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The immunization of mice, calves and pigs against *Salmonella dublin* and *Salmonella cholerae-suis* infections

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Following the development of live vaccines that protected chickens against *Salmonella gallinarum* infection (Smith, 1956*a*), attention has been turned to finding methods of immunizing calves against *S. dublin* infection and pigs against *S. cholerae-suis* infection, the principal salmonella infections of these two species of animals. Chickens were used in all the *S. gallinarum* studies. Those on *S. dublin* and *S. cholerae-suis* were rendered more difficult by the fact that it was not economically possible to use calves and pigs for the bulk of the experiments. These had to be performed on mice, part of the original plan being to find stable live vaccines which were avirulent for these animals but which protected them against oral infection with a fully virulent strain. Any such vaccines would finally be tested in calves or pigs. The limitations of this approach were realized when the vaccine most suitable in protecting mice against *S. dublin* infection, no. 17A, was found to be non-immunogenic in calves, whereas other vaccines which had been rejected initially because they possessed a considerable degree of virulence in mice were subsequently found to be both avirulent and immunogenic in calves.

This paper records the results of experiments to determine the immunogenicity of dead vaccines and antisera in mice, and the results obtained with live vaccines, experimentally with mice and both experimentally and against natural infections in the field with calves and pigs.

MATERIALS AND METHODS

Animals

Mice. Male White Swiss mice were used. They were fed on diet 41B (Oxoid) *ad lib.*

Pigs. Landrace × Large White pigs of both sexes were employed. They were kept under ordinary conditions of management and fed on a proprietary pig meal *ad lib.*

Calves. Unless otherwise stated, male Ayrshire calves were used. They had been weaned at approximately 1 week old and then fed solely on a proprietary liquid milk substitute twice daily. The ages of the mice, pigs and calves when their immunity to salmonella infection was challenged were 8, 11 and 6 weeks respectively.

Bacterial cultures

All vaccines, challenge inocula and agglutination suspensions were derived from smooth fully virulent strains of salmonellae, one strain each of *S. dublin*, no. 188, *S. cholerae-suis* var. *kunzendorf*, no. 195, *S. typhi-murium*, *S. gallinarum*, no. 9, and *S. pullorum* being used throughout. The smooth (S) and rough (R) nature of cultures were assessed by the acriflavine slide test (Braun & Bonestell, 1947).

Salmonella dublin *antisera*

Antisera were prepared in rabbits and calves. Those against dead bacteria were prepared by the multiple intravenous injection of a fully motile nutrient broth culture of strain 188 that had been killed at 56° C. for $\frac{1}{2}$ hr. Those against live bacteria were prepared by the multiple oral and subcutaneous administration of a 24 hr. nutrient broth culture of 188 to animals that had recovered from experimental infection. All sera were sterilized by filtration through a Seitz E.K. pad before use, and were given by subcutaneous injection 2 hr. before challenge.

Vaccines

The exact methods of preparing both live and dead vaccines will be referred to later; those for the 9S and 9R vaccines of *S. gallinarum* have been described previously (Smith, 1956*a*). All live vaccines were employed as single subcutaneous injections of 24 hr. nutrient broth cultures containing approximately 50×10^7 viable bacteria per ml. 3 weeks before challenge except in the experiments shown in Tables 5 and 8; 0·1 ml. were given to mice and 5·0 ml. to calves and pigs. Dead vaccines were employed as two subcutaneous injections of 0·1 ml. for mice and 5·0 ml. for pigs 3 and 2 weeks before challenge. Before vaccination the body temperature, appetite and general appearance of the calves and pigs were recorded and their faeces examined to confirm that they were healthy and free from salmonella infection. These examinations were continued at frequent intervals for 14 days after vaccination. Any animals that died after vaccination were examined for pathological lesions and their livers cultured bacteriologically.

Method of challenge

After an overnight fast, animals were given, by mouth, an aqueous suspension of an 18 hr. nutrient agar culture of the challenge strain to which was added a powder consisting of powdered chalk, 40%; colloidal kaolin, 43%; magnesium trisilicate, 17%. Each calf or pig was given 15 ml. of suspension containing 10^{10} viable organisms and 6 g. of the powder and each mouse 0·05 ml. of suspension containing 5×10^8 viable organisms and 0·03 g. of powder. To facilitate administration the mice were anaesthetized with ether and, when they were recovering, the infecting material was dropped into the mouth by means of a fine pipette.

The examinations on the calves and pigs performed after vaccination were continued after challenge. All animals, including mice, that died were examined for lesions and the liver and, frequently, other organs examined bacteriologically to confirm that they had died from the particular salmonella serotype with which they

had been challenged. The experiments on mice were terminated 19 days after challenge when deaths were uncommon, those on calves at 21 days when the survivors had recovered and those on pigs at 14 days when the survivors had either recovered almost completely or were so emaciated that destruction was necessary on humane grounds.

The examination of faeces and organs for salmonellae

Rectal swabs, taken so as to include a liberal portion of faeces, were cultured on deoxycholate–citrate agar before and after enrichment in selenite-F medium for 24 hr. at 37° C. The plates were incubated for 24 hr. at 37° C. and then examined for the presence of salmonellae. Portions of organs weighing about 2 g. were first ground with sterile sand in a mortar. Brilliant green broth (Smith, 1952) was substituted for selenite-F medium in the search for *S. cholerae-suis* because sodium selenite is very toxic for this serotype; brilliant green agar (Smith, 1952) was also used in addition to deoxycholate–citrate agar for the direct culture of specimens from pigs.

