Comparison of immune responses of two *Salmonella gallinarum* strains viewed as possible vaccines for fowl typhoid in Kenya

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ABSTRACT

BEBORA, LILLY C., NYAGA, P.N. & KIMORO, C.O. 1998. Comparison of immune responses of two *Salmonella gallinarum* strains viewed as possible vaccines for fowl typhoid in Kenya. *Onderstepoort Journal of Veterinary Research*, 65:67–73

The immune responses of two *S. gallinarum* strains, L46 and CN 180, were compared in 15-week-old cockerels.

The humoral and cell-mediated immune responses were assayed by means of the indirect haemagglutination test (IHA) and the macrophage migration inhibition test (MIT), respectively. Birds were vaccinated with the two vaccines, respectively, and bled for sera (for IHA) and cells (for MIT) every week up to the seventh week, post vaccination, then every alternate week, three times, and later once every month, for a total period of 37 weeks. Strain L46 was found to induce an immune response that was very similar to that of CN 180. Both gave good humoral and cellular responses.

Keywords: Fowl typhoid, immune response, *Salmonella gallinarum*, vaccines

INTRODUCTION

Salmonella organisms belong to the group of bacteria referred to as "facultatively intracellular organisms". These are organisms that can survive within macrophages without being degraded (Collins 1972a), i.e. fusion of the phagosome and the lysosome does not occur after phagocytosis, as happens under normal circumstances (WHO 1973). With facultatively intracellular organisms, which also include bacteria-like *Mycobacterium*, Brucella, Listeria, etc., fusion occurs only in hypersensitivity reaction, when macrophages are activated and become ferocious (Campbell 1976). Therefore, although *Salmonella* infections induce both humoral and cell-mediated immune responses (Pomeroy 1984), various workers have shown that cell-mediated immunity is the one responsible for protection against the invading organism (Mackaness 1970). It should be noted, however, that antibodies also play a role in protection (Cameron 1976; Davies & Kotlarski 1976). Dead vaccines tend to give rise to humoral immunity only (Solotorovsky & Soderberg 1972; Davies & Kotlarski 1976). Live vaccines (virulent or attenuated) give rise to both cell-mediated and humoral immunity (Mackaness 1967; Colins 1971).

In Kenya, an attenuated vaccine strain, CN 180, produced by The Veterinary Research Laboratories, Ministry of Livestock Development, Kabete, was used in the field for a long time, together with a formalin-killed vaccine which consisted of several *S. gallinarum* isolates pooled together. The killed vaccine was specifically recommended for far places because of its longer-lasting potency. The use of the attenuated vaccine was, however, stopped around the year 1990, owing to cases of fowl typhoid outbreaks even in birds vaccinated with the vaccine. It was this observation that necessitated the study of the immune response of the vaccine in birds, in order to solve the issue of

Accepted for publication 24 October 1997—Editor

the protectiveness (efficacy) of the vaccine. A new vaccine strain of *S. gallinarum* (L46) was also developed and its efficiency in inducing immune response in birds compared with that of CN 180.

Please note that the production of veterinary vaccines, which was originally done by the Veterinary Laboratories, Ministry of Livestock Development, Kabete, has now been taken over by the Kenya Veterinary Vaccines Production Institute (KEVEVAPI). KEVEVAPI currently produces only a killed (inactivated) fowl typhoid vaccine referred to as Fowlvax. Dosage: 1 mℓ intramuscularly.

MATERIALS AND METHODS

Birds

Fifteen-week-old cockerels, purchased and raised locally, were used in the study. They were obtained from a flock with no history of *Salmonella* infection, and were raised on *Salmonella*-free premises.

Vaccines

The already established *S. gallinarum* attenuated vaccine strain, CN 180, obtained from the Veterinary Research Laboratories, Ministry of Livestock Development, Kabete, and a newly developed *S. gallinarum* vaccine strain, L46, were used. The latter was selected from 20 *S. gallinarum* isolates recovered from clinical cases that were virulence-tested by means of the LD_{50} procedure (Reed & Muench 1938). Attenuation was accomplished by passaging 100 times (after every 2 d) on MacConkey agar. Both vaccine strains were of a smooth type.

