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The evaluation of a live salmonella vaccine in mice and chickens

By V. A. KNIVETT* and W. K. STEVENS
Twyford Laboratories Ltd., Twyford Abbey Road, London, N.W.10

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SUMMARY

Mice vaccinated with a live attenuated strain of *Salmonella dublin* were protected against heavy challenge infections of *S. dublin*, *S. typhimurium*, *S. choleraesuis* and *S. anatum*. Oral and subcutaneous vaccination were equally effective. When day-old chicks were orally vaccinated and subsequently challenged with *S. typhimurium*, the growth of the challenge organism was considerably reduced or eliminated from the livers of the vaccinated chicks whereas most of the non-vaccinated were heavily infected. Field trials with vaccinated day-old chicks showed that they suffered no setbacks in growth, stress, loss of appetite or adverse side effects.

INTRODUCTION

Many attempts have been made to control salmonellosis in animals with killed vaccines, but any protection was short-lived and ineffective against heavy infections. Specific live vaccines have been produced to protect chickens against *S. gallinarum* (Smith, 1956*a*), pigs against *S. choleraesuis* and calves against *S. dublin* (Smith, 1965); Rankin, Newman & Taylor (1966) showed that a live *S. dublin* vaccine (strain 51, Smith, 1965) would protect calves against *S. typhimurium*. Our findings show that this vaccine protects mice also against salmonella infections of antigenically related and unrelated serotypes, and are in agreement with the work of Smith (1956*b*), Botes (1965), Smith & Halls (1966) and Collins (1968).

Vaccination should be assessed against non-lethal as well as lethal salmonella infections (Collins, Mackaness & Blanden, 1966). Our aim was to show that vaccination reduced the severity of a non-lethal challenge infection in chickens, since they constitute the major reservoir of salmonellas in animals and are suitable laboratory models. The course of an infection was followed by examining groups of chickens at intervals after challenge and measuring the salmonella content of the livers. The infection (i.e. the growth of the pathogen in the liver) was less severe in vaccinated chickens than in controls.

* Present address: 11, Tregenna Court, 622 Harrow Road, Wembley, Middlesex, to which reprint requests should be sent.

MATERIALS AND METHODS

Mice

C. F. W. mice bred on the premises were fed on a pellet diet (E. Dixon and Sons Ltd., Ware, Herts.) and weighed 18–20 g. when vaccinated. Food and water were available *ad lib.*

Chickens

Day-old hybrid cockerel chicks, cross Rhode Island Red and Light Sussex (Scientific Animal Services, Elstree, Herts.) were housed in a brooder and at 14 days of age were transferred to wire-floor cages in batches of ten. They were fed on baby chick mash (British Oil and Cake Mills Ltd., Erith, Kent) containing no antibiotics. Food and water were available *ad. lib.*

Vaccine

Freeze-dried *S. dublin* A.T.C.C. No. 15480 (Strain 51, Smith, 1965) vaccine was reconstituted in sterile water when required and 0·2 ml. administered subcutaneously to mice and chickens. Alternatively 1·0 ml. of vaccine in an antacid suspension (Smith, 1965) was given orally to chickens or an appropriate dilution of the vaccine made so that each chicken would take approximately 5 ml. when the vaccine was administered in the drinking water. The vaccine doses are recorded with each experiment. Sensitivity of the vaccine strain to antibiotics was checked against Multodisk 30–9B (Oxoid Ltd., London, S.E.1).

Challenge routes

Oral. Animals were kept without food for 8 hr. and given by mouth an aqueous suspension of the challenge strain with an antacid powder (Smith, 1965). The challenge suspension was contained in 0·2 ml. for mice and 1·0 ml. for chickens.

Intraperitoneal. The same procedure was adopted as for the oral challenge but no antacid powder was added and the challenge dose was contained in 0·2 ml. for mice and chickens.

