Onderstepoort J. vet Res. 39(3), 139–146 (1972)

CHARACTERIZATION OF EIGHT ROUGH MUTANTS OF *SALMONELLA GALLINARUM*

C. M. CAMERON, W. J. P. FULS and LUCILLE VAN REENEN, Department of Bacteriology, Veterinary Research Institute, Onderstepoort

ABSTRACT

CAMERON, C. M., FULS, W. J. P. & VAN REENEN, LUCILLE. Characterization of eight rough mutants of *Salmonella gallinarum*. *Onderstepoort J. vet. Res.*, 39(3), 139–146 (1972).

The cultural, serological and immunological properties of eight rough mutants of *S. gallinarum* were compared with the parent smooth strains as well as with a reference rough mutant (*S. gallinarum* strain 9R).

The rough mutants could be readily differentiated from the smooth ones by means of their ability to agglutinate in acriflavine, but they could not be distinguished from each other although they were obviously not identical.

One of these mutants [5503(2)] was selected as a standard vaccine strain on the basis of its excellent immunizing properties and low virulence but some of the other mutants would also have been satisfactory.

The nature of the immunity induced by these mutants is discussed with particular reference to the possible role of cellular immunity and cytophilic antibody.

INTRODUCTION

It is common knowledge that *Salmonella* bacteria readily undergo smooth ⟶ rough (S ⟶ R) mutation. These mutations are due to genetic changes which result in a loss of specific cell wall 'O' polysaccharide antigenic determinants. Various degrees of 'R' mutation may occur and this results in a greater or lesser loss of 'O' polysaccharides and leads to the exposure of 'R' polysaccharides with their own specificity. These mutants can be characterized by the chemical composition of their cell walls and specific chemotypes are thus recognised (Lüderitz, Staub & Westphal, 1966).

Loss of 'O' antigens is accompanied by loss of virulence (Archer & Rowley, 1969) and 'R' mutants are able to induce a protective immunity. The immunogenicity of a particular mutant is, however, not directly related to the degree of cell wall degradation (Kenny & Herzberg, 1968; Edebo & Normann, 1970; Germanier, 1970; Jarolmen, Hewel & Kain, 1971).

These properties have been put to practical use and most effective vaccines have been developed against fowl typhoid (Smith 1956a & b) and calf paratyphoid (Botes, 1964).

The ideal strain selected for fowl typhoid vaccine should be of low virulence and yet induce a solid protection. In addition it should not stimulate the formation of appreciable amount of 'O' antibody as this would interfere with the standard BWD plate test. Furthermore, its properties should be stable and easily differentiated from 'S' strains as well as other 'R' mutants. Finally, the vaccine strain should be able to multiply in the host (Nakano & Saito, 1969), but it need not persist indefinitely (Germanier, 1970).

The object of this investigation was to find an 'R' mutant which would fulfil the above requirements. A number of mutants were therefore produced and studied, particular attention being paid to characteristics which would differentiate them from 'S' strains and from each other.

Particular attention was also paid to their suitability as vaccine strains in terms of safety and immunogenicity.

MATERIALS AND METHODS

Strains

The two virulent strains of *S. gallinarum* used in this study were isolated from extensive outbreaks of fowl typhoid in chickens. They were designated BV and KV respectively and maintained on Dorset egg medium (Cruickshank, 1965).

Strains 7139 and 5503 are rough mutants which were available in the laboratory (see below) while strain 9R was isolated from commercial vaccine* (Smith, 1956a).

Induction and selection of mutants

Three methods were used to obtain rough mutants from the virulent parent strains:

(a) *Ultra violet irradiation:* The two parent strains BV and KV were cultured on nutrient agar for 24 h at 37°C and the growth suspended in distilled water to give a density of approximately 5×10^8 bacteria per ml. Twenty ml of this suspension was placed in a 9 cm diameter glass petri plate and exposed to ultra violet light in an apparatus designed by French & Erasmus (Veterinary Research Institute, Onderstepoort, personal communication 1971). Samples (0,1 ml) were taken after 1; 2; 3; 4; 5; 10; 20 & 30 min. exposure and inoculated into tubes of nutrient broth containing 1,0; 0,5; 0,2 and 0,1% hyperimmune bovine anti *S. gallinarum* serum (see below).

The tubes were incubated overnight at 37°C and subcultures from those showing visible growth were made into tubes containing the same concentration of hyperimmune antiserum. This procedure was repeated once more.

Thereafter subcultures of each tube were made on nutrient agar plates and ten single colonies selected which were subcultured on to nutrient agar slopes. These cultures were tested for their ability to agglutinate in 1:5 000, 1:10 000, 1:20 000 and 1:40 000 acriflavine (Braun & Bonestell, 1947). Subcultures for single colonies were prepared from those cultures which showed agglutination and the process repeated until all ten colonies from a particular culture showed a homogeneous degree of agglutination in the different concentrations of acriflavine.

The mutants thus obtained were maintained on Dorset egg agar and also lyophilized.

(b) *Exposure to nitrous acid:* Buffered nitrous acid solution was prepared by adding 1,72 g sodium nitrite

*"Monevax" - Wellcome Research Laboratories, Beckenham, England

Received 7 July 1972 - Editor

to 100 ml 1M sodium acetate solution (pH 4,6) just before use. The BV and KV strains were incubated in broth overnight and 5 ml of each culture was centrifuged, and the sedimented bacteria suspended in 5,0 of the buffer solution.

Samples were taken at the same time intervals as indicated under the irradiation procedure and inoculated into nutrient agar broth containing 1,0% hyperimmune *S. gallinarum* antiserum.

Further selection of rough mutants was done exactly as outlined above for the ultra violet irradiation procedure.