Agglutination tests

The O and H antibody content of sera was estimated by conventional methods (Cruickshank, 1960).

RESULTS

(A) The immunization of mice against *Salmonella dublin* infection

Antisera

The effect of antisera in protecting mice against *S. dublin* infection is illustrated in Table 1. The control mice were given the pooled sera of rabbits before they were used to prepare the antisera that were given to the other mice. The table summarizes the results obtained in three separate experiments each with thirty mice per group and, in all three, antisera prepared against live *S. dublin* had a definite protective effect whereas that prepared against dead *S. dublin* had little or no effect. The serum given to the control mice contained no demonstrable antibodies to *S. dublin*. The anti-O titre of the serum prepared against live *S. dublin* was 1/1600 and the anti-H titre 1/32,000. The corresponding figures for the serum prepared against dead *S. dublin* were 1/3200 and 1/64,000.

In another experiment employing groups of thirty mice, heating to 58° C. for 1 hr. did not have a deleterious effect on the immunity conferred by antiserum prepared against live *S. dublin*. The difference in the mortality pattern between groups given heated and unheated antiserum and the group given normal serum was similar to that shown in Table 1.

Table 2 shows that the degree of immunity conferred by antisera against live *S. dublin* prepared in calves and in rabbits was similar. The H antibody titre of the calf serum, 1/16,000, was similar to that of the rabbit antiserum used in the previous experiments but the O titre, 1/40, was much lower.

Table 1. *The effect of rabbit antiserum in conferring immunity against* Salmonella dublin *infection in mice*

Time after infection (days)	Cumulative percentage mortality in 90 mice given: Antiserum against live *S. dublin*	Antiserum against dead *S. dublin*	Normal serum*
5	1	1	2
6	3	8	19
7	8	16	29
8	13	25	29
9	16	34	38
10	20	40	46
11	22	48	49
12	25	57	58
13	30	64	64
14	32	70	69
15	34	76	71
16	44	83	79
17	48	84	81
18	50	88	83
19	50	91	84

* The normal serum was pooled serum taken from rabbits before they were used for preparing antiserum against the live and dead (heat-killed) *S. dublin*. Sera in 1·0 ml. amounts were given subcutaneously to the mice 2 hr. before oral infection with *S. dublin*.

Table 2. *The effect of calf antiserum in conferring immunity against* Salmonella dublin *infection in mice*

Time after infection (days)	Cumulative percentage mortality in twenty-five mice given: Antiserum against live *S. dublin*	Normal calf serum
6	4	4
7	8	28
8	12	32
9	12	44
10	12	44
11	16	48
12	16	52
13	16	56
14	24	60
15	32	64
16	36	68
17	40	76
18	40	80

The dose of serum employed was 1·5 ml. For other details see Table 1.

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Dead vaccines

The immunizing ability of the following dead vaccines prepared from *S. dublin* 188, the fully virulent strain used for challenge, was tested in groups of twenty mice:

(1) Saline suspension of an 18 hr. nutrient agar culture killed by ethanol.

(2) Alum-precipitated bacterial suspensions prepared by the method of Henning (1953).

(3) Saline suspensions of 6 and 18 hr. nutrient agar cultures killed by ultraviolet light, with and without final precipitation with alum.

(4) Saline suspensions of 6 and 18 hr. nutrient agar cultures disrupted by ultrasonic rays and then rendered sterile by passing through a membrane filter.

(5) Nutrient broth cultures, 18 hr. old, killed by (*a*) heating to 56° C. for $\frac{1}{2}$ hr., (*b*) the addition of 0·06 % formaldehyde.

(6) Nutrient broth cultures, 6 hr. old, killed by: (*a*) neomycin, 500 μg./ml.; (*b*) polymixin, 500 μg./ml.; (*c*) penicillin, 500 units/ml.; (*d*) a saturated aqueous solution of furazolidone.

(7) A 6 hr. nutrient broth culture lysed by bacteriophage and rendered bacteria-free by filtration.

On challenge, the mortality rate in the control mice in these experiments was 90 % and that in the vaccinated groups varied from 70 to 100 %. In a final experiment in which the dose of the alum-precipitated vaccine was increased from the customary 0·1 to 0·25 ml., the dose employed by Henning (1953), the mortality rate in the vaccinated group of twenty-five mice was 52 % and in the similar-sized control group was 80 %.

Live vaccines

Live vaccines were prepared by a variety of methods. These included prolonged incubation of the initially fully virulent strains of *S. dublin* 188, in broth and liquid synthetic media held at different temperatures with and without occasional subculture. *S. dublin* O antiserum and bacteriophages were added to some of the cultures. At intervals, subcultures were made on plates of nutrient agar and colonies selected either at random, according to their morphology or according to their reaction to the slide acriflavine test. Those retained for assessment as vaccines were subcultured five times, single colonies being selected on each occasion, the final culture then being maintained on Dorset egg medium at 5° C. and by freeze-drying.

In all, fifty-five vaccines, some rough and some smooth, were tested on groups of ten to twenty mice. Most of them were rejected because of excessive virulence, because they reverted from avirulent to virulent during this initial test or during subsequent passage, or because they produced little or no immunity. Further tests revealed one vaccine, no. 17A, to be the most promising. However, when this vaccine was later shown to possess little or no immunogenicity for calves, attention was re-directed to some of the vaccines initially rejected because of excessive virulence for mice and one of them, no. 51, was found to be avirulent and immunogenic for calves.

All the studies reported below are concerned with the assessment of the immunity conferred against *S. dublin* infection by the two vaccines 17A and 51 and by *S. gallinarum*, 9, and its two variants, 9S and 9R, and by *S. pullorum*.