Challenge organisms

The challenge organisms, known to be pathogenic to mice, were chosen from four groups containing different antigens. They were Group B, *S. typhimurium* 1, 4, 5, 12:i:1, 2, (isolated from a human source in Hammersmith Hospital), Group C, *S. choleraesuis* 6, 7:c:1, 5. (N.C.T.C. No. 5735) and strain S418/68 (Reading) var *kunzendorf* (supplied by Dr W. J. Sojka of the Central Veterinary Laboratory, Weybridge, Surrey). Group D, *S. dublin* 1, 9, 12:gp:(N.C.T.C. No. 9676) and Group E, *S. anatum* 3, 10:eh:1,6. (N.C.T.C. No. F7078/66). These strains were grown overnight on nutrient agar and the challenge suspensions standardized on an E.E.L. colorimeter against a standard curve. The challenge dose was varied according to the particular strain of salmonella so as to kill all the non-vaccinated control mice in 5–6 days. Chickens were challenged with *S. typhimurium* var *copenhagen* (phage type No. 14) isolated from a poultry pro-

cessing plant. It was grown in broth at 37° C. on a swirler for a few hours or overnight and the appropriate dilution of the suspension administered. Viable counts were carried out to determine the number of organisms per dose.

Bacterial examination of tissue for salmonellas

Tissue was examined shortly after death or within a few hours. Either the whole liver of mice and young chicks or a 10 g. sample was examined. The sample was weighed, sterilized by dipping in 0·2% hypochlorite for 20 sec. and washed in sterile 0·2% sodium thiosulphate for a further 20 sec. Sterile techniques were employed subsequently. Tissue was sliced and then macerated to liberate bacteria at 14,000 rev./min. for 4 min. in 20 ml. 0·1% yeast extract in the 100 ml. vessel of the M.S.E. Homogeniser (Measuring and Scientific Equipment Ltd., Crawley, Sussex). Test suspensions of *S. dublin* survived this treatment without any loss of viability. Salmonellas are reported to survive better in diluents containing 0·1% yeast extract or peptone than in distilled water or saline.

Direct plating of the homogenized tissue was used to measure the number of live salmonellas per gram of tissue. Serial tenfold dilutions were made in 0·1% yeast extract to 10^{-4} or even higher dilutions, depending on the degree of infection expected. Suitable small volumes were spread on the surface of well dried plates of a selective medium which were incubated overnight at 37° C. If only very small numbers of organisms were expected, duplicate or quadruplicate plates were spread with undiluted suspension in order to examine an adequate amount of tissue. The remainder of the tissue was added to 50 ml. enrichment medium. Direct plating generally revealed salmonellas in all the infected samples. Some samples were positive after enrichment where the infection was minimal and no salmonellas were detected on direct plates. Liver samples often revealed almost pure cultures of salmonellas so that identification presented no difficulty.

Media

Agar plates

The medium contained (per l.) 10 g. yeast extract (Oxoid L21); 2·5 g. bile salts (Oxoid L55); 2 g. tripotassium citrate monohydrate; 5 g. sodium thiosulphate pentahydrate; 15 g. agar (Oxoid No. 1 L11); 2·5 g. lactose; 2·5 g. sucrose; 20 mg. neutral red; 0·2 g. ferric citrate; alkali to pH 7·0 and distilled water to 1 l. The medium was autoclaved at 10 lb. pressure for 10 min. Lactose, sucrose and neutral red were sterilized separately by filtration through a Millipore filter and ferric citrate was autoclaved separately. These constituents were added to the molten agar just before the plates were poured.

Enrichment medium

The selenite mannitol broth contained (per l.) 5 g. peptone (Evans); 4 g. mannitol; 4 g. sodium biselenite; 12 g. dipotassium hydrogen phosphate, acid to pH 6·8 and distilled water to 1 l. The medium was dispensed in 10 or 50 ml. amounts, and steamed for 5 min.

The enrichments were incubated overnight at 43° C. and plated on the selective agar medium. A further enrichment was made by inoculating 0·04 ml. into 10 ml. selenite, and was usually plated after 8 hr. incubation.

Field trials

Examination of poultry houses for salmonellas

After intercrop disinfection and cleansing, twelve dust samples were examined from each house, three each from the floor, walls, beams and ventilators. As each house was restocked, swabs were taken from the chick boxes. Samples of the food and some chicks were also taken to the laboratory for examination. Enrichments were made of liver and caeca for salmonellas and aliquots of the homogenized liver were plated.

Vaccination

Freeze-dried vaccine was added to the drinking water, allowing one font containing 2–3 pints of water to 200 chicks. This quantity was drunk within 3–4 hr. and it is estimated that each chick received 10^7–10^8 cells of the vaccine strain.

