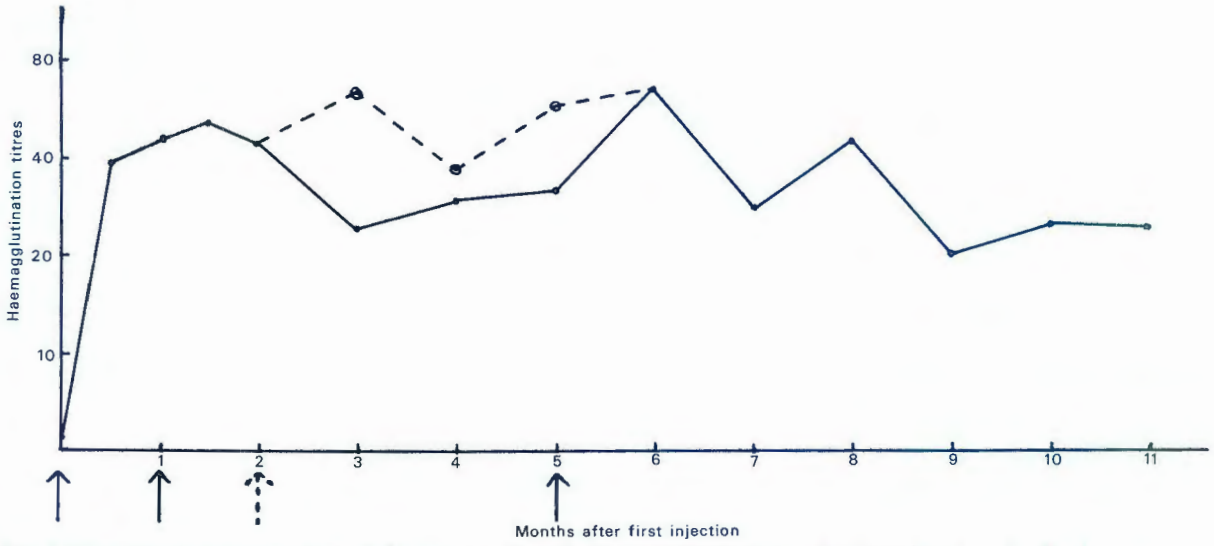

FIG. 6 Mean haemagglutination titres of sheep sera to *P. multocida* strain DI (Type D) after administration of polyvalent vaccine. (Arrows indicate administration of vaccine and broken line indicates response of sheep which received three initial injections).

TABLE 3 *Haemagglutination (HA) titres and passive protection values of sera from immunized sheep challenged with* P. multocida *strain A14g (Type A)*

	Sheep Number	HA titres at time of challenge	Result of challenge	Logs protection obtained in mice with pooled sera collected before challenge
Immunized group	24148	40	Survived	4.6
	24550	20	Survived	
	24395	40	Survived	
	24998	20	Survived	
	24232	20	Survived	
	24547	20	Died	
Control group	24810	10	Died	0.0
	24877	10	Died	
	24164	10	Died	
	24921	10	Died	

A similar experiment was conducted in which the sheep were challenged with strain DI (Table 4).

Although 50 per cent of the immunized sheep survived the challenge dose, it is obvious that the immunity obtained against strain DI is not nearly as solid as that obtained against strain A14g. There was no essential difference between the protective value of sera of immunized sheep that died and the controls. Apparently an increase of 0.3 logs is sufficient to afford some protection.

Immune response to P. haemolytica

Attempts to conduct immunity tests with *P. haemolytica* in either mice or rabbits invariably resulted in failure. The pathogenicity of *P. haemolytica* for laboratory animals is notoriously poor (Carter, 1967) and it was impossible to infect laboratory animals consistently and effectively. Consequently the passive protection values of sheep sera could not be determined, and only the average haemagglutination titres obtained for the four strains over a period of 44 weeks are shown (Fig. 7). The titres varied considerably among the strains and in some instances three initial injections depressed the antibody level and delayed the secondary response.