Characteristics of vaccines 17A and 51

Vaccine 17A was obtained by submitting *S. dublin* 188 to fourteen passages of 3–10 days duration in nutrient broth at 18° C. and finally selecting a rough colony. Vaccine 51 was obtained by spreading an 18 hr. broth culture of 188 over the surface of a dried nutrient agar plate and placing a drop of a suspension of salmonella anti-O phage no. 1 (Felix & Callow, 1943) upon it. The plate was incubated at 37° C. for 24 hr. and a phage-resistant colony from within the zone of lysis selected.

Colonies of 17A and 51 had a rough appearance and suspensions agglutinated immediately and completely when submitted to the slide acriflavine test. Broth cultures of 17A, after incubation at 37° C. for 18 hr., consisted of a coarse granular deposit and a clear supernatant fluid; the deposit in the case of 51 was finer and more powdery and the supernatant fluid was turbid. On slide testing, 17A slowly agglutinated spontaneously in normal saline but 51 formed a stable suspension. The biochemical reactions of 17A and 51 were those of 188 itself, a typical strain of *S. dublin*.

The virulence for mice of live S. dublin, S. gallinarum *and* S. pullorum *vaccines*

The virulence for mice of vaccines 17A and 51, their parent strain of *S. dublin* 188, vaccines 9S, 9R and their parent strain of *S. gallinarum* 9, and *S. pullorum* is shown in Table 3, from which it can be seen that 17A possessed very little virulence and that vaccine 51 and, to a lesser extent, 9S had a considerable degree of virulence which was, however, less than that of their parent strains. The lesions found in mice that died after vaccination with 17A, 51 and 9S were the same as those produced by fully virulent salmonellae, and included the characteristic white necrotic regions in the liver. The cultures isolated from the dead mice appeared to have undergone no change and further passage experiments did not show any increase in virulence.

The immunizing effect in mice of live vaccines against S. dublin

The immunity to *S. dublin* infection of mice that survived vaccination with different cultures is illustrated in Table 4. Those vaccinated with 51 were completely immune and those vaccinated with either 17A or *S. gallinarum* 9S had a substantial immunity; the mice vaccinated with either *S. gallinarum* 9R or *S. pullorum* were fully susceptible to infection with *S. dublin*.

The effect of vaccinating mice with 17A at different times before and after challenge with S. dublin

The immunity possessed by groups of forty mice vaccinated with 17A at different times before and after challenge with *S. dublin* is illustrated in Table 5. An appreciable immunity was detected in mice vaccinated before and on the day

Table 3. *The virulence for mice of live vaccines*

Time after injection (days)	Cumulative percentage mortality of mice given: *S. dublin* 17A (255)*	*S. dublin* 51 (109)	*S. dublin* 188 (20)	*S. gallinarum* 9S (125)	*S. gallinarum* 9R (36)	*S. gallinarum* 9 (20)	*S. pullorum* (30)
2	0	0	5	1	0	0	0
3	1	1	10	1	0	0	0
4	1	6	55	1	0	0	0
5	2	21	85	2	0	0	0
6	3	39	100	14	0	15	7
7	3	44	100	22	0	40	7
8	5	55	100	27	0	50	7
9	6	62	100	29	0	50	7
10	6	70	100	29	0	50	10
11	6	72	100	29	0	50	10
12	6	72	100	29	0	50	10
13	6	72	100	29	0	50	10
14	6	77	100	29	0	50	10
15	6	79	100	29	0	50	10
16	6	81	100	29	0	50	10
17	6	81	100	29	0	50	10
18	6	82	100	29	0	50	10

The dose of each vaccine given by subcutaneous injection was approx. 5×10^7 viable bacteria.

* The numbers of mice per group are shown in parentheses.

Table 4. *The immunizing effect of live vaccines against* Salmonella dublin

Time after challenge with *S. dublin* 188 (days)	Cumulative percentage mortality in mice vaccinated with: *S. dublin* 17A (144)*	*S. dublin* 51 (20)	*S. gallinarum* 9S (41)	*S. gallinarum* 9R (26)	*S. pullorum* (27)	None (126)
6	1	0	0	4	4	6
7	3	0	3	16	4	15
8	7	0	8	16	16	24
9	8	0	8	20	26	35
10	14	0	15	28	30	41
11	17	0	18	44	48	48
12	19	0	23	44	48	55
13	19	0	23	44	55	61
14	20	0	25	48	55	66
15	21	0	25	54	60	68
16	21	0	25	63	63	70
17	21	0	25	69	67	73
18	23	0	25	71	70	75

* The numbers of mice per group are shown in parentheses.

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of challenge, although it was never as good as in mice vaccinated with 17A at 3 weeks before challenge (Table 4). The groups vaccinated 3 and 5 days after challenge appeared to be unaffected by vaccination, their mortality pattern closely resembling that of the control group.