RESULTS

Mouse protection tests

Four groups of 10 mice were vaccinated orally or subcutaneously with a single dose of vaccine. Each group was challenged intraperitoneally 14 days later together with non-vaccinated controls with a pathogenic strain of salmonella and the deaths recorded daily for 10 days (Table 1).

Table 1. *Survival of mice against lethal salmonella infections*

Challenge dose (10^6)	Vaccination (oral or subcutaneous)	Vaccine dose (10^6)	Survivors out of 10 at 10 days		Day on which all non-vaccinated mice were dead
			Vaccinated	Non-vaccinated	
S. choleraesuis (100 cells)	Oral	40	6	0	6
	s.c.	5	6	0	6
S. dublin (50 cells)	Oral	50	6	0	5
	s.c.	25	8	0	5
S. typhimurium (1·25 cells)	Oral	50	5	0	5
	s.c.	25	9	0	5
S. anatum (250 cells)	Oral	50	4	0	6
	s.c.	25	7	0	6

The results show the number of mice surviving at 10 days after challenge. In most cases, half or more than half of the vaccinated mice survived a heavy challenge infection which had killed all the controls in 5 or 6 days. Oral vaccination was almost as effective as subcutaneous vaccination. There was no significant difference in response of the vaccinated mice to challenge by serotypes which were related or unrelated antigenically to the vaccine strain.

Table 2. *Progress of an experimental* S. choleraesuis *var* kunzendorf *infection in livers of mice*

Days after challenge	Log_{10} No. of salmonellas/g. of liver										Deaths	
	Vaccinated					Non-vaccinated					Vaccinated	Non-vaccinated
2	0	0	+	1·14	2·34	0	+	+	2·70	4·22	1	0
3	–	–	–	–	–	–	–	–	–	–	1	0
4	–	–	–	–	–	–	–	–	–	–	2	6
5	–	–	–	–	–	–	–	–	–	–	0	10
6	0	3·16	3·85	4·06	4·73	5·29	5·41	6·77	6·86	> 7	0	6
7	–	–	–	–	–	–	–	–	–	–	1	5
8	2·38	2·29	2·33	2·59	5·32	4·37	6·47	8·06	8·26	9·0	0	0
9	–	–	–	–	–	–	–	–	–	–	0	7
15	2·21	2·77	> 3	3·45	> 5	4·91	None left				1	–
18	0	+	3·27	3·42	5·61	.	.	.	.	.	2	–
25	2·41	2·42	3·30	4·12	5·96	.	.	.	.	.	2	–
32	0	0	2·58	5·89	–	.	.	.	.	.	0	–

Progress of an experimental S. choleraesuis *var* kunzendorf *infection in livers of mice*

Forty-four mice were vaccinated subcutaneously with a single dose of 10^7 cells of vaccine. Fourteen days later, these and 50 non-vaccinated mice were challenged intraperitoneally with about 100 cells from an overnight nutrient broth culture of *S. choleraesuis* var *kunzendorf* after passage through a pig.

At 2, 6, 8 and 15 days after challenge, five mice from each group were killed for measurement of the salmonella content of the liver (on day 15 there was only one left for this purpose in the control group). In the vaccinated group five mice were also killed on days 18 and 25, and the last remaining four on day 32. The salmonella counts in the livers, together with the number of mice that died as a result of the challenge dose are shown in Table 2. There were more deaths amongst the non-vaccinated controls than in the vaccinated mice.

We have assessed the degree of infection in the surviving mice by measuring the salmonella content of the liver by direct plate count. The mesenteric lymph nodes might contain the highest concentration of salmonellas but a quantitative estimation would be impracticable. The figures show a 400-fold difference in the salmonella content of the livers from the two groups.

Salmonella infections in unvaccinated chickens

Lethal challenge infections are often used to test the efficacy of vaccination and the presentation of the results is simple (death or survival). A natural salmonella infection is not necessarily lethal and we have compared the number of salmonellas per gram of a selected tissue (the liver) in vaccinated and non-vaccinated chickens. The liver was selected because salmonellas frequently collect in this organ.