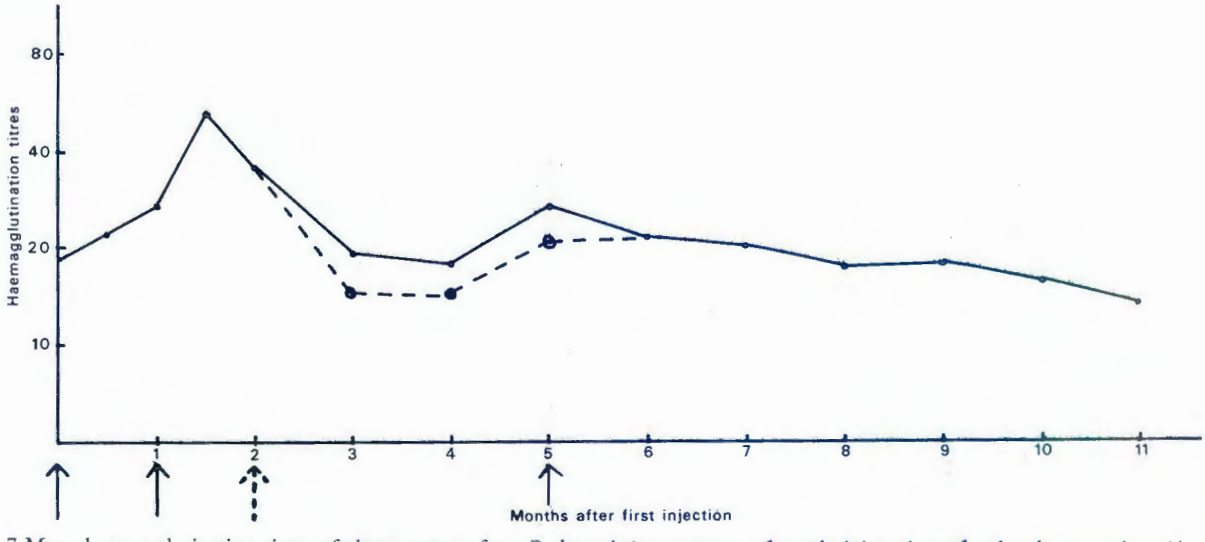

FIG. 7 Mean haemagglutination titres of sheep sera to four *P. haemolytica* serotypes after administration of polyvalent vaccine. (Arrows indicate administration of vaccine and broken line indicates response of sheep which received three intial injections).

TABLE 4 *Haemagglutination titres and passive protection values of sera from immunized sheep challenged with* P. multocida *strain DI (Type D)*

	Sheep Number	HA titres at time of challenge	Result of challenge	Logs protection obtained in mice with pooled sera collected before challenge
Immunized group	24488	40	Survived	2.2
	22751	20	Survived	
	24481	20	Survived	
	23649	40	Survived	
	24216	80	Survived	
	25329	80	Survived	
	24562	20	Died	1.9
	24194	20	Died	
	24997	20	Died	
	23396	80	Died	
	24529	320	Died	
Control group	24539	10	Survived	n.t.
	24493	10	Died	1.9
	24842	10	Died	
	26775	10	Died	
	26781	10	Died	
	24226	10	Died	

n.t. = not tested

Serological response of immunized sheep after challenge

The haemagglutination titres of immunized sheep at the time of challenge as well as their titres to the challenge strain 14 days after exposure are shown in Table 5. Both *P. multocida* strains A14g and DI stimulated a marked increase in the haemagglutination titres but the response elicited by all the serotypes of *P. haemolytica* was poor.

TABLE 5 *Serological response of immunized sheep after challenge with strains of* P. multocida *and* P. haemolytica

Sheep number	Challenge strain	Haemagglutination titres	
		At time of challenge	14 days after challenge
1	*P. multocida* A14g Type A	20	640
2	*P. multocida* A14g Type A	20	240
3	*P. multocida* A14g Type A	20	80
4	*P. multocida* DI Type D	40	640
5	*P. multocida* DI Type D	80	640
6	*P. multocida* DI Type D	80	640
7	*P. haemolytica* 129 Type 1	20	20
8	*P. haemolytica* 129 Type 1	20	80
9	*P. haemolytica* 4367 Type 4	10	20
10	*P. haemolytica* 4367 Type 4	10	10
11	*P. haemolytica* 14834 Type 6	20	10
12	*P. haemolytica* 14834 Type 6	10	10
13	*P. haemolytica* HI Type 7	20	80
14	*P. haemolytica* HI Type 7	10	40

DISCUSSION

The results presented in this paper show that sheep can be effectively immunized with polyvalent vaccine and are well protected against challenge with the homologous strain of *P. multocida*. To what extent this vaccine will afford protection to other strains of the same serotype is, however, not known. Namioka & Murata (1964) have shown that full cross immunity does not necessarily occur between strains of the same serotype and it has also been found that in the case of fowl cholera a positive serological test does not always indicate immunity (Heddleston, Watko & Rebers, 1964). On the other hand cross immunity to heterologous strains could be demonstrated in the absence of serological cross reactions (Heddleston & Watko, 1965). Evidence has also been presented by Yaw & Kakavas (1957) that the protective somatic antigens of strains of the same serotype may differ. Therefore, in order to assess the practical effectiveness of the present vaccine extensive immunological experiments will have to be done using a large number of *P. multocida* isolates from natural cases.