Table 5. *The effect of vaccinating groups of* 40 *mice with* Salmonella dublin *live vaccine* 17*A at different times before and after oral challenge with* Salmonella dublin

Time after challenge (days)	Cumulative percentage mortality in mice vaccinated on the following days in relation to challenge: Before 10	Before 7	Before 5	Before 3	Before 1	0	After 1	After 3	After 5	Controls
6	3	8	0	0	3	3	5	3	8	8
7	5	8	3	5	10	12	23	28	30	25
8	10	13	5	8	25	15	38	38	35	38
9	13	20	8	10	25	18	43	43	38	43
10	13	30	13	23	28	25	45	48	38	60
11	15	38	13	23	30	25	58	63	45	68
12	18	43	15	30	33	30	58	63	53	78
13	20	43	15	35	35	30	60	70	63	85
14	25	45	18	40	35	33	60	70	65	87
15	28	45	25	43	38	38	63	75	70	87
16	33	45	25	45	38	43	65	80	78	87
17	33	45	33	50	43	45	68	83	80	87
18	38	45	40	53	50	50	68	83	83	90

Table 6. *The effect of different doses of* Salmonella dublin *live vaccine* 51 *in conferring immunity against* S. dublin *infection in mice*

	Vaccinal dose (viable organisms): 50,000	5000	500	0
No. of mice	30	27	25	23
Percentage died from vaccination	57	48	8	0
No. of survivors challenged	13	14	23	23
Percentage died from challenge	0	0	13	83

The immunizing ability of vaccine 17*A against heterologous strains of* S. dublin

When twenty mice vaccinated with 17A were challenged with a strain of *S. dublin* epidemiologically unrelated to 188, five died compared with seventeen of twenty control mice. When the experiment was repeated using another challenge strain, five vaccinated and sixteen control mice died.

The influence of dose size on the virulence and immunizing ability of vaccine 51

Since vaccine 51 in its usual dose of 5×10^7 viable organisms killed a considerable proportion of the mice injected with it, the virulence and immunizing ability of smaller doses were assessed. The results (Table 6) indicated that doses much smaller than the customary vaccinal dose were lethal for mice and that only when the numbers of viable organisms were reduced to 500 was the virulence greatly

decreased, a procedure that was accompanied by a slight mortality on challenge with the fully virulent strain.

The presence of agglutinins in the sera of mice vaccinated with 17*A and* 51

No O-agglutinins to *S. dublin* were detected by tube tests in a 1 in 10 dilution of the sera of fifteen mice 3 weeks after vaccination with 17A or 51. The H titres of the sera varied from 1/160 to 1/640.

(B) The immunization of mice against *Salmonella cholerae-suis* infection

Dead vaccines

Only one dead vaccine was tested. This was an alum-precipitated vaccine prepared from *S. cholerae-suis* 195 and used in the manner described by Henning (1953) for *S. dublin*. Of twenty vaccinated mice, fourteen died after challenge; seventeen of twenty control mice died.

Live vaccines

These were prepared in a similar manner to the live *S. dublin* vaccines except that the parent strain was *S. cholerae-suis* 195. Eight vaccines were tested in mice and two, nos. 3 and 6, were retained for further study because they satisfied the criteria initially sought for in the *S. dublin* vaccines except that they were still appreciably virulent for mice.

Characteristics of vaccines 3 *and* 6

Vaccine 3 was produced by the same procedure as the *S. dublin* vaccine 17A and vaccine 6 by the same procedure as vaccine 51. Both vaccines 3 and 6 were judged to be rough on colonial appearance. Broth cultures of vaccine 3, after 24 hr. at 37° C., consisted of a powdery deposit and a clear supernatant fluid; a powdery deposit was also present in the case of vaccine 6 but the supernatant fluid was turbid. Suspensions of both vaccines agglutinated immediately and completely when submitted to the slide acriflavine test. They also agglutinated slowly in normal saline, vaccine 6 being the slower. The colonies of 3 and 6 on deoxycholate–citrate agar were smaller than those of their parent strain 195. After 24 hr. incubation on this medium at 37° C., colonies of 195 had a diameter of 2 mm., whereas the diameter of those of 3 and 6 were 1 and 0·5 mm. respectively. After 48 hr. incubation the colonies of 195 were flat with an uneven edge and 3–4 mm. diameter; those of 3 and 6 had a 'poached egg' appearance and their diameters were approximately 1·5 and 1 mm. respectively.

The virulence and immunizing ability of vaccines 3 *and* 6 *in mice*

Experiments to assess the virulence and immunizing ability of vaccines 3 and 6 are summarized in Table 7, from which it can be seen that both vaccines possessed a considerable degree of virulence for mice, greater in 3 than in 6, and that the survivors had a considerable degree of immunity against oral infection with *S. cholerae-suis* 195. The mortality pattern from vaccination and challenge was

similar to that shown previously in the case of the *S. dublin* vaccine 51 and *S. dublin* 188 respectively. All strains isolated from mice dead from vaccination were rough.

Table 7. *The virulence and immunizing ability of* Salmonella cholerae-suis *vaccines* 3 *and* 6 *in mice*

	Vaccinated with			
	3	6	195*	Unvaccinated
No. of mice	70	139	30	80
Percentage died from vaccination	63	44	100	0
No. of survivors challenged	26	78	0	80
Percentage died from challenge	15	14	0	84

* 195 is the fully virulent strain from which vaccines 3 and 6 were derived. It was also the strain used for oral challenge.

Table 8. *The effect of vaccinating groups of* 20 *mice with* Salmonella cholerae-suis *vaccine* 6 *at different times before and after oral challenge with* S. cholerae-suis

	No. of days vaccinated in relation to challenge									
	Before						After			
	10	7	5	3	1	0	1	3	5	Unvaccinated
Percentage died from vaccination*	40	35	55	35	5	0	0	0	0	0
Percentage died from challenge*	5	15	20	25	80	80	100	100	95	75
Total % died	45	50	70	60	85	80	100	100	95	75

* Assessed by performing the acriflavine test on the colonies isolated from the livers of the dead mice.

