Genetic manipulation of *Salmonella* serotype Bovismorbificans to aromatic-dependence and evaluation of its vaccine potential in mice

T. K. S. MUKKUR, B. A. D. STOCKER* and K. H. WALKER†

CSIRO Division of Animal Health, McMaster Laboratory, Private Bag No. 1, Glebe, New South Wales 2037, Australia, *Department of Medical Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305–5402, USA, and †New South Wales Department of Agriculture and Fisheries, Elizabeth MacArthur Agricultural Institute, Regional Veterinary Laboratory, Menangle, New South Wales 2568, Australia

Summary. The generation of smooth aromatic-dependent *Salmonella* serotype Bovismorbificans (Group C2, O6, 8) from a smooth wild-type parent strain by transduction with phage P1, and conjugation with *Salmonella* serotype Typhimurium carrying F'-8gal is described. The smooth aromatic-dependent *S.* serotype Bovismorbificans was non-lethal for mice at an oral challenge dose of 2×10^9 cfu (equivalent to 200 LD₅₀ of the parent, wild-type strain). The safety of the auxotrophic mutant was further substantiated by comparing its multiplication kinetics *in vivo* with that of its virulent parent organisms. Mice immunised with live, smooth aromatic-dependent *S.* serotype Bovismorbificans by either the oral or intraperitoneal (i.p.) route were protected against oral challenge with virulent *S.* serotype Bovismorbificans; the degree of protection was significantly better ($p < 0.05$) at a challenge dose of 100 or 200 LD₅₀ in mice receiving two rather than one vaccination. In contrast, mice immunised with three doses of the formalin-killed virulent, parent organisms by the i.p. route were not protected, in spite of high antibody titres. Only those mice immunised with the live, smooth aromatic-dependent *S.* serotype Bovismorbificans i.p. developed significant ($p < 0.01$ – 0.05) delayed-type hypersensitivity.

Introduction

Salmonellosis of ruminant animals is recognised as an important disease in most countries and causes serious economic losses, particularly in animals subjected to stressful conditions, such as those encountered during shipping over long distances^{1,2} or intensively-managed livestock enterprises.³ Furthermore, it constitutes a serious public health hazard not only for the consumers of meat and meat products but also for the worker associated with the handling of these products such as abattoir employees.^{4,5}

Although a wide variety of *Salmonella* serotypes has been reported from outbreaks of clinical salmonellosis, most episodes can be attributed to a cluster of selected serotypes. The most common isolates from cases of salmonellosis in cattle in Australia, in order of frequency, are *S.* serotypes Dublin, Typhimurium, and Bovismorbificans. In sheep, they are *S.* serotypes Typhimurium, Bovismorbificans and Havana.² Our aim is to develop a composite salmonella vaccine containing aromatic-dependent derivatives of the above-mentioned pathogens and to evaluate its efficacy in reducing the mortality of sheep during transit

over long distances. Previously we reported that sheep immunised with *S.* serotype Typhimurium *aroA* by either the oral or intramuscular route were highly protected against oral challenge with virulent organisms.⁶ In this communication, we describe the manipulation of virulent *S.* serotype Bovismorbificans to aromatic-dependence and the vaccine potential of the *S.* serotype Bovismorbificans *aroA* thus generated in experimental murine salmonellosis as a model system in the first instance.

Materials and methods

Bacterial strains and phage lysates

Virulent *S.* serotype Bovismorbificans strain CS400/SL5747 used in this investigation was isolated from a dead sheep. Other bacterial strains and phage lysates used in the generation of *S.* serotype Bovismorbificans *aroA* are shown in table I. The virulent parent strain and derivatives with mutation in the *aroA* gene were maintained as cultures from Brain Heart Infusion Broth (BHI) in glycerol at -70°C in a Biofreezer. Derivatives with mutations in the *gal* operon were characterised by determining the ability of mutant organisms to ferment galactose in MacConkey's agar