Preparation of antigens

The crude antigen used for the indirect haemagglutination test (IHA) and the macrophage migration inhibition test (MIT) was prepared according to the procedure of Neter, Bertram & Arbesman (1952). Briefly, a 24 h culture of *S. gallinarum* was boiled for 2–2,5 h and centrifuged at 5 000 x *g* for 15 min at room temperature. The supernatant, containing between 2,5 and 5,9 μg/mℓ of protein by Lowry's method (Lowry, Rosebrough, Farr & Randall 1951), was collected, preserved with thiomersal and stored at 4 °C.

For the rapid whole-blood plate test (RWBPT), the antigen was obtained from Iffa Merieaux, London, through a local chemist (Monks, Nairobi, Kenya).

Collection and processing of sheep red-blood cells

Blood for IHA was obtained from sheep. Approximately 50 mℓ of blood was collected into a flask containing 50 mℓ of Alsever's solution and centrifuged at 750 x *g* for 10 min. The supernatant was decanted and the red-blood cells (rbc) were washed three times with saline (0,85 % NaCl) prior to being coated with antigen, according to the method of Neter *et al.* (1952) The undiluted crude antigen (prepared earlier) was added to the rbc pellet to make a 2,5 % rbc suspension and incubated at 37 °C for 1 h, with regular stirring. The rbc's were then washed three times with physiological saline and finally made up to a 2 % suspension. It was stored in 5 mℓ volumes at 4 °C until used.

Collection of serum for IHA and cells for MIT

Chicken blood was obtained by puncture of the wing vein. Blood to be used for IHA was collected into sterile universal bottles without anticoagulant. After incubation at 37 °C for 1 h, the clotted blood samples were centrifuged at 750 x *g*, at room temperature, for 10 min. Serum from each blood sample was collected into a Bijou bottle and stored at –20 °C until used. The blood to be used as a source of leucocytes was collected into a sterile universal bottle containing 15 cc of heparinized calcium-magnesium-free Dulbecco phosphate buffer (H-DPB). The heparin was added at the rate of 20 units per 1 mℓ of DPB. About 5 cc of blood was added to H-DPB to give a dilution of 1:4.

Procedure for IHA

This was done according to the method of Herbert (1973). Microtitre 'U' plates (Cooke Engineering Co., Alexandria, V.A. San Mateo, California) were used. Doubling dilutions of the test sera were made in 50 μℓ aliquots and equal volumes of coated erythrocytes added. Positive reaction appeared as a complete carpet of haemagglutinated cells covering the bottom of the wells. Negative reaction (no haemagglutination) appeared as a compact button or small ring at the centre of the well bottom. The titre was taken as the highest dilution giving complete haemagglutination.

Procedure for MIT

The capillary-tube technique of the MIT was applied according to the method given by Timms (1974). Peripheral blood, diluted 1:4, was used as the source of leucocytes, and Ficoll/Hypaque gradient solution (Nyegaard & Co. A/s Oslo, Norway) as the separating medium. Cell enumeration was done with the aid of the improved Neubauer haemacytometer (Bright Line Hemacytometer, American Optical Company, Scientific Instrument Division, Buffalo N.Y. 14215) and 0,05 % trypan blue intravital stain. The cell concentration was adjusted to 1,5–2,0 x 10^7 cells/mℓ (Falk & Zabriskie 1971). Non-heparinized capillary tubes, 75 mm long and with an internal diameter of 1,0–1,2 mm, were used for packing and loading the cells in the cell migration chambers.

The test was done in two sets—the test chamber which contained the growth medium (minimum essential medium) plus the antigen, and the control chamber containing the medium only. After overnight incubation at 37 °C on a flat surface, the cell migration was viewed under a low-power microscope and the migration fronts projected onto Whattman filter paper (18,5 mm). For each system, three tracings were made on the paper. These were cut and weighed, and the average mass for each set of papers was determined. The percentage migration inhibition (% MI) was calculated according to the formula of Timms (1974):

$$\%MI = \left\{ \frac{1 - \text{wt of paper from chamber with antigen}}{\text{Wt of paper from chamber without antigen}} \right\} \times 100$$

The area of the migration was taken to be directly proportional to the mass of the paper traced (Timms 1974).

Procedure for RWBPT

This was done according to Snoeyenbos (1984). Briefly, a drop of blood from the suspected bird was placed on a white tile, and a drop of the blue-stained smooth *S. pullorum* antigen was added. The mixture was thoroughly stirred and the tile rocked up and down. In positive cases, blue clumps appeared within 2 min.