Table 9. *The virulence and immunizing ability of* Salmonella cholerae-suis *vaccines when administered orally to mice*

	Vaccinated with		
	3	6	Unvaccinated
No. of mice	20	20	20
Percentage died from vaccination	50	30	0
No. of survivors challenged with *S. cholerae-suis* 195	10	14	20
Percentage died from challenge	15	15	85

The vaccines were administered orally in exactly the same manner as the challenge dose was 3 weeks later.

The effect of vaccinating mice with vaccine 6 *at different times before and after challenge with* S. cholerae-suis

The numbers of mice that died in groups of twenty given vaccine 6 at different times before and after challenge with *S. cholerae-suis* 195 is shown in Table 8. The reaction to the acriflavine test of the strain isolated from the livers of the dead mice was the factor used to decide whether death was due to vaccination or challenge. On this basis, most of the dead mice that had been vaccinated 3 or more days before challenge were considered to have died from vaccination and all

but one of the dead mice vaccinated after this time had died from the challenge infection. As expected, the total mortality was least in the mice vaccinated 10 days before challenge. In those vaccinated 1–5 days after challenge it was higher than in the unvaccinated controls.

The effect of oral administration of vaccines 3 *and* 6

The results of giving vaccines 3 and 6 orally to mice 3 weeks before challenge with *S. cholerae-suis* is shown in Table 9. The vaccines were administered in exactly the same manner as the challenge strain. Both vaccines had a considerable lethal effect and the survivors exhibited a reasonably high level of immunity.

The presence of agglutinins in the sera of mice given vaccines 3 *and* 6

No O-agglutinins to *S. cholerae-suis* were detected by tube tests in a 1 in 10 dilution of the sera of thirteen mice 3 weeks after vaccination with 3 or 6.

Table 10. *The immunity conferred by live vaccines against challenge in mice with heterologous species of* Salmonella *and other bacteria*

Vaccine	Challenge organism	No. of vaccinated mice challenged	Mortality following challenge (%)	No. of unvaccinated mice challenged	Mortality following challenge (%)
S. cholerae-suis 6	*S. dublin* 188	29	3	24	96
S. dublin 51	*S. cholerae-suis* 195	37	3	20	55
S. dublin 51	*S. typhi-murium*	27	4	21	86
S. dublin 51	*Erys. rhusiopathiae*	25	100	25	100
S. dublin 51	*E. coli*	22	69	19	88

The immunity conferred by the live vaccines against infections in mice with heterologous salmonella serotypes and other bacteria

The results of administering live vaccines to mice and 3 weeks later challenging the immunity of the survivors against bacteria other than the one from which the vaccine they had been given was derived is illustrated in Table 10. The dose of vaccine was reduced from the usual 5×10^7 living organisms to 5×10^6 and this resulted in a vaccinal mortality of from 33–45 %. The immunity of the survivors to salmonella infection was determined in the usual way. That against *Escherichia coli* was determined by the intraperitoneal injection of 10^8 viable organisms of a 24 hr. nutrient broth culture, approximately 3 times the LD 50, and that against *Erysipelothrix rhusiopathiae* by the subcutaneous injection of 10^7 viable organisms of a 24 hr. broth culture. The *S. cholerae-suis* vaccine 6 conferred a high degree of immunity against *S. dublin* and so did *S. dublin* vaccine 51 against *S. cholerae-suis* and *S. typhi-murium*. Vaccine 51 produced no apparent immunity against *Erys. rhusiopathiae*, the mice in the vaccinated and the control groups exhibiting a similar mortality pattern, deaths commencing on the third day and concluding on the fifth day after infection. It also produced no apparent immunity against infection with *E. coli*.

(C) THE IMMUNIZATION OF CALVES AGAINST *SALMONELLA DUBLIN* INFECTION

Experimental studies

The results of experiments on the efficacy of antiserum and vaccines 17A, 51 and 9S in protecting calves against *S. dublin* infections are summarized in Table 11. The O and H titres of the antiserum, which was prepared in calves given live cultures of *S. dublin* 188, were 1/40 and 1/16,000 respectively. None of the calves showed any signs of ill-health after vaccination. Appetite and body temperature were unaltered and the faeces remained normal in consistency and were not found

Table 11. *The immunizing ability of live vaccines and antisera against* Salmonella dublin *infection in calves*

Immunizing agent	No. of calves used	Cumulative mortality on the following days after challenge with *S. dublin* 188						No. of survivors
		2	3	4	5	6	7	
Antiserum* (450 ml.)	5	1	2	3	3	4	5	0
Vaccine 17 A	8	0	2	6	6	6	8	0
Vaccine 9 S	7	0	0	0	2	4	4	3
Vaccine 51	5	0	0	0	0	1	1	4
Vaccine 51 + 9 S	2	0	0	0	0	0	0	2
Controls	14	4	10	13	13	13	14	0

* Prepared in calves against live *S. dublin* and given 2 hr. before challenge.

to contain the vaccinal or any other salmonellae. At 3 weeks after vaccination no O antibodies against *S. dublin* were detected in the sera of the calves. The sera of those vaccinated with 17A and 51, but not with the non-motile 9S, possessed H antibody titres of 1/400–1/1600. No *S. dublin* O or H antibodies were found in the sera of the unvaccinated calves. Within 1–3 days of challenge all the calves appeared unwell, their body temperature rose to 104–107° F., their appetites were impaired, they had diarrhoea and their faeces contained *S. dublin*. These signs were most prominent in the control calves and in those given antiserum or vaccine 17A. Apart from some possible slight difference in survival time, the disease appeared equally severe in these three groups of calves. In the others the disease was less severe, particularly in those vaccinated with 51 or 51 and 9S. Although only two calves were given both 51 and 9S there was no real evidence to indicate that a combination of both vaccines produced a better immunity than 51 alone. Three weeks after challenge all the surviving calves appeared reasonably well and *S. dublin* was not found in their faeces. Two of the calves vaccinated with 51 and one each vaccinated with 9S or 9S and 51 were killed at this time; salmonellae were not isolated from their organs.