Table I. Bacterial strains

Strain no.	Description*	Origin or reference
(a) <i>Salmonella</i> serotype Bovismorbificans		
CS400/SL5747	Wild-type, tetracycline-resistant	From fatal enteritis in sheep
CS401/SL5750	As SL5747 but <i>galE</i>	From SL5747 by selection for resistance to 2-deoxygalactose
CS402/SL5752	As SL5750 but tetracycline-sensitive	From SL5750 by selection on Böchner medium ¹⁰
CS403/SL5780	As SL5752 but CRR426 [<i>aroA554::Tn10</i> (Tc-sens) non-rev] <i>zbj-903::Tn10 galE galT</i> and/or <i>galK</i> †	From SL5752 by transduction from SL2838, with selection for tetracycline-resistance
CS404/SL5785	As SL5752 but CRR [<i>aroA</i>] CRR[<i>zbj-903::Tn10</i> (Tc-sens)] <i>galE galT</i> and/or <i>galK</i>	From SL5780 by selection on Böchner medium ¹⁰
CS405/SL5794	As SL5785 but Gal ⁺ because carrying F'-8 <i>gal</i> +	From SL5785 by conjugational cross with SL3550, with selection for <i>gal</i> +
(b) <i>Salmonella</i> serotype Typhimurium		
SL1479†	UCD108-11 CRR426 [<i>aroA554::Tn10</i> (Tc-sens, non-rev)]	<i>S.</i> serotype Typhimurium UCD <i>aroA</i> ¹²
SL2838†	7471 <i>galE705</i> CRR426 [<i>aroA554::Tn10</i> (Tc-sens, non-rev)] <i>zbj-903::Tn10</i>	P1-sensitive stable <i>aroA zbj::Tn10</i> strain used as transductional donor ¹³
SL3550†	LT2 <i>trp metA galE541/F'8 gal</i> +	See Reference 14

* CRR, Tn10-generated complex rearrangement mutation causing phenotypic characters indicated in parenthesis: Tc-sens, sensitive to tetracycline; non-rev, unable to revert to *aro* + ; *zbj::Tn10*, silent insertion of Tn10 at minute 20 of chromosome (co-transducible with *aroA*).

† The failure of strain SL5780 to recover wild-type phage sensitivity pattern when grown with galactose (and glucose) is presumed to result from a coincidental mutation in the *gal* operon, affecting function of gene *galT* or *galK* or both.

‡ Additional characters of these strains, not here relevant, omitted.

base with D-galactose 0.5% and were grown in BHI with D-galactose for preservation at -70°C .

Generation of aromatic-dependent *S. serotype Bovismorbificans*

Various steps used in the generation of aromatic-dependent *S. serotype Bovismorbificans* are shown in table I and are discussed in the *Results* section.

Determination of LD50

The LD50 of the virulent *S. serotype Bovismorbificans* strain CS400/SL5747 was determined in BALB/c mice as described elsewhere.⁷

Immunisation and challenge experiments

BALB/c, female mice (6–8 weeks old) were immunised with *S. serotype Bovismorbificans aroA* (CS405/SL5794) either orally (one or two doses of 10^{10} cfu/mouse administered 7 days apart) or intraperitoneally (i.p.) (one dose of 10^5 cfu/mouse or two doses of 10^5 and 10^6 cfu administered 7 days apart). Additional groups of mice were immunised i.p. with formalin-killed *S. serotype Bovismorbificans* (three doses of 10^7 , 10^8 and 10^9 cfu given at intervals of 7 days). All immunising doses were prepared by washing bacteria grown in BHI with sterile physiological saline and resuspension in the same diluent so that the total cfu to be administered was contained in a total volume of 0.5 ml. All mice (including unimmunised control mice) were challenged at 4 weeks post-immunisation with 20, 100 or 200 LD50 of virulent *S. serotype Bovismorbificans* (CS400/SL5747) by gavage with 0.5 ml of a

culture grown in BHI with shaking at 37°C for 18 h and after adjustment of pH to 8.5. Mice were observed for 15 days after challenge and mortality was recorded. The number of mice in each group was 8–18.