(c) *Subculture in hyperimmune serum or minimal medium:* The mutants obtained by these methods were prepared by Botes (1965, personal communication). Mutant 7139 was isolated from a smooth strain of *S. gallinarum* which had been subcultured 57 times in the presence of 5% hyperimmune serum, while mutant 5503 was recovered after passaging a smooth strain on minimal medium 70 times.

Both mutants were subcultured and single colonies examined for their ability to agglutinate in acriflavine. Repeated selection was carried out in this way until a homogeneous population was obtained.

Agglutination in acriflavine

Solutions of acriflavine were prepared in distilled water to give doubling dilutions from 1:500 to 1:40 000. In order to assess the ability of cultures to agglutinate in these solutions, a loop of culture was mixed with a large drop of acriflavine solution on a microscope slide. The slide was rotated gently and the degree of agglutination recorded after 30 sec. The interpretation was arbitrarily based on the rapidity and intensity of the reaction.

Growth inhibition

The inhibitory effect of malachite green and crystal violet was examined by a modification of the procedure described by Schmidt, Schlecht & Westphal (1969).

Sixty ml of D1,5 agar (Schlecht & Westphal, 1966) was poured into 14 cm diameter petri plates and allowed to solidify in a slanted position at 4°C overnight. Hereafter 60 ml of D1,5 agar containing either 50; 75; 100; 200 or 400 μg/ml of dye was overlaid on the slant and the plates again kept at 4°C overnight. The following morning the plates were kept at 37°C for 6 h when they were streaked down the centre of the dye gradient from a 6 h old actively growing culture. The plates were incubated at 37°C overnight and the distance of growth measured.

Biochemical tests

A full range of biochemical tests for identification of *Salmonella* was done on each mutant according to the methods of Edwards & Ewing (1962).

Antibiotic sensitivity

The sensitivity of the parent strains and the mutants was determined on AST agar (Oxoid)* using the disc method. Sensitivity discs (Difco)** containing low concentrations of antibiotics were used.

Serological methods

(a) *Preparation of hyperimmune antiserum:* The hyperimmune *S. gallinarum* antiserum was prepared in bovines by Botes (1965, Personal communication). A formalinized broth culture of *S. gallinarum* was used and 10 ml of this culture injected subcutaneously three times at weekly intervals.

The animals were bled 10 days after the last injection. The sera were pooled, a few drops of chloroform added as preservative, and stored at 4°C.

Antisera to the parent strains and the mutants were prepared in rabbits. The procedure used was based on the findings of Schlecht & Westphal (1967). For this purpose bacteria were grown on D1,5 agar at 37°C overnight and suspended in 0,85% NaCl to a density of 1 to 2 $\times$ 10^{10} bacteria per ml. The suspension was heated to 94°C for 3 h and cultures made to ascertain that all the organisms were dead.

Two rabbits were immunized with each strain. They were given 0,25 ml; 0,5 ml and 1,0 ml of the suspension intravenously at 5 day intervals and were bled 4 days after the last injection. The sera were stored at —20°C.

(b) *Haemagglutination tests:* Sheep erythrocytes (SRBC) were sensitized with polysaccharide extracts of *S. gallinarum* as described by Landy, Sanderson & Jackson (1965). The polysaccharide was prepared according to the procedure outlined by Buxton & Allan (1963) and treated with alkali as described by Ravin, Rowley, Jenkin & Fine (1960).

Sera were titrated and the test itself conducted according to the technique described by Buxton (1959) using Cooke microtitre equipment.* The haemagglutination (HA) titre was taken as the last well showing complete agglutination.

(c) *Haemagglutination inhibition:* Polysaccharides prepared from the parent strains and the mutants were dissolved in saline (0,2 mg/ml) and serial twofold dilutions prepared in 0,05 ml volumes using Cooke microtitre equipment.

Immune serum to strain BV prepared in rabbits with an HA titre of 1:2 048 was diluted 1:500, and 0,05 ml added to each well. The mixtures were allowed to stand at room temperature for 30 min and 0,05 ml SRBC sensitized with polysaccharide from strain BV was added to each well. The end point of the titration was taken as the highest dilution of polysaccharide which gave complete inhibition of agglutination. Each polysaccharide preparation was tested nine times and the end point taken as the mean dilution giving total inhibition of haemagglutination.

(d) *Tube agglutination tests:* Serial twofold dilutions of serum in 0,85% NaCl were prepared in Dryer tubes in 0,5 ml volumes starting at a dilution of 1 in 5. Antigen was prepared by inactivating a suspension of *S. gallinarum* in 80% alcohol at 55°C for 2 h. The killed organisms were collected by centrifugation and resuspended in 0,85% NaCl to a density corresponding to Brown's opacity tube No. 3.** Antigen (0,5 ml) was added to each tube and the tubes incubated at 42°C for 6 hours. The end point was taken as the highest dilution of serum showing complete agglutination.

(e) *Absorption of BV pooled antisera:* The rabbit antiserum was absorbed with the different strains essentially as described by Edwards & Ewing (1962).

Bacteria for absorption were grown in Mason tubes on D1,5 agar at 37°C overnight and harvested by means of a curved glass rod. The serum was diluted and 5 ml aliquots absorbed as follows:

*Oxoid Ltd., London
**Difco Laboratories, Detroit, Michigan

*Cooke Engineering Company, Alexandria, Virginia, USA
**Burroughs Wellcome & Co., London

Aliquot	Dilution of serum	Number of tubes used for absorption
1.	1 : 5	1
2.	1 : 10	1
3.	1 : 10	2
4.	1 : 10	5
5.	1 : 10	10

After absorption of the antiserum with the various strains at different levels as indicated above, the residual titre was assayed by haemagglutination and tube agglutination methods.