The results of a further experiment to assess the immunizing ability of vaccines 51 and 9S are summarized in Table 12. In this experiment the calves were not Ayrshires, the breed used in the previous experiment, but male Friesians reared intensively for beef production. There were selected for the final experiment,

despite their cost, because clinical *S. dublin* infection is most common in intensive beef production units. These calves had been maintained on a diet consisting solely of solid concentrated food for a week before challenge when approximately 6 weeks of age, having been vaccinated 3 weeks previously. Neither the vaccinal strains nor any other forms of salmonellae were found in the faeces of these animals before challenge and they all remained in a good state of health. Only H antibodies against *S. dublin* were found in the sera of those vaccinated with 51. The course of the disease after challenge was less severe in the control animals than in those used in the previous experiment. Not only was the death-rate lower but the survival time of those that died was much longer. None of the animals died in the early bacteraemic phase as most of the control calves did in the previous experiment. Most that died became emaciated and suffered from a diarrhoea in which the faeces often contained pieces of necrotic mucous membrane. The disease was definitely most mild in those given vaccine 51 and the death rate was nil.

S. dublin was only isolated from the faeces of one of the surviving calves after the 17th day of infection, at which time most of them were in reasonably good condition.

Table 12. *The immunizing ability of live vaccines against* Salmonella dublin *infection in intensively-reared calves*

Vaccine	No. of calves used	Cumulative mortality on the following days after challenge						No. of survivors
		7	8	9	10	11	11+	
51	10	0	0	0	0	0	0	10
9 S	10	1	1	2	2	2	3	7
None	10	0	1	2	3	5	6	4

Field studies

Field studies were performed from October 1963 to August 1964 in an intensive beef production unit that had previously experienced a considerable amount of clinical *S. dublin* infection. The calves, male Friesians, had been born on farms in south-west England and were brought in batches to the unit in Essex when approximately 1 week old. One-third of the calves were vaccinated in the usual manner with 51, one-third with 9S and the remainder with a heat-killed broth culture of a coagulase-negative staphylococcus; the owner of the unit was not made aware of the particular vaccine any calf received. In the first half of the experiment the calves were vaccinated at 3 weeks of age but in the second half, owing to the fact that clinical *S. dublin* infection began to occur in the young age groups, they were vaccinated on the day after their arrival at the unit. None of them showed any signs of ill-health that could be attributed to vaccination. Subsequently the owner recorded the rectal temperature and took a rectal swab of all animals that became unwell. Immediately after this the owner was permitted, on economic grounds, to treat these animals with furazolidone, which he usually did. The swab was brought to the laboratory for bacteriological examination. In the absence of any other diagnosable disease, all the ill animals from which

S. dublin was isolated from the faeces were recorded as suffering from clinical *S. dublin* infection. Clinically, these animals formed a fairly clear-cut group in that they were dull, disinclined to move or eat, had temperatures of 104–107° F. and usually had diarrhoea. The results (Table 13) showed that, apart from calves that developed the clinical disease within 1 week of vaccination, vaccine 51 and, to a lesser extent, 9S had a beneficial effect on reducing the incidence of clinical *S. dublin* infection. The vaccination history of sick calves from whose faeces *S. dublin* was not isolated but whose rectal temperatures were 104·5° F. or higher was also analysed; of 36, seven had been vaccinated with vaccine 51, eight with 9S and 21 with the dead staphylococcus.

Table 13. *The incidence of clinical* Salmonella dublin *infection in vaccinated calves in an intensive beef unit*

Vaccine	No. of calves vaccinated	No. that developed infection in relation to vaccination	
		Within 1 week	After 1 week
51	312	5	3
9 S	312	2	9
Dead coagulase-negative staphylococcus	312	5	35

On six occasions during the period November 1963 to June 1964 the faeces of most of the healthy calves under 6 months of age in the unit were examined for the presence of *S. dublin*. Smooth strains of *S. dublin* were found in sixty-three (4·9 %) of the 1293 rectal swabs examined. There was little variation in the incidence of positive swabs from occasion to occasion. Ten of them were from calves vaccinated with 51, twenty from calves vaccinated with 9S and thirty-three from calves vaccinated with the dead staphylococcus. Rough strains of *S. dublin* were found in the faeces of one calf vaccinated with 51 and in one vaccinated with the dead staphylococcus; they were not found at repeat examinations two days later.

(D) THE IMMUNIZATION OF PIGS AGAINST *SALMONELLA CHOLERAE-SUIS* INFECTION

Experimental studies

The results of challenging the immunity to *S. cholerae-suis* infection of pigs given either one of the live vaccines 3 and 6 or a dead alum-precipitated vaccine are summarized in Table 14. None of the pigs showed any signs of ill-health after vaccination, their appetites were unimpaired and they continued to gain weight in the normal manner. The highest body temperature recorded in the individual pigs in the 3 weeks before challenge varied from 103 to 105° F. (median 104° F.) in the unvaccinated control group and in the vaccine 3 group and from 102·5 to 104·8° F. (104·1° F.), in the vaccine 6 group. During this period neither the vaccinal strain nor any other form of *S. cholerae-suis* was isolated from the faeces of the pigs. Immediately before challenge, the sera of four pigs in each live vaccine

group and the control group were examined for antibodies against the strain of *S. cholerae-suis*, 195, with which they were to be challenged. No O antibodies were found in any of the sera diluted 1 in 10. No H antibodies were found in the sera of the control pigs; the sera of the pigs in the two vaccinated groups contained similar levels of H antibodies, the titres ranging from 1/200 to 1/3000.