Determination of multiplication of *S. serotype Bovismorbificans aroA* in vivo

The kinetics of survival of *S. serotype Bovismorbificans aroA* (CS405/SL5794) in mice were determined after inoculation by the i.p. (1×10^5 cfu/mouse) or oral (5×10^8 cfu/mouse) route, over a period of 27 days after inoculation. *S. serotype Bovismorbificans aroA* were enumerated in liver, spleen, mesenteric lymph nodes (MLN) and intestine. Organs were homogenised with a Colworth Stomacher 400, sterile physiological saline (1.0 ml) was added and the homogenate was spread-plated for viable counts on BHI agar, with the exception of intestine samples for which Xylose Lysine Deoxycholate Medium (XLD) was used.

Immunological procedures

Antibody titres were determined by an enzyme-linked immunosorbent assay.⁸ Antigen (100 μg of protein) used for coating the ELISA plates (M129B, Dynatech) was prepared by ultrasonication of virulent *S. serotype Bovismorbificans* followed by centrifugation at 27 000 *g* to remove the cell debris. The ELISA titres were expressed as end-point antibody titres. The cut-off point was determined by an average of the optical densities at 405 nm for four antigen-free wells, with a high activity mouse-specific anti-*S. serotype Bovismorbificans* serum used at a dilution identical with the starting dilution of individual test samples.

ELISA titres were the sample dilutions determined by interpolation from the cut-off point.

Cutaneous hypersensitivity was measured by injecting the ultrasonicated antigen preparation described above (10 µg of protein in 5 µl of saline) into the foot pads of four mice and measuring their thickness with vernier calipers 48 h after inoculation. Cutaneous hypersensitivity was calculated as the mean increase in the foot-pad thickness (mm) after antigen injection in one hind foot minus that obtained after injection of saline in the other hind foot, measured 48 h after injection.

Statistical analyses

The significance of differences between mean log₁₀-transformed values for ELISA titres and hypersensitivity measurements was determined by Student's *t* test. Significance of protection in the mouse immunisation and challenge experiments was assessed by Fisher's Exact test.

Results

Generation of aromatic-dependent *S. serotype Bovismorbificans aroA*

The wild-type strain of *S. serotype Bovismorbificans*, CS400/SL5747, was prototrophic, smooth (as indicated by resistance to rough-specific phages and by reaction with *Salmonella* O 6,8 serum) and sensitive to Felix O phage; it was resistant to tetracycline. *Salmonella* of O groups other than A, B and D do not adsorb phage P22 which, therefore, cannot be used to transduce genes to or from such strains. Another general transducing phage, P1, is, generally, ineffective on smooth *Salmonella* strains but active on strains with the mutation *galE*, because when grown on medium not supplemented with galactose they make "rough" LPS of type Rc (galactose-deficient), appropriate for adsorption of phage P1.⁹ Galactose-negative (non-fermenting) mutants of the wild-type *S. serotype Bovismorbificans* strain were, therefore, isolated by selection for ability to grow on defined medium containing 2-deoxygalactose, which inhibits the growth of galactose-positive strains. Two of 20 such mutants tested were resistant to Felix O phage (indicating an LPS core defect) and sensitive to phage C21 which, like phage P1, acts efficiently on strains making type Rc LPS. These two mutants were sensitive also to rough-specific phages 6SR and Br60. The presence of galactose (plus glucose, to prevent galactose toxicity) in the medium used for testing phage sensitivities restored the phage sensitivity pattern of the wild-type parent strain, indicating loss of *galE* function but retention of *galK* and *galT* functions, so that exogenous galactose could be used for synthesis of UDP galactose, the precursor of the galactose units of LPS. One of the two *galE* mutants, CS401/SL5750, was plated on autoclaved chloram-