Serological response of chickens to rough mutants of S. gallinarum

Groups of twelve, 12-week old New Hampshire chickens were injected with each of the mutants. Every bird was given approximately 10^8 live bacteria subcutaneously and bled 14 days later. The sera were assayed by means of the standard BWD slide agglutination test using strained antigen, tube agglutination and haemagglutination.

Pathogenicity of 'R'-mutants for 7-day old chickens

The pathogenicity of the virulent parent strains and the 'R'-mutants was investigated in 1-week old chickens. The strains to be tested were grown on D1,5 agar at 37°C overnight, collected and suspended in 1,0% tryptone water* to a density of 5×10^9 organisms per ml. Tenfold serial dilutions were made in tryptone water and four chickens were given 0,2 ml of each dilution intravenously. Deaths were recorded daily for 7 days.

Immunization and challenge of adult chickens

The immunizing properties of the 'R'-mutants were assayed in groups of 12-week old New Hampshire chickens. Bacterial suspensions were prepared as for the pathogenicity experiments and dilutions made in tryptone water to give 2×10^7, 5×10^7, 1×10^8 and 5×10^8 live organisms per ml.

For each strain, four chickens were given 1,0 ml of each dilution subcutaneously and challenged 2 weeks later. In a repeat experiment four more birds were immunized with 10^8 organisms of some of the strains.

* Tryptone (Oxoid) – 100 g
$Na_2HPO_4.12H_2O$ – 20 g
NaCl – 30 g
Dist. water – to 1000 ml

The birds were challenged *per os* as described by Smith (1956a) except that the dose was approximately 2×10^8 live bacteria instead of 5×10^7. Deaths were recorded for 14 days and the aetiology confirmed by bacteriological examination.

Results

A *Mutants obtained*

Only a single mutant was obtained by ultra violet irradiation. This mutant originated from strain BV and was designated BUV. Many more mutants were obtained by the nitrous acid method. Of these, two originated from strain BV and two from strain KV. They were designated B1, B2, K1 and K2 respectively. The procedures used to isolate these particular mutants are summarized in Table 1.

B *Agglutination of mutants in acriflavine solutions*

The degree of agglutination exhibited by the mutants was determined repeatedly at the time of isolation. Mutant 5503 was found to be composed of two slightly different types. The one agglutinated in 1:10 000 acriflavine while the other did not and they were designated 5503(1) and 5503(2) respectively.

The other mutants also differed in their reaction in acriflavine (Table 2). Mutants B1 and K2 agglutinated strongly in 1:10 000 acriflavine while mutants B2 7139 and 9R did not. Mutants BUV and K1 gave intermediate reactions.

These characteristics were fairly stable although some changes did occur on storage. The ability to agglutinate remained almost unchanged after a year's storage on Dorset egg medium or when the cultures were lyophilized. Cultures in semisolid agar, however, tended to become rougher and this method of storage was therefore unsatisfactory.

C *Cultural properties of mutants of* S. gallinarum

(i) *Growth inhibition by crystal violet:* The results presented in Fig. 1 show that the growth of all the mutants is inhibited at a dye concentration of 75 μg/ml and they can be differentiated from the parent strains on this basis. Although the growth of mutants BUV and 9R is inhibited to a lesser extent than the other mutants, these differences are not large enough to differentiate between them and the other mutants.

(ii) *Biochemical properties:* The parent strains as well as all the mutants had biochemical properties typical for *S. gallinarum* and could therefore not be differentiated on this basis.

TABLE 1 Summary of the procedures used to isolate 'R' mutants of *S. gallinarum*

Mutant	Method of induction	Method of Selection
BUV	UV light 2 min	Subculture in 1,0% immune serum 3 x. Single colonies selected 3 x
B1	HNO_2 1 min	Subculture in 1,0% immune serum 1 x. Single colonies selected 3 x
B2	HNO_2 2 min	Subculture in 1,0% immune serum 1 x. Single colonies selected 3 x
K1	HNO_2 20 min	Subculture in 1,0% immune serum 1 x. Single colonies selected 3 x
K2	HNO_2 5 min	Subculture in 1,0% immune serum 1 x. Single colonies selected 3 x
*7139	—	Subculture in 5% immune serum 57 x. Single colonies selected 2 x
*5503(1)	—	Subculture in minimal medium 70 x. Single colonies selected 2 x
*5503(2)	—	Subculture in minimal medium 70 x. Single colonies selected 2 x

*Botes (1965).

TABLE 2 *Effect of storage on the agglutination of 'R' mutants of* S. gallinarum *in acriflavine*

Mutant	Acriflavine concentration	At time of preparation	Dorset egg 1 year	Lyophilized 1 year	Semi solid agar 1 year
BUV	1: 5 000	++++	++++	++++	++++
	1:10 000	++	+	+	++++
	1:20 000	—	—	—	++++
B1	1: 5 000	++++	++++	++++	++++
	1:10 000	++++	+	+	++
	1:20 000	—	—	—	—
B2	1: 5 000	++++	++++	++++	++++
	1:10 000	—	++	++	++++
	1:20 000	—	—	—	+++
K1	1: 5 000	++++	++++	++++	++++
	1:10 000	+	+++	++++	++++
	1:20 000	—	—	+	—
K2	1: 5 000	++++	++++	++++	++++
	1:10 000	++++	++	++	++++
	1:20 000	—	—	—	—
7139	1: 5 000	++	+++	++	Died
	1:10 000	—	—	—	
	1:20 000	—	—	—	
5503(1)	1: 5 000	++++	++++	++++	++++
	1:10 000	++++	++	—	++
	1:20 000	—	—	—	—
5503(2)	1: 5 000	+++	+++	+++	++
	1:10 000	—	—	+	—
	1:20 000	—	—	—	—
9 R	1: 5 000	++++	++++	++++	+++
	1:10 000	—	—	—	—
	1:20 000	—	—	—	—