Table 14. *The immunizing ability of vaccines against* Salmonella cholerae-suis *infection in pigs*

Vaccine	No. of pigs used	Cumulative mortality on the following days after challenge									No. of survivors		
		5	6	7	8	9	10	11	12	13	With severe lesions	With mild lesions	With no lesions
3	12	0	0	0	1	1	1	1	1	1	0	0	11
6	12	0	0	0	0	0	0	0	0	0	0	1	11
Dead alum-precipitated*	4	0	0	0	1	1	1	1	1	1	2	0	1
None	12	1	2	2	2	3	4	4	4	7	4	0	1

* Prepared according to the method of Henning (1953).

Within 2 days of challenge all the pigs appeared unwell. They refused food, some vomited and their body temperatures varied from 105 to 108° F. The body temperatures of the pigs given live vaccines remained above 105° F. for 1–3 days and their appetites were impaired for up to 6 days. However, with the exception of the one pig given vaccine 3 that died and the one pig given vaccine 6 which had lesions when killed at the end of the experiment (Table 14), the general health of the pigs given live vaccines was greatly improved at 7 days after challenge and by 14 days they appeared normal. By contrast, the unvaccinated control pigs had very high body temperatures for 3–4 days and, with the exception of one that was normal at the end of the experiment, the remaining eleven ate little or no food and their general condition continued to deteriorate until they died or were killed at the termination of the experiment, the survivors by then being in an extremely emaciated condition and unlikely to recover. All these survivors are recorded in Table 14 as having severe lesions which principally consisted of necrotic enteritis involving the whole of the large intestine. The designation 'mild lesions' implied the presence of small areas of necrotic enteritis in the large intestine which were resolving. Although only four pigs were vaccinated with the dead alum-precipitated vaccine it was apparent that their immunity was much less than that in the pigs given vaccines 3 and 6.

At the end of the experiment, *S. cholerae-suis* was not isolated by direct culture from the liver, bile, spleen or from a mesenteric lymph node of eight pigs examined that had been given vaccine 3 or of eight pigs examined given vaccine 6. Preliminary enrichment of approximately 2 g. of each of these materials revealed the presence of *S. cholerae-suis* in the mesenteric lymph node of one of the pigs given vaccine 6.

Field studies

From November 1963 to June 1964 field studies were conducted on a very large pig farm some 300 miles from the laboratory, a farm in which cases of clinical *S. cholerae-suis* infection of a bacteraemic type had been occurring for several years. On this farm, all weaning was performed on one day only of every week, the weaned pigs, approximately 8 weeks of age, then being mixed together. One-third of all the pigs weaned in any week were given vaccine 3, another third vaccine 6 and the remainder were left as unvaccinated controls. No complaints were received of any ill effect of vaccination. No treatment was given to any pigs that became unwell and the spleens of all that died were sent to our laboratory by post. Pigs from whose spleens *S. cholerae-suis* was isolated in plentiful culture were recorded as having died from *S. cholerae-suis* infection. The results are summarized in Table 15, from which it can be seen that both vaccine 3 and vaccine 6 had a controlling effect on the incidence of fatal cases of *S. cholerae-suis* infection.

Table 15. *The effect of vaccination on the incidence of fatal* Salmonella cholerae-suis *infection in a herd of pigs*

Vaccine	No. of pigs vaccinated	No. that died from: All causes	No. that died from: *S. cholerae-suis* infection
3	384	32	9
6	384	28	10
None	384	70	40

The deaths attributed to *S. cholerae-suis* infection occurred from 9 to 68 (median 22) days after vaccination. Several pigs, including unvaccinated ones, died, presumably from other causes, within 1 week of the vaccination time; this mortality was no higher than at other periods. A rough strain of *S. cholerae-suis*, probably vaccine 3, was found in plentiful culture in the spleen of one pig that died 5 days after it had been given this vaccine. Neither of the two vaccines was isolated from any of the other spleens by direct culture.

DISCUSSION

A great deal of doubt exists as to the relative importance of cellular and humoral immunity in salmonella infection. Although the protection produced in mice in the present work by a large dose of antiserum prepared in rabbits or calves against live *S. dublin* was far from complete and inferior to that produced by live vaccines it was sufficient to indicate that humoral immunity may play some part in salmonella infection. The administration of antiserum, however, had very little practical application in *S. dublin* infection in calves. The fact that both antiserum prepared against dead *S. dublin* and dead vaccines had very little protective effect on mice suggests that the immunogenic factors present in the live bacteria were destroyed by the killing processes. The negative results with these materials which either contained or gave rise to considerable amounts of O and H antibodies and the fact that the live rough *S. dublin* and *S. cholerae-suis* vaccines provoked no O

antibodies and the live smooth *S. gallinarum* vaccine 9S provoked no H antibodies yet all produced a reasonably good immunity provides confirmatory evidence for the view that the conventional O and H antigens are not concerned in salmonella immunity. The observation that mice surviving vaccination with the *S. choleraesuis* variant 6 were resistant to *S. dublin* infection and mice surviving vaccination with the *S. dublin* variant 51 were resistant to *S. choleraesuis* infection (Table 10) indicates that *Salmonella* species bearing little resemblance as far as ordinary *in vitro* antigen–antibody tests are concerned may be closely related immunogenically. It cannot be argued that the immunity produced by these variants was non-specific in character because the mice vaccinated with variant 51 were fully susceptible to both *Escherichia coli* and *Erysipelothrix rhusiopathiae* infections. On the other hand, immunogenic factors were not common to all members of the *Salmonella* groups since the mice vaccinated with *S. pullorum* were fully susceptible to *S. dublin* infection yet *S. pullorum* and other members of the O '9' group immunized chickens against *S. gallinarum* infection (Smith, 1956*b*).