phenicol/fusaric-acid medium¹⁰ which impedes the growth of tetracycline-resistant *Salmonella* and *Escherichia coli* strains. Nine of 10 resistant mutants from this selection were found to be tetracycline-sensitive. One of them, CS402/SL5752, was treated with phage P1 grown on an *S. serotype* Typhimurium strain, SL2328, which is *galE* and has a transposon-generated non-reverting mutation at *aroA* and an adjacent silent Tn10 insertion, *zbf-903::Tn10*.¹¹ Selection was made for tetracycline resistance. One of 34 tetracycline-resistant colonies tested was found to be aromatic-dependent by co-transduction of *aroA* of the donor with its Tn10 insertion. This transductant, CS403/SL5780, had the same phage-sensitivity pattern as its parent, which showed that it still had the *galE* mutation, but its phage sensitivities were not altered when tested on galactose-supplemented medium. This probably indicates that it had lost *galK* or *galT* functions, or both, by a second mutation affecting the *gal* operon. As tetracycline resistance is not desirable in a live vaccine strain, a tetracycline-sensitive mutant, CS404/SL5758, was next isolated from CS403/SL5780 by the same method as before. Finally, to restore wild-type LPS character (smooth), strain CS404/SL5780 was given an F' factor, F'8, which includes the *gal* + operon of *E. coli*, by a conjugational cross with a *S. serotype* Typhimurium strain carrying this plasmid. A galactose-positive transconjugant, now smooth by the criterion of phage sensitivity pattern and O-antigen character and still aromatic-dependent, was labelled CS405/SL5794. This strain was employed as a live vaccine in the trials. No reversion to aromatic-independence has been detected in strain CS405/SL5794, as was to be expected since its transposon-generated *aroA* mutation, originally used to construct a *S. serotype* Typhimurium live vaccine strain, SL1479, has given no revertants in extensive trials of that strain.¹²

Strain CS405 did not produce galactose-negative segregants at a rate detectable by streaking broth cultures on MacConkey agar base with added galactose, indicating either a fairly stable maintenance of the F' factor in this strain or, less probably, recombination of the wild-type *gal*⁺ (of *E. coli* origin) of the F' factor with the mutant *gal* region of the salmonella chromosome.

In-vivo safety of various derivatives of *S. serotype Bovismorbificans* in mice

The oral LD50 of the parent virulent *S. serotype* Bovismorbificans (CS400/SL5747) was calculated to be 1 × 10⁷ cfu. As can be seen from table II, a significant percentage of mice survived when challenged with 200 LD50 dose-equivalents of that calculated for the wild-type *S. serotype* Bovismorbificans, i.e., 2 × 10⁹ cfu of the *galE* (CS402/SL5752), *galE galK* and/or *galT*, *aroA* (CS404/SL5785) or *aroA Gal*⁺ (CS405/SL5794) smooth derivatives of *S. serotype* Bovismorbificans.

Table II. Relative safety *in vivo* of *S. serotype Bovismorbificans* derivatives administered to mice by the oral route

Derivative	Challenge dose (cfu)	Total number of mice surviving (%)/total number challenged
<i>galE</i> (CS402/SL5752)	2.0×10^9 *	8(67)/12
<i>galE galT</i> and/or <i>galK, aroA</i> (CS404/SL5785)	2.0×10^9	12(100)/12
<i>aroA Gal</i> ⁺ (CS405/SL5794)	2.0×10^9	12(100)/12
Wild-type, parent	2.0×10^9	0(0)/12

* 2.0×10^9 cfu represent 200 LD₅₀ dose-equivalents of that calculated for the wild-type parent organism.