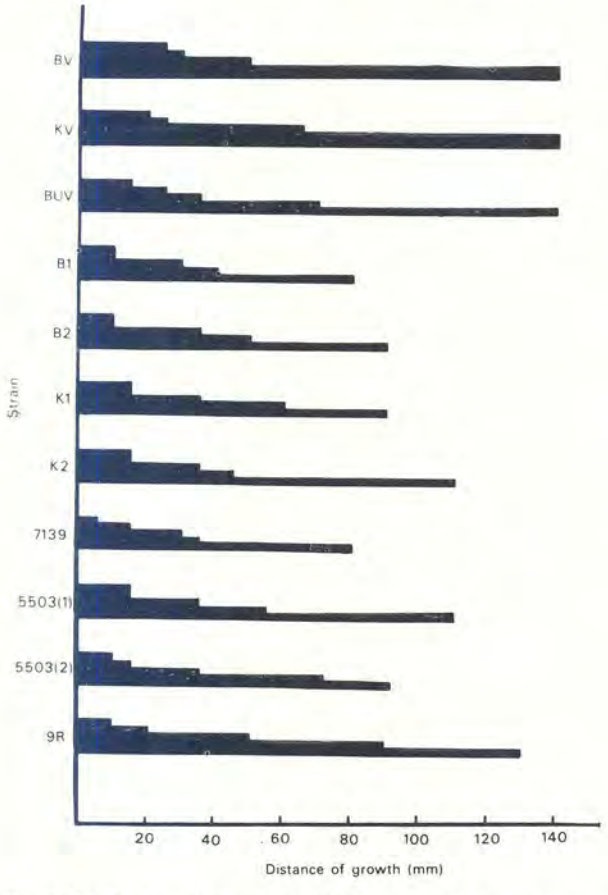

FIG. 1 Inhibition of growth of 'R'-mutants of *S. gallinarum* on media containing crystal violet. (400; 200; 100; 75 or 50 μg/ml)

(iii) *Antibiotic sensitivity:* All the mutants as well as the parent strains were equally resistant or sensitive to the antibiotics listed in Table 3.

TABLE 3 *Antibiotic sensitivity of parent and mutant strains of* S. gallinarum

Antibiotic	Concentration per disc	Degree of inhibition
Erythromycin	2 mcg	—
Kanamycin	5 mcg	++++
Neomycin	5 mcg	++++
Tetracycline	5 mcg	++
Aureomycin	5 mcg	+++
Terramycin	5 mcg	+
Streptomycin sulphate	2 mcg	—
Dihydrostreptomycin	2 mcg	—
Penicillin	2 units	—
Phenethicillin	3 units	—
Ampicillin	2 mcg	—
Methicillin	5 mcg	—
Oxacillin	2 mcg	—
Cloxacillin	1 mcg	—
Chloromycetin	5 mcg	++
Coly-Mycin	2 mcg	+
Bacitracin	2 units	—
Polymyxin	50 units	++
Furadantin	50 mcg	++++
Sulphadiazine	50 mcg	—
Bactrin	25 μg	++++

D *Serological properties of mutants of* S. gallinarum

(i) *Antibody response of chickens to mutants of* S. gallinarum: A single injection produced some degree of antibody response when measured by means of haemagglutination tests (Table 4). When a tube

TABLE 4 *Serological response of 12-week old New Hampshire chickens to 10^8 live organisms of 'R' mutants of* S. gallinarum

Mutant	BWD Slide test	Tube agglutination		Haemagglutination	
	Number positive	Number positive	Average titre of positives	Number positive	Average titre of positives
BUV	0/12	0/12	—	4/12	1:5
B1	0/12	2/12	1:10	3/12	1:4
B2	0/12	0/12	—	7/12	1:4
K1	0/12	nt	.	nt	.
K2	5/12	5/12	1:208	8/12	1:25
7139	0/12	nt	.	nt	.
5503 (1)	0/12	1/12	1:10	8/12	1:5
5503 (2)	2/12	2/12	1:180	7/12	1:22
9R	0/12	0/12	—	9/12	1:6

nt = not tested

agglutination test was used no antibodies could be detected in the sera of chickens injected with mutants BUV, B2 or 9R while five out of 12 chickens responded to mutant K2. Antibodies could only be detected by means of the standard BWD slide tests in those groups which had been injected with mutants K2 and 5503(2). In the latter case only 2 out of 12 chickens were positive.

(ii) *Cross reactions as measured by means of a haemagglutination test:* Immune sera were prepared in rabbits and the titres determined against all the mutants and the parent strains. The results are summarized in Table 5.

The parent strains (BV and KV) reacted in a high titre with the homologous antisera and to a greater or lesser degree with the antisera to most of the mutants. This indicates that they all have some residual 'S' polysaccharide and are therefore able to induce some 'S' antibody notably the mutants BUV, and B2. On the other hand the majority of mutants only reacted either in a low titre with their homologous antisera or, more often, not at all. Furthermore, with the exception of mutants B1 and B2 they did not react with antisera of the parent strains.

This lack of reactivity of the mutants might be due to unsatisfactory sensitization of the SRBC. In an attempt to overcome this possibility, treatment of the polysaccharide with alkali was omitted as suggested by Ciznar & Shands (1970) but similar results were obtained.

Other authors have, however, used slightly different test procedures which may give better results (Kenny & Schlecht, 1971). The use of a milder extraction procedure might also be beneficial for 'R' polysaccharides (Galanos, Lüderitz & Westphal, 1969).