Mice that survived vaccination with variant 51 were more resistant to challenge with the fully virulent *S. dublin* 188 than were those vaccinated with variant 17A (Table 4). This might be because the severer infection that followed vaccination with 51 constituted a greater immunogenic stimulus than the milder infection that followed vaccination with the less virulent 17A. In view of the greater tolerance of calves than mice to these vaccines, it is conceivable that the failure of 17A to protect calves against challenge with 188 (Table 11) was because the infection produced in them by 17A was too mild to constitute an adequate immunogenic stimulus whereas the infection produced in them by 51, although nothing like as severe as that produced in mice, was sufficient to produce a reasonable immunity. It is noteworthy in this respect that the reduction of the dose of variant 51 in mice to a point at which the vaccinal mortality was greatly reduced (500 organisms) was accompanied by an immunity to challenge with 188 which, although of a high order, was less than that following the administration of larger doses (Table 6).

The experimental and field studies in calves and pigs indicated that the live vaccines may have a practical application in the control of salmonella infection in these species. The results point to 51 being better than 9S in preventing *S. dublin* infection in calves and, since it was highly effective against experimental infection in mice with *S. typhi-murium*, it is conceivable that 51 may have a beneficial effect in controlling disease in calves caused by this organism—the second most important salmonella type causing disease in these animals. The studies revealed little difference between vaccines 3 and 6 in controlling experimental and natural *S. cholerae-suis* infection in pigs. However, since vaccine 3 was found in plentiful culture in the spleen of one dead pig in the field trial—the significance of which is in doubt, particularly as the spleens had been sent to the laboratory by post—vaccine 6 is to be preferred in further studies.

The rapidity with which immunity developed in mice after injection of the live vaccine 17A resembled the 'interference' type phenomenon reported after vaccination with 9S in experimental *S. gallinarum* infection in chickens (Smith, 1956*a*). Vaccine 17A never produced any ill effect related to its time of administration.

An ill effect was noted when mice were injected with vaccine 6 shortly after oral infection with *S. cholerae-suis* and this was probably associated with the considerable degree of virulence of vaccine 6 for these animals. Since vaccine 51 was avirulent for calves and vaccines 3 and 6 avirulent for pigs, it is unlikely that these vaccines would cause harm when administered to infected animals during outbreaks of salmonella infection; they might well be beneficial. Vaccine 51 was, in fact, administered to some infected calves during the field studies; the losses were certainly no higher than in the controls. However, this point and others can only be settled by further use of these vaccines.

SUMMARY

1. Antisera prepared in rabbits or calves against live *Salmonella dublin* gave mice some degree of protection against oral infection with this organism. Both antiserum prepared against heat-killed *S. dublin* and dead vaccines prepared in a variety of ways produced little or no immunity.

2. A rough variant of *S. dublin* of low virulence for mice, no. 17A, produced a reasonably good immunity against oral infection with *S. dublin* in mice but not in calves. Mice that survived injection with another rough variant that possessed a considerable degree of virulence for these animals, no. 51, were immune to oral infection with *S. dublin*. Experimentally and naturally, this variant and, to a lesser extent, 9S, a smooth variant of *S. gallinarum* of reduced virulence, produced an appreciable degree of immunity in calves against *S. dublin* infection; none of the calves injected with these variants showed any signs of ill-health as a result.

3. Two rough variants of *S. cholerae-suis*, nos. 3 and 6, possessed a considerable degree of virulence for mice; those that survived were resistant to oral infection with *S. cholerae-suis*. Experimentally and naturally, both variants produced an appreciable degree of immunity in pigs.

4. Mice that survived vaccination with the rough *S. cholerae-suis* variant no. 6 were resistant to oral infection with *S. dublin*. Those that survived vaccination with the rough *S. dublin* variant no. 51 were resistant to oral infection with *S. cholerae-suis* and *S. typhi-murium*; they were fully susceptible to parenteral administration of *Escherichia coli* and *Erysipelothrix rhusiopathiae*.

5. Vaccination with *S. cholerae-suis* variants 3 and 6 and *S. dublin* variant 51 provoked the formation of H but not O antibodies. These variants were never found to mutate from rough to smooth *in vitro* or *in vivo*.

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ADDENDUM

From 1 July 1964 all 575 calves brought into the beef production unit were vaccinated with vaccine 51. By 30 December only six had shown signs of clinical *S. dublin* infection that could be associated with vaccine failure; three of them had also been suffering from 'virus' pneumonia. The last case occurred on 18 November. On 2 September 120 of the younger calves in the unit were examined and smooth strains of *S. dublin* were isolated from the faeces of ten and rough strains from the faeces of two; all twelve calves appeared healthy. On 22 December all 422 calves in the unit were examined and a smooth strain of *S. dublin* was isolated from the faeces of one calf, a persistent excretor after clinical infection, and rough strains from two. The rough strains isolated from calves in this unit were probably strain 51 itself; they were never found in calves at repeat examinations.