The safety of *S. serotype Bovismorbificans aroA* (CS405/SL5794) *in vivo* was further substantiated by comparing its multiplication kinetics *in vivo* with that of its virulent parent when administered by oral or i.p. routes. As shown in table III, *S. serotype Bovismorbificans aroA Gal*⁺ (CS405/SL5794) was present in the liver, spleen, MLN and intestine (only in small numbers) regardless of the route of administration. By day 27 after oral administration, the mutant organisms were detectable in the intestine (in small numbers) but not in liver, spleen or MLN. On the other hand, when the organisms were administered by the i.p. route, the mutant *aroA* organisms were detectable in the spleen and MLN (not liver or intestine) of one out of three mice, but only by enrichment cultures. When mice were inoculated with the virulent parent organisms (CS400/SL5747) by the oral route, the organisms were detectable by direct plating on day 1 in the intestine. By day 4 they were detectable by direct plating in all organs and the total cfu showed a significant increase on day 7. On day 14, the total cfu showed a decline but one out of three mice had died. The remaining inoculated mice died on day 16 and no more animals were available for enumeration of organ counts. When

Table III. Multiplication of *S. serotype Bovismorbificans aroA Gal*⁺ strain (CS405/SL5794) *in vivo* in mice inoculated by oral or i.p. routes

Day after inoculation	Mean cfu (SD)/organ in			
	liver	spleen	MLN	intestine
<i>Oral route</i>				
1	0[1/3E*]	0	0[1/3E]	$58(50) \times 10^3$
4	17(29)	0[1/3E]	33(50)	0[3/3E]
7	407(352)	87(78)	33(33)	$3.74(5.2) \times 10^3$
14	17(29)	20(35)	87(64)	0[3/3E]
27	0	0	0	60
<i>I.p. route</i>				
1	105[1/3E]	4[2/3E]	133(188)	0[1/3E]
4	83(76)	70[1/3E]	107(159)	70(32)
7	7(3)	48[1/3E]	167(148)	0[3/3E]
14	17(29)	20(35)	87(64)	0[3/3E]
27	0	0[1/3E]	0[1/3E]	0

* E denotes recovery of salmonella organisms by an enrichment procedure whereas the figures shown within [] represent the proportions of mice from the organs of which salmonella organisms were isolated by enrichment culture.

mice were inoculated i.p. with the virulent parent, the organisms were detected by direct plating on day 1 from the liver, spleen, MLN and intestine. The total number of cfu increased in these organs on day 4. By day 7 after inoculation, all but two mice were dead (table IV).

Immunisation-challenge experiments

Table V shows that immunisation of mice with three doses of formalin-killed virulent *S. serotype Bovismorbificans* imparted no protection against oral challenge with virulent organisms. In contrast, mice immunised with live *S. serotype Bovismorbificans aroA Gal*⁺ (CS405/SL5794) by either the oral or i.p. route were significantly protected. Protection appeared to be better in mice immunised with two doses

Table IV. Multiplication of the parent virulent *S. serotype Bovismorbificans* (CS400/SL5747) *in vivo* in mice inoculated by oral or i.p. routes

Day after inoculation	Mean cfu (SD) (10^3)/organ in			
	liver	spleen	MLN	intestine
<i>Oral route</i>				
1	0	0	0[3/3E*]	23.4(26.10)
4	2.03(1.13)	2.6(2.0)	6.2(3.6)	9967(7596)
7	2180.1(3484.7)	48.3(52.7)	10.0(7.65)	91667(70868)
14	832.5	9.6	1.95	78000
<i>I.p. route</i>				
1	0.11(0.047)	0.05(0.029)	0.029(0.22)	0.09(0.03)
4	171.3(129)	285(208)	77(77)	23.3(31.3)
7	1565	1245	588	1587

* Isolation by enrichment; the proportion shown in [] represents the number of mice in which salmonellae were isolated by enrichment divided by the total number of mice used on that day after inoculation.

† Average of two mice only because no more mice were alive after this interval.

Table V. Protection of mice immunised with *S. serotype Bovismorbificans aroA* Gal⁺ strain (CS405/SL5794) against oral challenge with virulent organisms (CS400/SL5747)

Vaccine preparation	Route of immunisation	Number of vaccine doses administered	Number of mice surviving (%) / total number of mice challenged with		
			20 LD50	100 LD50	200 LD50
None (unimmunised)	NA	NA	2(13)/15	0(0)/16	0(0)/16
Formalin-killed virulent, parent (CS400/SL5747)	I.p.	3	2(25)/8	0(0)/8	0(0)/8
Live, derivative, <i>aro</i> ⁻ (CS405/SL5794)	I.p.	1	15(83)/18†	8(44)/18*	7(39)/18*
	I.p.	2	8(100)/8†	12(75)/16§	12(71)/17§
	Oral	1	6(75)/8*	3(30)/10	2(20)/10
	Oral	2	7(87)/8*	6(75)/8	3(38)/8

NA, not applicable.