(iii) *Haemagglutination inhibition:* According to the results of these experiments, which are illustrated in Fig. 2, polysaccharide from all the mutants with the exception of 7139 was able to inhibit haemagglutination of SRBC sensitized with polysaccharide from strain BV. In this respect mutant BUV was the most efficient, suggesting a close relationship to the smooth strains.

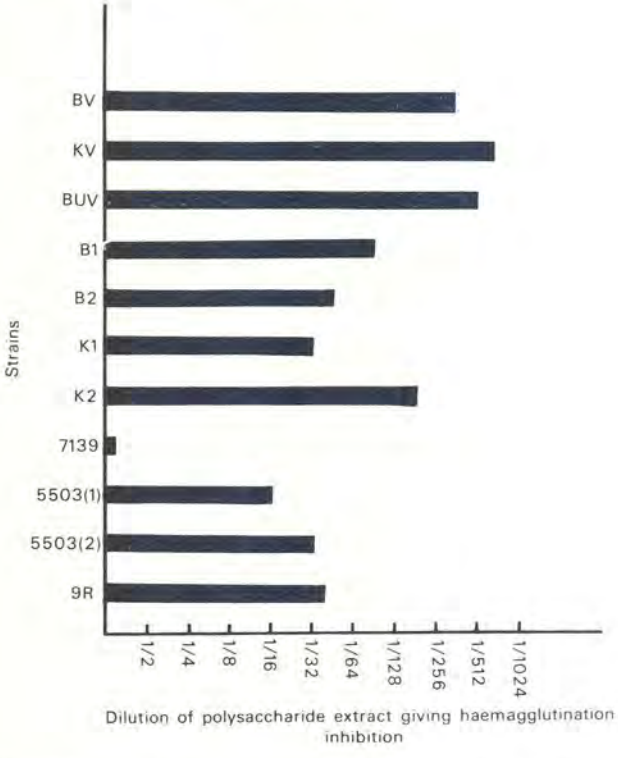

FIG. 2 Haemagglutination inhibition by polysaccharide extracts of 'R'-mutants of *S. gallinarum*

(iv) *Absorption tests:* Antiserum to strain BV prepared in rabbits was absorbed with whole bacteria of the different mutants and the residual titres assayed by means of a haemagglutination test as well as a tube agglutination test.

According to the results of the haemagglutination test mutants K1, K2 and 7139 were ineffective

TABLE 5 *Serological cross reactions of parent and of 'R' mutants of* S. gallinarum *measured by haemagglutination*

Antigens	Antisera										
	BV	KV	BUV	B1	B2	K1	K2	7139	5503(1)	5503(2)	9R
BV	1:2560	1:640	1:320	1:40	1:320	1:5	1:20	0	1:10	1:5	1:20
KV	1:2560	1:1280	1:640	1:160	1:640	1:320	1:40	1:10	1:80	1:160	1:40
BUV	0	0	0	0	0	0	0	0	0	0	0
B1	0	1:10	1:10	1:20	1:10	1:10	1:10	0	1:10	1:10	0
B2	10	1:40	1:40	1:160	1:60	1:80	1:40	0	1:40	1:40	1:20
K1	0	0	0	1:10	0	1:10	0	0	1:10	0	1:10
K2	0	0	0	0	0	0	0	0	0	0	0
7139	0	0	0	0	0	0	0	0	0	0	0
5503(1)	0	0	0	0	0	0	0	0	0	0	0
5503(2)	0	0	0	0	0	0	0 0	0	0	0	0
9R	0	0	0	0	0	0	0	0	0	0	0

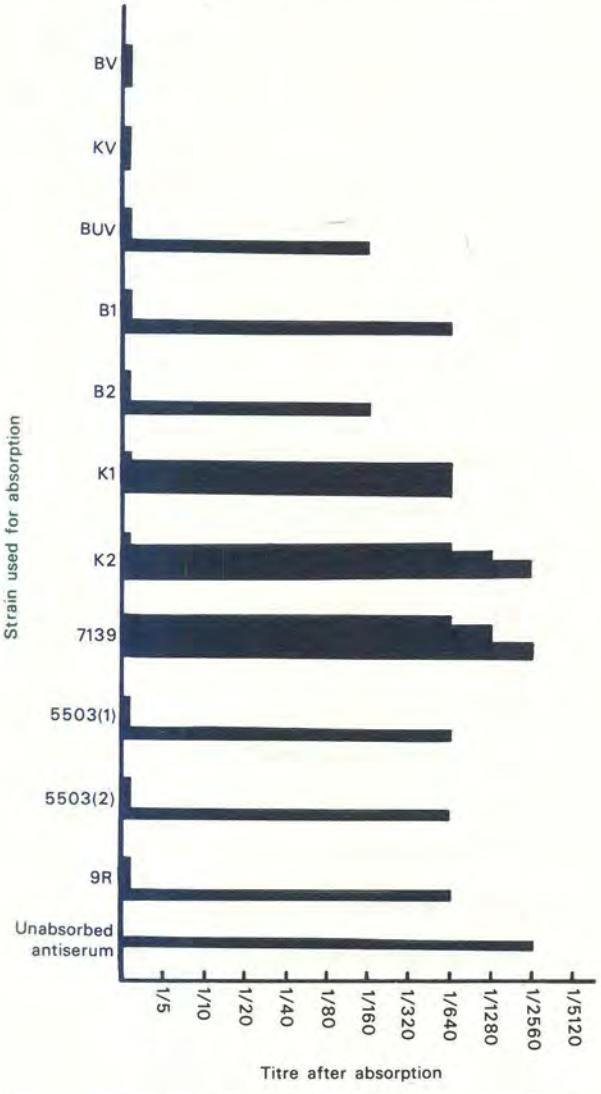

FIG. 3 Residual haemagglutination titres of *S. gallinarum* BV antiserum after absorption with whole bacteria