Chickens were challenged intraperitoneally at four weeks of age with graded doses of *S. typhimurium* and two weeks later they were killed and the livers examined. The number of *S. typhimurium* cells found per gram of liver ranged

Table 3. *The number of* S. typhimurium *found in liver after intraperitoneal challenge*

(Five chickens were given the same challenge, and the livers were examined 2 weeks later, and the results are expressed as the number of *S. typhimurium* cells/g. wet weight of liver.)

No. of *S. typhimurium cells* in challenge dose				
40	200	1,000	5,000	25,000
0	40	16	10	125
+	50	18	70	205
750	1,000	405	1,040	375
2,870	3,000	6,350	2,000	900
$> 10^4$	$> 10^4$	u/c	7,150	1,060

\+ No salmonellas were isolated on direct plating but were isolated after enrichment.
0 No salmonellas were isolated after direct plating or enrichment.
u/c The plates were overcrowded and the colonies uncountable.

from none to several millions and bore no relation to the size of the original challenge dose (Table 3) which had spanned a 600-fold range. Five chickens receiving the same challenge dose had such widely different numbers of salmonellas that an average figure would have been meaningless.

Other chickens, varying in age from one to twelve days, were challenged orally with *S. typhimurium*. Each group of four birds received a 1000-fold range of challenge dose. The birds were killed eleven to fourteen days after challenge and the livers examined. The number of salmonellas per gram of liver was independent of the size of the challenge dose (Table 4) and varied widely from one bird to another.

Table 4. *The number of* S. typhimurium *found in liver after oral challenge*

Four chicks received a 1000-fold range of challenge. The livers were examined subsequently and the results are expressed as the number of *S. typhimurium* cells/g. wet weight of liver.

Age of chick at challenge (days)	Age of chick at examination (days)	*S. typhimurium** challenge dose (10^6 cells)	Range of challenge dose			
			× 1	× 1/10	× 1/100	× 1/1000
1	15	0·7	25,000	1,116	240	1,225
2	16	0·8	345	2,200	65	11,000
4	18	0·5	140	35	0	10,000
8	19	0·5	270	6,650	4,930	1,330
10	22	0·55	345	315	8	310
12	24	0·3	165	1,335	90	1,910

* A viable count was performed on each broth culture and tenfold dilutions were made.

Vaccination

Twenty-five chickens were vaccinated orally and 25 subcutaneously at 2 weeks of age, with a range of doses of *S. dublin* vaccine. Two weeks later these and 25 non-vaccinated controls were challenged with different amounts of *S. typhimurium*. They were examined 3 weeks after challenge and the number of *S. typhimurium* per gram of liver estimated (Table 5). All chickens appeared to be in good health and without any visible signs of infection. The challenge organism was considerably reduced or eliminated from the livers of the vaccinated chickens, whereas most of the non-vaccinated were heavily infected. Within the range tested, vaccination was equally effective whether administered orally or subcutaneously and was independent of the size of the vaccine dose.

A more detailed inspection of these results shows the number of salmonellas per gram of liver was much lower in the vaccinated than in the non-vaccinated chickens. The proportion of vaccinated and non-vaccinated chickens falling into various ranges of infection is shown in Table 6.

The figures obtained for the controls (non-vaccinated) (Table 5) are assembled and arranged in order of increasing salmonella content. From this series the proportion of livers whose salmonella content does not exceed a stated value can be plotted as a cumulative frequency diagram or 'ogive' (Fig. 1). The line represents the range of infection amongst the results examined.

The results from the vaccinated chickens are similarly plotted. If vaccination had not been effective, then the two lines would have been superimposed. In Fig. 1 the plot for vaccinated birds is shifted away from the controls down towards the abscissa, a distance of approximately 3 logarithmic units, i.e. a 1000-fold decrease.

Table 5. *The number of* S. typhimurium *cells found in livers of vaccinated and non-vaccinated chickens after a* S. typhimurium *challenge*

(Chickens were vaccinated at 2 weeks of age, challenged 2 weeks later and examined after 3 weeks. Results expressed as the number of *S. typhimurium* cells/g. wet weight of liver.)