Significance of protection in immunised *vs.* unimmunised mice: § $p < 0.001$; † $p < 0.01$; * $p < 0.05$.

rather than one dose. This was particularly evident at challenge doses of 100 and 200 LD50 (table V). The percentage survival of unimmunised control mice in the above experiments ranged from 0% to 13%. It is interesting that mice surviving immunisation with the *galE* mutant of *S. serotype Bovismorbificans* (CS402/SL5752) were significantly ($p < 0.01$) protected against challenge-infection with the virulent parent, but mice immunised with the *galE galK* and/or *galT* mutant *aroA* strain (CS404/SL5785) were not (data not shown).

Immunological response

Mice immunised with either two doses of live mutant (CS405/SL5794) or three doses of formalin-killed virulent organisms by the i.p. route developed high antibody ELISA titres, whereas those immunised with one dose of live mutant organisms by the i.p. route had low antibody titres (table VI). On the other hand, no antibody titres were detectable in mice immunised orally with one or two doses of the mutant

aroA strain. Mice immunised with the live, *S. serotype Bovismorbificans aroA* (CS405/SL5794) by the i.p. route (one or two doses) developed a significant cutaneous hypersensitivity which was determined to be characteristic of a delayed-type hypersensitivity response (i.e., type IV cell-mediated immune response represented by a predominantly lymphocytic infiltration, determined histologically). Those immunised by the oral route or with formalin-killed virulent parent organisms did not show this response (table VI).

Discussion

Recent experiments in our laboratory revealed that while mice immunised with *S. serotype Typhimurium aroA*, administered either orally or parenterally, were partially protected against experimental challenge infection with virulent *S. serotype Bovismorbificans*, sheep immunised orally were unprotected (Mukkur and Walker, unpublished data). Therefore, it was

Table VI. Immunological response of mice immunised with either live *S. serotype Bovismorbificans aroA* Gal⁺ strain (CS405/SL5794) or formalin-killed virulent parent organisms 4 weeks after immunisation

Vaccine strain	Route of immunisation	Number of vaccine doses	ELISA titre (units/ml, SEM)	Cutaneous hypersensitivity (mm, SEM)
Live CS405/SL5794	i.p.	1	1126 (236) ^a	0.57 (0.12) ^d
Live CS405/SL5794	i.p.	2	20 094 (9860) ^b	0.86 (0.08) ^e
Live CS405/SL5794	Oral	1	Not detectable	ND
Live CS405/SL5794	Oral	2	Not detectable	0.17 (0.02) ^f
Formalin-killed CS400/SL5747	i.p.	3	11 767 (3958) ^c	0.20 (0.07) ^g
None	Unimmunised	NA	Not detectable	0.13 (0.05)

ND, not done. NA, not applicable

Values significantly different from each other: b *vs.* a ($p < 0.01$); c *vs.* a ($p < 0.001$); d *vs.* f and g ($p < 0.05$); e *vs.* f and g ($p < 0.01$).

considered necessary to generate *S. serotype Bovismorbificans aroA* by genetic manipulation and to evaluate its vaccine potential in mice in the first instance. Whereas the O-specific phage P22 has been used to transduce genes from one *Salmonella* strain to another within or between Groups A, B and D, the generalised transducing phage P1 was used for transduction of genes from *S. serotype Typhimurium aroA* made *galE* to *S. serotype Choleraesuis* (Group C1) made *galE*.¹¹ In this investigation, this process led to the generation of *aroA*, *galE galK* and/or *galT* derivatives (CS404/SL5785 or 5786) which were rough. However, the *aroA* strain CS404/SL5785 could be made Gal⁺ and smooth by conjunction with *S. serotype Typhimurium* carrying F'8-*gal* (SL3550).