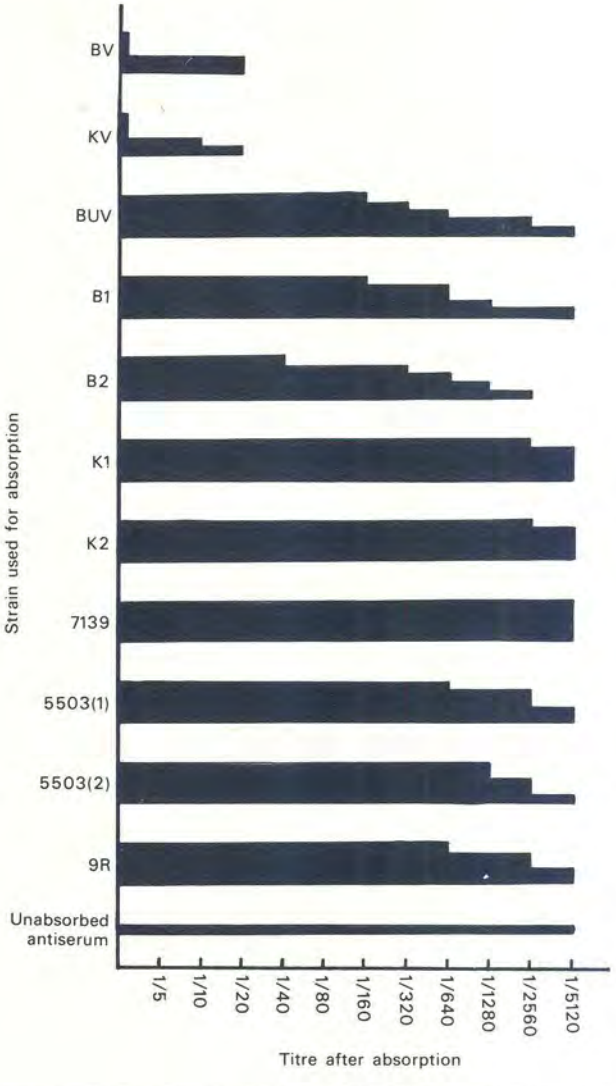

FIG. 4 Residual agglutination titres of *S. gallinarum* BV antiserum after absorption with whole bacteria

in absorbing antibodies while the other mutants were quite effective (Fig. 3).

Similar results were obtained with agglutinin assays but the differences were less clear cut (Fig. 4).

These results also indicate that mutants K1; K2 and 7139 are not serologically closely related to the smooth parent strain.

E. *Biological and immunological characteristics of mutants of* S. gallinarum

(i) *Virulence of mutants:* The results of titrations of the mutants in 1-week old chickens are presented in Table 6.

All the mutants were markedly less virulent than strain BV. The most virulent mutants were K1 and

TABLE 6 *Comparative virulence of 'R' mutants of* S. gallinarum *for 7-day old New Hampshire chicks*

	Mutants									
	BV	BUV	B1	B2	K1	K2	7139	5503(1)	5503(2)	9R
Dose	Deaths out of four									
10^9	4	4	4	4	4	4	4	4	4	0
10^8	4	2	2	3	4	4	3	1	0	0
10^7	4	2	2	3	4	4	3	1	1	0
10^6	4	2	1	4	3	4	4	0	0	0
10^5	4	0	0	0	4	4	0	0	0	0
10^4	4	0	0	0	2	2	0	0	0	0
10^3	4	0	0	0	0	0	0	0	0	0

TABLE 7 *Immunizing properties of 'R' mutants of* S. gallinarum

	Mutants used for immunization									
	BUV	B1	B2	K1	K2	7139	5503(1)	5503(2)	9R	Control
	Survivors after challenge									
5×10^8	4/4	4/4	3/4	4/4	4/4	2/4	4/4	3/4	3/4	
1×20^8	8/8	6/8	8/8	3/4	8/8	2/4	6/8	8/8	5/8	
5×10^7	4/4	3/4	3/4	nt	4/4	nt	3/4	4/4	4/4	
2×10^7	3/4	2/4	4/4	nt	4/4	nt	2/4	4/4	3/4	
0	—	—	—	—	—	—	—	—	—	2/20

K2; an intermediate position was occupied by BUV, B1, B2 and 7139 and the least virulent mutants were 5503(1), 5503(2) and 9R.

(ii) *Immunizing properties:* The immunizing properties of the mutants were tested at four different levels. As shown by the results summarized in Table 7 all the mutants except 7139 gave a very good immunity at dosages of 5×10^7 or higher.

SUMMARY AND DISCUSSION

The results presented in this paper confirm earlier reports on the excellent immunity to fowl typhoid which can be obtained when chickens are immunized with a vaccine prepared from suitable avirulent 'R' mutants (Smith, 1956a; Botes, Veterinary Research Institute, Onderstepoort, unpublished data, 1965).

The 'R' mutants can be easily differentiated from the parent strains. This can be most readily accomplished by the acriflavine test and is confirmed by a series of serological investigations. The results of these experiments show that, although the mutants described are not identical, their characteristics overlap considerably and they cannot be clearly differentiated individually. This can probably only be accomplished by chemical analysis (Schmidt, Schlecht, Lüderitz & Westphal, 1969) or phage typing (Davies & Sojka, 1971).

The mutant 5503(2) was selected for routine vaccine production because it produced very solid immunity and according to the acriflavine test its degree of roughness remained stable. With the exception of the reference mutant 9R it had the lowest virulence of all the mutants examined.

Its only disadvantage was that it gave a positive BWD reaction in a few chickens, but with practical experience it has been found that such reactions disappear soon and do not interfere with routine testing (Coetzee, Veterinary Research Institute, Onderstepoort, personal communication 1970).