Similar problems are encountered with *P. haemolytica*. The organism has a low pathogenicity for laboratory animals (Carter, 1967) and immunological experiments with this organism are difficult and of doubtful validity (Knight, Biberstein & Allison, 1969). In order to conduct proper experiments it would be necessary to mix the organism with mucin or other substances which enhance its pathogenicity (Wessman, 1967). Biberstein & Thompson (1965) have shown that there is a close correlation between serotype and immunity, but Knight *et al.* (1969) found that some strains which were serologically different gave cross immunity, while other strains which were serologically identical did not. This latter finding supports a previous report in which it was shown that immunity could not always be correlated with the haemagglutination titre and that another antigen is probably also involved in eliciting immunity (Cameron, 1966).

One of the major shortcomings of the present vaccine is the fact that three initial injections are required to induce an immune response of reasonable duration. We have observed that strains differ widely in their antigenicity and a thorough search for strongly antigenic strains may provide a solution for the problem.

Another possibility for improving the vaccine would be to investigate the use of oil adjuvants more thoroughly. The adjuvant we used did not give particularly good results but the use of oil adjuvants is advocated by Carter (1961) and excellent results have been obtained with oil adjuvant vaccines for immunization against haemorrhagic septicaemia (Bain & Jones, 1955; Bain, 1956, 1962). It is known that the composition of oil adjuvants is critical (Roerink, 1966) and this aspect could probably be fruitfully investigated.

The duration of the immune response could possibly be lengthened by using live attenuated strains such as have been used with success in immunization against *P. tularensis* (McCoy & Chapin, 1912) by Nutter (1969), *P. pseudotuberculosis* (Pfeiffer, 1889) by Thal, Hanko & Knapp (1964), *P. pestis* (Lehman & Neumann, 1896) using *P. pseudotuberculosis* as antigen (Thal, Knapp & Hanko, 1967), and *Salmonella dublin* (White, 1930) by Botes (1964). The variants of *P. multocida* which have been described by Carter (1957), Yaw, Briefman & Kakavas (1956) and Heddleston *et al.* (1964) and the variants of *P. haemolytica* found by Biberstein, Meyer & Kennedy (1958) and Wessman (1964), may be useful in this respect. In fact Heddleston *et al.* (1964) have shown that loss of the capsule may result in marked loss of virulence but not of immunogenic antigens.

Another feature of our experiments was the absence of a classical booster effect after administration of the second and third initial injections and the injection given 3 months later. This was especially evident in the

case of the *P. haemolytica* strains. Similar phenomena have also been found by Clasener (1967). This phenomenon is due to the fact that antibody production is related to the level of circulating antibodies and that in this instance the ratio of existing antibodies to injected antigen was probably not optimal (Myers, 1969).

It is noteworthy that after challenge with virulent *P. multocida*, the animals which survived developed antibody titres of between 1/640 and 1/1280. This rapid and spectacular increase in antibody titre is probably an important aspect of the defence mechanism in immunized animals. Such a reaction would have been of particular value in the case of *P. haemolytica* where the post-vaccinal titres often remained rather low, but it was found that even after injection of live bacteria there was only a slight and irregular elevation of the haemagglutination titres.

Summary

Immunization of sheep with polyvalent *Pasteurella* vaccine resulted in a sound immunity to challenge with *P. multocida* strain A14g (Type A) and a marked increase in resistance to challenge with strain DI (Type D). The vaccine also induced a very good immunity in mice to these two strains.

When three initial injections were given to sheep, a good antibody response was produced which lasted for 20 weeks. A booster injection at this stage resulted in an increase in both haemagglutination titres and passive protection values which persisted for a further 24 weeks. In view of the fact that exposure of immunized animals to infection results in rapid antibody production, even a low titre should contribute materially to protection against infection.

The antibody response to *P. haemolytica* strains was poorer and varied from strain to strain.

The use of strains with greater antigenic properties with a superior oil adjuvant, or live vaccines prepared from avirulent strains, may give better results.

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