Regardless of the route used for the immunisation of mice, the rough strain (CS404/SL5785) did not impart any protection (data not shown) whereas the *aroA* Gal⁺ strain (CS405/SL5794) imparted significant ($p < 0.001-0.05$) protection. The percentage protection obtained was significantly better ($p < 0.05$), particularly at a challenge dose of 100 or 200 LD₅₀ when mice were vaccinated more than once. Mice immunised with live, *S. serotype Bovismorbificans aroA* Gal⁺ by the oral route or formalin-killed virulent

parent (CS400/SL5747) did not develop any delayed-type hypersensitivity, whereas those immunised with the live, *aroA* Gal⁺ strain (CS405/SL5794) by the i.p. route showed a significant development. Since elicitation of delayed-type hypersensitivity is an indicator of cell-mediated immune response, the failure of the formalin-killed vaccine to impart protection against salmonellosis confirmed a role for cellular immunity in the prevention of this disease, as suggested previously.¹⁵ However, since mice immunised by the oral route which did not develop any delayed-type hypersensitivity were also protected, an important role for the localised cellular or humoral immune factors, or both, was also indicated. Although the nature of these local protective factors is not known at the present time, similar observations were made previously in sheep immunised with *S. serotype Typhimurium aroA* by the oral route.⁶

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References

1. Jelinek PD, Franklin DA, Iveson JB. The recovery of salmonella from sheep that died during transportation by ship. *Aust Vet J* 1982; **58**: 170-171.
2. Richards RB, Norris RT, Dunlop RH, McQuade NC. Causes of death in sheep exported live by sea. *Aust Vet J* 1989; **66**: 33-38.
3. Wray C, Sojka WJ. Reviews of the progress of dairy science: bovine salmonellosis. *J Dairy Rs* 1977; **44**: 383-425.
4. Smeltzer T, Thomas R, Collins G. Salmonellae on posts, hand-rails and hands in a beef abattoir. *Aust Vet J* 1980; **56**: 184-186.
5. Samuel JL, Eccles JA, Francis J. Salmonella in the intestinal tract and associated lymph nodes of sheep and cattle. *J Hyg (Camb)* 1981; **87**: 225-232.
6. Mukkur TKS, McDowell GH, Stocker BAD, Lascelles AK. Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*. *J Med Microbiol* 1987; **24**: 11-19.
7. Litchfield JT, Wilcoxon F. A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 1949; **96**: 99-113.
8. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol* 1972; **109**: 129-135.
9. Ornellas EP, Stocker BAD. Relation of lipopolysaccharide character to P1 sensitivity in *Salmonella typhimurium*. *Virology* 1974; **60**: 491-502.
10. Bochner BR, Huang H-C, Schieven GL, Ames BN. Positive selection for loss of tetracycline resistance. *J Bacteriol* 1980; **143**: 926-933.
11. Nnalue NA, Stocker BAD. Tests of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *Salmonella choleraesuis*. *Infect Immun* 1987; **55**: 955-962.
12. Smith BP, Reina-Guerra M, Hoiseth SK *et al.* Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. *Am J Vet Res* 1984; **45**: 59-66.
13. Nnalue NA, Stocker BAD. The effects of O-antigen character and enterobacterial common antigen content on the in-vivo persistence of aromatic-dependent *Salmonella* sp. live-vaccine strains. *Microb Pathog* 1987; **3**: 31-44.
14. Kuo T-T. Genetics of *Salmonella typhimurium*: rough mutants, transduction by phage ES18 and suppression of proline auxotrophy. Ph.D. Thesis, Stanford University, California. 1969: 210 pp.
15. Collins FM, Mackaness GB, Blanden RV. Infection-immunity in experimental salmonellosis. *J Exp Med* 1966; **124**: 601-619.