Experimental procedure

Nine of the 15-week-old cockerels, randomly selected, were divided into three groups of three chickens each. In group 1, consisting of cockerels 8, 19 and 21, the birds were vaccinated intramuscularly with the already-established vaccine strain, CN 180, at a dosage of 1 mℓ (1 000 organisms) per bird. In the second group, with cockerels 23, 10 and 20, the birds were vaccinated intramuscularly with *S. gallinarum* strain L46 at a dosage of 1 000 organisms per bird. Group 3 contained cockerels 12, 15 and 17 and it served as a control. These three groups were kept in cages located in separate rooms.

Each bird was bled, for both serum and cells (leucocytes), once every week for 7 weeks, then every alternate week for 6 weeks, and then once a month until the termination of the experiment. The serum was screened with the aid of IHA, and the cells, mainly lymphocytes and macrophages, were used to run MIT. RWBPT was carried out on whole blood.

RESULTS

There was an immune response to both vaccines. The MIT response (Table 1), as measured by the percentage macrophage migration inhibition, fluctuated throughout the experimental period, reaching an average maximum of 65 % in CN 180-vaccinated birds, and 76 % in L46-vaccinated birds, 9 weeks post vaccination. L46 seemed to be more effective than CN 180 in maintaining the response. It is interesting that the control birds also showed some MIT responses up to 20 %.

Overall, the L46-vaccinated birds showed higher IHA titres than the CN 180-vaccinated birds (Table 2), the mean peak titre of 44 117 being demonstrated on week 17 post vaccination. The titre fluctuated a great deal throughout the experimental period. The CN 180-vaccinated birds showed a more gradual increase in titre and there were fewer fluctuations than in L46-vaccinated birds. The control birds also gave titres up to 16 for *S. gallinarum*.

Statistical analysis of both MIT and IHA responses for the two vaccine strains was done by means of a two-way ANOVA. Owing to the nature of the data, an arcsine transformation was done for the MIT responses, whereas for the IHA, a log transformation was carried out. Both responses showed that there was no statistical difference between vaccine strains CN 180 and L46 at $P \leq 0,05$ level [MIT:F = 3,18 ($P = 0,10$) at 1 and 6 degrees of freedom; IHA:F = 2,82 ($P = 0,10$) at 1 and 36 degrees of freedom].

However, when the two groups of vaccinated birds were compared with the unvaccinated birds (controls), they showed a significant statistical difference at $P = 0,05$ level for L46: MIT:F = 30,88 at 1 and 6 degrees of freedom ($P < 0,015$), IHA:F = 20,11 at 1 and 36 degrees of freedom ($P = 0,005$), and for CN 180: MIT: F = 17,82 at 1 and 6 degrees of freedom ($P = 0,056$), IHA:F = 131,20 at 1 and 36 degrees of freedom ($P < 0,005$).

One of the birds vaccinated with L46 (bird 10) developed a good immmunity as indicated by high MIT values (Table 1), IHA titres (Table 2), and also its strong positive agglutinations with RWBPT, from 4 weeks onwards, while the other birds gave negative results (Table 3). Bird 23, also from the L46-vaccinated group, gave an unusually high IHA titre of 131,072 at week 17 post vaccination (Table 2). Although the IHA titre of this bird was higher than that of bird 10, at this particular time bird 23 gave a negative RWBPT result while bird 10 gave a positive RWBPT result (Table 3).

DISCUSSION

Since *Salmonella* organisms, like any other facultatively intracellular organisms, can survive within normal macrophages without being digested, antibodies play a minor role or first line of defence in getting rid of the organisms. The major role or second line of defence, as reviewed earlier, lies on the cell- mediated immunity which manifests itself as delayed hypersensitivity (DHS). When DHS develops,

macrophages are activated and become more ferocious, hence being able to get rid of any organism inside them (Mackaness 1970). Therefore the number of such organisms in an organ will decrease only when DHS has developed, and activated macrophages have formed.