Vaccination	Vaccine dose. No. of *S. dublin* cells (10^6)	Challenge dose. No. of *S. typhimurium* cells 25,000	5,000	1,000	200	40
Oral	5,000	+	0	4	+	4
	2,500	0	0	0	+	0
	1,250	0	+	–	14	0
	625	+	210	0	8	0
	312	6	+	0	0	+
Subcutaneous	2,000	0	0	30	0	4
	1,000	0	0	0	0	0
	500	0	0	0	20	0
	250	3	0	15	9	0
	125	0	0	10	0	0
Controls	Non-vaccinated	375	1,040	u/c	3,000	0
	Non-vaccinated	205	7,150	405	1,000	+
	Non-vaccinated	900	70	6,350	$> 10^4$	$> 10^4$
	Non-vaccinated	1,060	2,000	16	55	2,870
	Non-vaccinated	125	10	18	40	750

u/c Uncountable, plates overcrowded.
\+ No salmonellas were isolated on direct plating, but were isolated after enrichment.
0 No salmonellas were isolated after direct plating or enrichment.

Table 6. *The proportion of chickens with various ranges of infection*

Range of infection (No. of salmonellas/g. liver)	Proportion of infected chickens (%) Vaccinated	Non-vaccinated
0	58	4
0–10	90	12
0–100	96	32
0–210	100	40
0–1,000	100	60
0–5,000	100	80
0–10,000	100	88

Progress of an experimental infection

Twelve chickens were vaccinated by mouth (5×10^8 cells of the vaccine strain) when 5 days old and 14 days later were challenged intraperitoneally, together with 12 non-vaccinated controls, with 470 *S. typhimurium* cells. The course of the

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infection was traced by examining the livers of vaccinated and non-vaccinated chickens at intervals after challenge (Fig. 2). The experimental infection increased rapidly to a high level in the controls and then slowly regressed. The infection was considerably reduced in vaccinated chickens. With one exception, the infection in the vaccinated chickens did not exceed 70 salmonellas per gram, whereas at the peak of infection some non-vaccinated controls had more than 3000 salmonellas

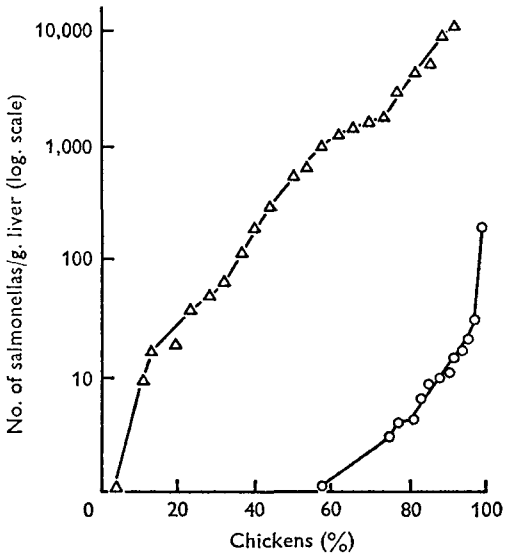

Fig. 1. Effect of vaccination on the salmonella content of livers of infected chickens. The number of salmonellas/g. liver was measured in vaccinated (○) and non-vaccinated (△) chickens.

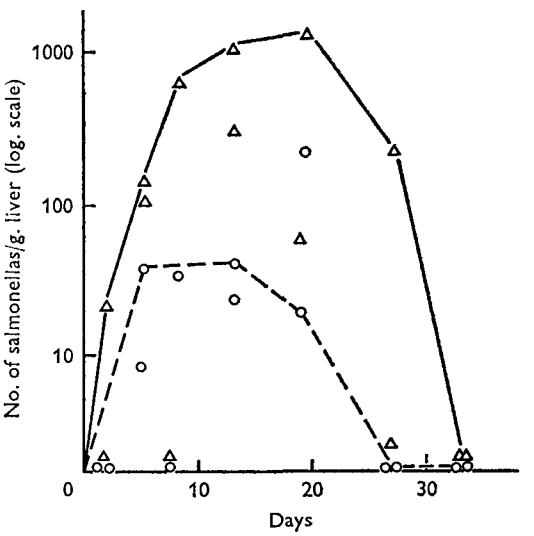

Fig. 2. Progress of a *S. typhimurium* infection in livers of vaccinated (○) and non-vaccinated (△) chickens.

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per gram of liver. A comparison between the two groups is shown as an ogive in Fig. 3. Again vaccination had displaced the vaccinated curve downwards more than 1 logarithmic unit. No salmonellas were found in 5/12 vaccinated chickens compared with 1/12 of the controls. Of the vaccinated chickens, 92 % had no more than 100 salmonellas per gram of liver compared with 25 % of the controls. Half the vaccinated chickens had fewer than 10 salmonellas per gram, whereas half the controls had more than 400 per gram. One quarter of the controls had more than 1000 salmonellas per gram of liver.