The nature of immunity to fowl typhoid is not known. A solid immunity is obtained following immunization with 'R' mutants in the virtual absence of serum antibodies. Conventional serum antibodies and humoral immune mechanisms may nevertheless be involved, but are not detectable by standard serological techniques which only involve cell wall antigens. In fact, several authors have isolated labile immunogenic antigens from Salmonellae (Jenkin & Rowley, 1965; Kawakami, Osawa & Mitsuhashi, 1966; Milne & Collins, 1966; Venneman & Bigley, 1969), and unless such antigens are employed in serological tests, the corresponding antibodies responsible for protection will not be detected.

Many authors, however, consider the immunity to *Salmonella* infections following administration of live vaccine to be cellular in nature (Mitsuhashi, Kawakami, Yamaguchi & Nagai, 1958; Collins, 1969) and mediated through activated macrophages (Mitsuhashi, Sato & Tanaka, 1961; Mackaness, Blanden & Collins, 1966; Blanden, Mackaness & Collins, 1966).

The concept of cellular immunity is widely accepted as being an important feature in protection against infection (Mackaness & Blanden, 1967) but the exact mechanism is not yet understood. Jenkin, Rowley & Auzins (1964) and Turner, Jenkin & Rowley (1964) have stressed the importance of antibody and subsequently showed that this antibody is cytophilic in nature (Rowley, Turner & Jenkin, 1964). They conclude that immunity to *S. typhimurium* infection in mice is dependent on an increased bactericidal activity of the macrophage population which requires specific antibody for its expression (Rowley, Auzins & Jenkin, 1968). The role of cytophilic antibody in the expression of cellular immunity is also supported by Kurashige, Osawa, Kawakami & Mitsuhashi (1965, 1967).

If it was possible to establish the nature of the immunity to fowl typhoid, an *in vitro* test could possibly be developed to assess the immune status of an immunized chicken. Such a test would allow a time study of the immune response in experimental animals and this possibility is currently being investigated.

ACKNOWLEDGEMENT

We wish to thank Dr L. Coetzee for his suggestions during the course of this work and Mr G. J. de Ridder for generously supplying the experimental chickens.

REFERENCES

ARCHER, J. R. & ROWLEY, D., 1969. A quantitative comparison of the antigenic structure of a virulent and an avirulent strain of *Salmonella typhimurium*. *Immunology*, 17, 551–558.

BLANDEN, R. V., MACKANESS, G. B. & COLLINS, F. M., 1966. Mechanisms of acquired resistance in mouse typhoid. *J. exp. Med.*, 124, 585–600.

BOTES, H. J. W., 1964. Calf paratyphoid immunity: Evaluation of formol-killed and live attenuated vaccines. *Bull. Off. int. Epiz.*, 62, 22–25.

BRAUN, W. & BONESTELL, AILEEN, E., 1947. Independent variation of characteristics in *Brucella abortus* variants and their detection. *Am. J. vet. Res.*, 8, 386–390.

BUXTON, A. & ALLAN, D., 1963. Studies on immunity and pathogenesis of Salmonellosis. I. Antigen-antibody reactions on circulating leucocytes of chickens infected with *Salmonella gallinarum*. *Immunology*, 6, 520–529.

BUXTON, A., 1959. The *in vivo* sensitization of avian erythrocytes with *Salmonella gallinarum* polysaccharide. *Immunology*, 2, 203–210.

CIZNAR, I. & SHANDS, J. W., 1970. Effect of alkali on the immunological reactivity of lipopolysaccharide from *Salmonella typhimurium*. *Infec. Immun.*, 5, 549–555.

COLLINS, F. M., 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in non-vaccinated mice challenged by various routes. *J. Bact.*, 97, 667–675.

CRUICKSHANK, R., 1965. Medical Microbiology, 7th Ed. E. & S. Livingstone Ltd., Edinburgh and London.

DAVIES, G. & SOJKA, W. J., 1971. Identification of a vaccine strain of *S. dublin*. *Vet. Rec.*, 88, 432–433.

EDEBO, L. & NORMANN, B., 1970. Virulence and immunogenicity of mutant strains of *Salmonella typhimurium*. *Acta path. mibrobiol. scand. Section B.*, 78, 75–84.

EDWARDS, P. R. & EWING, W. H., 1962. Identification of Enterobacteriaceae, 2nd Ed. Burgess Publishing Co., Minneapolis, Minnesota, U.S.A.

GALANOS, C., LÜDERITZ, O. & WESTPHAL, O., 1969. A new method for the extraction of *R. lipopolysaccharides*. *Eur. J. Biochemistry*, 9. 235–249.

GERMANIER, R., 1970. Immunity in experimental Salmonellosis. I. Protection induced by rough mutants of *Salmonella typhimurium*. *Infec. Immun.*, 2, 309–315.

JAROLMEN, H., HEWEL, D. & KAIN, E., 1971. Immunogenicity of R factor-carrying *Salmonella*. *Infec. Immun.* 4, 274–280.

JENKIN, C. R. & ROWLEY, D., 1965. Partial purification of the "protective" antigen of *Salmonella typhimurium* and its distribution amongst various strains of bacteria. *Aust. J. exp. Biol. med. Sci.*, 43, 65–78.

JENKIN, C. R., ROWLEY, D. & AUZINS, I., 1964. The basis for immunity to mouse typhoid. 1. The carrier state. *Aust. J. exp. Biol. med. Sci.*, 42, 215–228.

KAWAKAMI, M., OSAWA, N. & MITSUHASHI, S., 1966. Experimental salmonellosis. VII. Comparison of the immunizing effect of live vaccine and materials extracted from *Salmonella enteritidis*. *J. Bact.*, 92, 1685–1689.