In this study, MIT and IHA were assayed to monitor the development of immunity after vaccination of birds with the two vaccines. MIT was taken as the *in vitro* correlate of DHS (Timms 1979) and IHA as one of the sensitive serological tests for detecting antibody titres (Wray, Morris & Sojka 1975). Since cell-mediated immunity plays the major role in protection against the invading *Salmonella* organism (Collins 1974), in this study, the MIT results were taken as better indicators of the protective ability of the respective vaccine than the IHA results.

When the 15-week-old cockerels were vaccinated with *S. gallinarum* strains L46 and CN 180, and their immune response was monitored by MIT and IHA over a period of 37 weeks, they showed a good response to the two vaccines. The response, as measured by the percentage of macrophage-migration inhibition, compared well in L46 and CN 180, al-

TABLE 1 Immune response (MIT) for cockerels vaccinated with *Salmonella gallinarum* strains on 180 and L46, given as percentage migration inhibition

Bird no.	Weeks post vaccination									
	1	2	4	5	7	9	13	17	21	37
Control birds										
12	16	20	–	16	–	–	–	–	–	–
15	NI	–	NI	–	–	NI	–	NI	–	NI
17	NI	–	NI	–	–	NI	NI	NI	–	NI
Birds vaccinated with CN 180										
8	NI	–	–	61	–	–	67	8	–	NI
19	8	30	4	47	–	65	47	46	52	–
21	19	32	14	68	–	64	83	–	35	14
Birds vaccinated with L46										
23	6	–	–	74	–	74	96	20	43	29
10	26	17	–	72	–	79	45	61	–	81
20	38	34	–	68	–	–	61	NI	44	–

– = Not done, or set up spoilt
NI = No inhibition
MIT = Macrophage migration inhibition test

TABLE 2 Immune response (IHA) for cockerels vaccinated with *Salmonella gallinarum* strains CN 180 and L46

Bird no.	Weeks post vaccination									
	1	2	4	5	7	9	13	17	21	37
Control birds										
12	16	–	16	16	–	–	–	16	16	–
15	–ve	–ve	–ve	–	–ve	–ve	–ve	–ve	–ve	–ve
17	–ve	–ve	–ve	–	–	–ve	–	–ve	–	–ve
Birds vaccinated with CN 180										
8	32	16	256	128	64	128	64	256	128	512
19	64	64	256	64	64	64	128	128	512	256
21	32	128	128	64	64	128	256	512	512	1 024
Birds vaccinated with L46										
23	128	64	32	256	128	128	512	131 072	256	512
10	16	32	1 024	1 024	2 048	1 024	512	1 024	256	1 024
20	64	64	128	64	256	256	64	256	128	64

– = Not done
–ve = Negative results IHA indirect haemagglutination test

TABLE 3 Immune response (RWBPT) for cockerels vaccinated with *Salmonella gallinarum* strains CN 180 and L46

Bird no.	Weeks vaccination									
	1	2	4	5	7	9	13	17	21	37
Control birds										
12	–	–	–	–	–	–	–	–	–	–
15	–	–	–	–	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	–	–
Birds vaccinated with CN 180										
8	–	–	–	–	–	–	–	–	–	–
19	–	–	±	–	–	–	–	–	–	–
21	–	–	–	–	–	–	–	–	–	–
Birds vaccinated with L46										
23	–	–	±	–	–	–	–	–	–	–
10	–	±	+	+	+	+	+	+	+	++
20	–	±	±	–	–	–	–	–	–	–

RWBPT = Rapid whole-blood plate test
– = Negative
± = Suspiciously positive
+ = Positive

though there were times when L46 seemed to be more effective than CN 180 in maintaining the response. Overall, L46-vaccinated birds showed higher IHA titres than CN 180-vaccinated birds. The titres also fluctuated a great deal throughout the experimental period. The CN 180-vaccinated birds showed a more gradual increase in titre and there were fewer fluctuations than in L46-vaccinated birds. The IHA results therefore compared well with the MIT results where birds vaccinated with L46, in general, gave a higher immune response than those vaccinated with CN 180. However, the difference is not statistically significant (MIT:F = 3,18, $P = 0,10$ at 1 and 6 degrees of freedom, IHA:F = 2,82, $P = 0,10$ at 1 and 36 degrees of freedom).