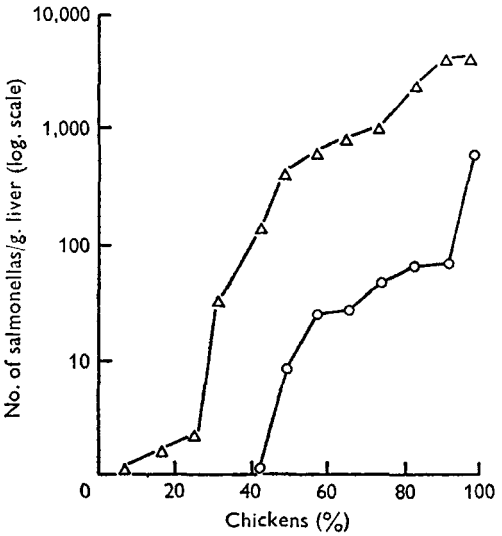

Fig. 3. Effect of oral vaccination on the salmonella content of livers of *S. typhimurium* infected. The number of salmonellas/g. liver was measured in vaccinated (○) and non-vaccinated (△) chickens.

A comparison of oral and subcutaneous vaccination

Chickens 3 days old were vaccinated orally with 1×10^8 cells of *S. dublin* vaccine in antacid suspension, and chickens 9 days of age were vaccinated with 1×10^7 by subcutaneous injection. Together with control non-vaccinated chickens they were challenged at 18 days of age with *S. typhimurium* either 250 organisms intraperitoneally or 1×10^6 orally. Four chickens from each group were examined at intervals until the infection could not be detected in the controls.

The distribution of infection in both groups is shown as a cumulative frequency diagram in Fig. 4. The effect of vaccination was to displace the vaccinated curve as in Figs. 1 and 3 about 2 logarithmic units. Oral and subcutaneous vaccination were equally effective.

Effect of vaccine in drinking water

Since oral and subcutaneous vaccination were equally effective, the vaccine might also be effective if added to the drinking water thus eliminating the need for injection. The vaccine was resuspended in sufficient drinking water so that each

day-old chick would receive 10^8 viable cells by drinking 5 ml. of water over a period of several hours. At the same time other chicks were vaccinated with a similar dose of vaccine *per os*.

Groups of chickens from each method of vaccination together with control non-vaccinated chickens were challenged orally with *S. typhimurium*. This experiment gave results similar to those in Fig. 4 and showed that oral and drinking water vaccination were equally effective.

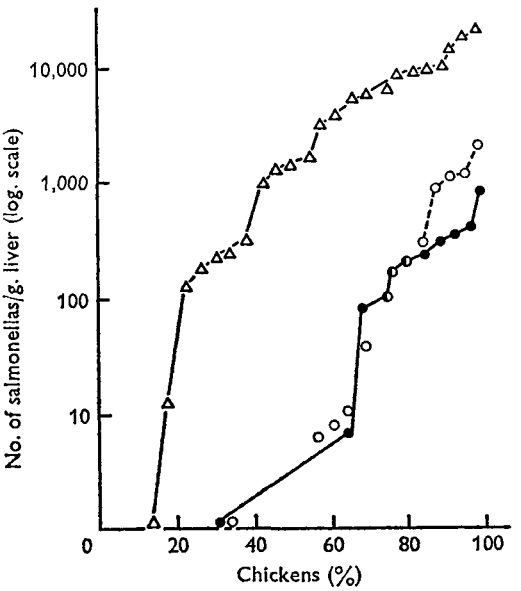

Fig. 4. Effect of subcutaneous or oral vaccination on the salmonella content of *S. typhimurium* infected chickens. The number of salmonellas/g. liver was measured in non-vaccinated (△), orally vaccinated (○) and subcutaneously vaccinated (●) chickens.

Some properties of the vaccine in chickens

Use of the vaccine in the field

Litter and dust were examined from several farms and two of these from which *S. typhimurium* was isolated were chosen for trials. Newly hatched chicks received the vaccine in the drinking water on arrival. Some tagged control chicks were segregated and did not receive any vaccine, but were allowed to mingle with the vaccinated birds 10–14 days later. Subsequently some livers from each group were examined for the presence of *S. typhimurium*.