KENNY, KATHRYN & HERZBERG, M., 1968. Antibody response and protection induced by immunization with smooth and rough strains in experimental salmonellosis. *J. Bact.*, 95, 406–417.

KENNY, KATHRYN & SCHLECHT, S., 1971. Antibody response in rabbits to *Salmonella minnesota* R-mutants. *Zbl. Bakt. Hyg., I. Abt. Orig.*, A217, 183–197.

KURASHIGE, S., OSAWA, N., KAWAKAMI, M. & MITSUHASHI, S., 1965. Cell bound antibody in mouse mononuclear phagocytes immunized with live vaccine of *Salmonella enteritidis*. *Japan J. Bact.*, 20, 131.

KURASHIGE, S., OSAWA, N., KAWAKAMI, M. & MITSUHASHI, S., 1967. Experimental salmonellosis. X. Cellular immunity and its antibody in mouse mononuclear phagocytes. *J. Bact.* 94, 902–906.

LANDY, M., SANDERSON, R. P. & JACKSON, ANNE, L., 1965. Humoral and cellular aspects of the immune response to the somatic antigen of *Salmonella enteritidis*. *J. exp. Med.*, 122, 483–504.

LÜDERITZ, O., STAUB, A. M. & WESTPHAL, O., 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. *Bact. Rev.*, 30, 192–255.

MACKANESS, G. B., BLANDEN, R. V. & COLLINS, F. M., 1966. Host-parasite relations in mouse typhoid. *J. exp. Med.*, 124, 573–583.

MACKANESS, G. B. & BLANDEN, R. V., 1967. Cellular Immunity. *In:* Progress in Allergy. Vol. 11. Edited by P. Kallos and B. H. Waksman. S. Karger, Basel & N.Y.

MILNE, MARGARET & COLLINS, F. M., 1966. Heat-labile antigens of *Salmonella enteritidis*. 1. Extraction of antigens. *J. Bact.*, 92, 543–548.

MITSUHASHI, S., KAWAKAMI, M., YAMAGUCHI, Y. & NAGAI, M., 1958. Studies on the experimental typhoid. (1) A comparative study of living and killed vaccines against the infection of mice with *S. enteritidis*. *Japan J. exp. Med.*, 28, 249–258.

MITSUHASHI, S., SATO, I. & TANAKA, T., 1961. Experimental salmonellosis. Intracellular growth of *Salmonella enteritidis* ingested in mononuclear phagocytes of mice and cellular basis of immunity. *J. Bact.* 81, 863–868.

NAKANO, M. & SAITO, K., 1969. Chemical components in the cell wall of *Salmonella typhimurium* affecting its virulence and immunogenicity in mice. *Nature, London*, 222, 1085–1086.

RAVIN, H. A., ROWLEY, D., JENKIN, C. R. & FINE, J., 1960. On the absorption of bacterial endotoxin from the gastro-intestinal tract of the normal and shocked animal. *J. exp. Med.*, 112, 783–792.

ROWLEY, D., TURNER, K. J. & JENKIN, C. R., 1964. The basis for immunity to mouse typhoid. 3. Cell-bound antibody. *Aust. J. exp. Biol. med. Sci.*, 42, 237–248.

ROWLEY, D., AUZINS, I. & JENKIN, C. R., 1968. Further studies regarding the question of cellular immunity in mouse typhoid. *Aust. J. exp. Biol. med. Sci.*, 46, 447–463.

SCHLECHT, S. & WESTPHAL, O., 1966. Wachstum und lipopolysaccharid (O-Antigen) -gehalt von Salmonellen bei züchtung auf agarnährböden. *Zbl. Bakt. I. Orig.*, 200, 241–259.

SCHLECHT, S. & WESTPHAL, O., 1967. Über die Herstellung von Antiseren gegen die Somatished (O-) Antigene von Salmonellen. I. Mitteilung: Untersuchungen über Agglutinistiter. *Zbl. Bakt. I. Orig.*, 204, 335–355.

SCHMIDT, G., SCHLECHT, S., LÜDERITZ, O. & WESTPHAL, O., 1969. Untersuchungen zur typisierung von Salmonella-R-Formen. I. Mitteilung. Mikrobiologische und serologische Untersuchungen an *Salmonella minnesota*-Mutanten. *Zbl. Bakt. I. Orig.*, 209, 483–496.

SCHMIDT, G., SCHLECHT, S. & WESTPHAL, O., 1969. Untersuchungen zur typisierung von *Salmonella*-R-Formen. 3. Mitteilung. Typisierung von *S. minnesota*- Mutanten mittels chemischer Agenzien. *Zbl. Bakt. I. Orig.*, 212, 88–96.

SMITH, H. W., 1956a. The use of live vaccines in experimental *Salmonella gallinarum* infection in chickens with observations on their interference effect. *J. Hyg., Camb.*, 54, 419–432.

SMITH, H. W., 1956b. The immunity to *Salmonella gallinarum* infection in chickens produced by live cultures of members of the *Salmonella* genus. *J. Hyg. Camb.*, 54, 433–439.

TURNER, K. J., JENKIN, C. R. & ROWLEY, D. ,1964. The basis for immunity to mouse typhoid. 2. Antibody formation during the carrier state. *Aust. J. exp. Biol. med. Sci.* 42, 229–236.

VENNEMAN, M. R., & BIGLEY, NANCY, J., 1969. Isolation and partial characterization of an immunogenic moiety obtained from *Salmonella typhimurium*. *J. Bact.*, 100, 140–148.

Printed in the Republic of South Africa for the Government Printer, Pretoria, by Heer Printing Co. (Pty) Ltd., Pretoria.