Farm No. 1. Although five out of six broiler houses on this farm had previously been infected, no evidence for infection appeared during the trial. *S. typhimurium* was isolated from only one out of 95 chicks examined, but was not isolated from dust, litter, food or swabs taken from the newly hatched chicks. The thorough cleansing and disinfection had removed the infection.

Farm No. 2. One hundred chicks from the main flock were brought back to the laboratory and half of them were vaccinated and half left as controls. Two weeks

later they were all challenged with 10^6 *S. typhimurium* cells and subsequently examined. In the laboratory trial, the challenge organism was detected only after enrichment in three out of fifty livers from vaccinated chickens whereas it was present on the direct plates of twelve of the controls. On the farm, however, *S. typhimurium* was detected in only one out of one hundred chickens examined.

Tolerance

Twenty-five 1-day-old chicks tolerated 50 times the recommended vaccine dose and no deaths were recorded. More than 160,000 day-old chicks have been vaccinated on farms and in laboratory tests and no ill effects have been observed.

Persistence of vaccine strain

To determine how rapidly the vaccine strain was destroyed, chickens were orally vaccinated with one or five times the recommended vaccine dose (10^8 cells). Cloacal swabs or livers were examined at intervals up to 40 days. The vaccine strain was isolated from 55 % of chickens examined from 1 to 4 days, in 25 % from 5 to 8 days and less than 1 % from 9 to 40 days.

Effect of passaging the vaccine strain

Chickens were vaccinated and the organism re-isolated and 5×10^8 cells injected subcutaneously into fresh chickens. After four such passages the organism remained unchanged and no ill effects were noted in any of the chickens.

Viability of the vaccine strain in the drinking water

The viability of the vaccine strain was measured 4 hr. after the freeze-dried organisms were re-constituted in distilled water or London tap water supplemented with varying amounts (0–0·2 %) of sodium metaphosphate (Calgon). The viability of the vaccine strain fell rapidly in London tap water and no survivors were found after 4 hr in comparison with distilled water where 80 % of the cells remained viable. The addition of Calgon (0·05; 0·1 and 0·2 %) to London tap water produced 24, 64 and 76 % of the initial viable count. Owing to the variability of water supplies and the presence of bactericidal agents, it is recommended that 0·2 % Calgon be added routinely to avoid undue loss of viability of the vaccine strain.

Antibiotic sensitivity

The vaccine strain was resistant to erythromycin, novobiocin, cloxacillin, penicillin, streptomycin, tetracycline and sulphafurazole, but it was sensitive to chloramphenicol, ampicillin, neomycin, kanamycin and furazolidone. The effect of furazolidone in the diet of vaccinated birds was studied. The results suggested that furazolidone should not be introduced earlier than 4 days after vaccination. The introduction of furazolidone later than 4 days after vaccination appeared to be beneficial and these birds were less infected than the controls not receiving the drug.

DISCUSSION

The effect of vaccination was to reduce the salmonella content of chicken livers significantly. Reductions from tenfold to a 1000-fold were observed. The liver was chosen for quantitative examination for three reasons; it provided a convenient way of assessing the number of salmonellas per gram; it contained the highest concentration of salmonellas, and infected chicken livers may cause outbreaks of food poisoning. This work has shown that vaccination could reduce this hazard.

Until recently, live vaccines were not favoured because of the fear that they might become virulent. With the use of the *S. dublin* vaccine in calves, Hall & Taylor (1970) found little evidence of the strain appearing in human or animal sources and concluded that there was no danger to the general public.

Chickens are most susceptible to invasion and infection by salmonellas during the first few days of life and become more resistant to infection as they grow older. Since vaccination with a live oral vaccine is equivalent to a mild infection, it should be given as early as possible after hatching in order to anticipate a natural challenge. Incorporating the vaccine in the drinking water avoids the need for injections.

Because salmonellosis in flocks is sporadic we did not encounter a field challenge whilst testing the vaccine on farms. However the laboratory trial which was carried out in parallel with the field trial on farm 2 confirmed our earlier findings and further work on these lines with Houghton Poultry Research Station will be published elsewhere.

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