The fluctuation of titres in serological tests has been reported by other workers (Gordon & Garside 1944; Gordon & Buxton 1945). Although positive responses to MIT and IHA in control birds may mean that the birds had been exposed to the organism or to a cross-reacting antigen, titres to IHA have been detected in animals shown not to have been exposed to the respective antigen (Magwood & Bigland 1962; Wray *et al.* 1975). False positives with regard to MIT have also been reported (Rocklin 1976; Timms & Alexander 1977).

The fact that both L46 and CN 180 gave good immune responses, indicated that the apparent breakdown in immunity observed in the field may not have been caused by a lack in the efficacy of the vaccine. However, more work, including protective studies both in the laboratory and in the field, needs to be done to assess the actual degree of protection rendered by the respective vaccines under laboratory and field conditions. Assuming that the vaccine was properly handled by the farmers in terms of storage, dosage, application, etc. at field level, one cannot rule out the fact that field conditions could have had an effect on the efficiency of the live CN 180 vaccine.

Other possible reasons for the recurrence of vaccination breakdowns experienced in the field, can only be speculated upon. It is possible that new virulent strains of *S. gallinarum* may have emerged, as has been shown in another study on various *S. gallinarum* isolates Bebora 1987). It is also possible that birds developing the disease, even though having been previously vaccinated, may have encountered overwhelmingly large challenge doses that overcame any resistance that might have developed as a result of vaccination. Controlled experiments on typhoid fever in human volunteers, have shown this hypothesis to be true (Hornick & Woodward 1967). Such studies have shown that the incidence of disease and apparent level of protection against a standardized oral dose of virulent *S. typhi* varies with the size of the challenge inoculum. An inoculum of more than 10^7 viable organisms will overwhelm any immunity induced by previous exposure to killed typhoid vaccine (Hornick & Woodward 1967). This is consistent with the clinical finding that typhoid fever may occur in recently convalescent individuals (Marmion, Naylor & Steward 1953). It has also been demonstrated that mice vaccinated with a living attenuated vaccine can still be superinfected (Collins 1972b). Therefore, even during convalescence, resistance to re-infection may decline so rapidly that second (although usually milder) attacks of the disease can occur if the infectious dose is large enough (Collins 1972b) or if the virulence of the reinfecting strain is very high (Marmion

et al. 1953). Hobson (1957a, 1957b) and Collins (1968) observed that salmonella carriers have a high degree of resistance to re-infection and this state usually lasts as long as the primary infection persists. These observations led Collins (1971) to conclude that antityphoid immunity may not be absolute, even under ideal conditions, except, perhaps, in the case of a permanent typhoid carrier. The same may be the case with fowl typhoid infection in chickens. This is in keeping with Reitman's (1967) statement that an ideal vaccine is one that retains the protective antigens of the bacterial cell, some of which might be extremely labile even to the extent of being non-demonstrable by present means of testing.

The current knowledge that the pathogenicity of *Salmonella* organisms is mediated by plasmids ranging in mass from 30–138 megadalton (Terakado, Sekizaki, Hashimoto & Naitoh 1983; Nakamura, Sato, Ohya, Suzuki & Ikeda 1985; Barrow, Simpson, Lovell & Binns 1987) explains why an active infection (virulent organism; this includes the carrier state in man or bird) gives better protection than an attenuated vaccine. The plasmid may be coding for specific proteins connected with pathogenicity to which the immune response will produce totally protective immunity. These plasmids may be lost during passage on artificial media (Ewing 1986), hence contributing to the attenuation of the respective strains. However, they may be acquired through conjugation with a strain possessing the respective plasmid (Yamamoto, Honda, Miwatani & Yokota 1984). In this case, the non-pathogenic strain may acquire pathogenicity. The latter observation may be a limiting factor to the usage of live attenuated vaccines, and could be a possible cause of some of the vaccination breakdowns mentioned earlier, experienced in the field.

ACKNOWLEDGEMENTS

The excellent technical assistance of Messrs Zachary Munene and Francis Njoroge from the University of Nairobi, and Mr Aggrey Ambani from the Ministry of Livestock Development, is gratefully acknowledged.

This study was supported by funds provided by the National Council for Science and Technology.

REFERENCES

BARROW, P.A., SIMPSON, J.M., LOVELL, M.A. & BINNS, M.M. 1987. Contribution of *Salmonella gallinarum* large plasmid toward virulence in fowl typhoid. *Infection and Immunity*, 55:388.

BEBORA, L.C. 1987. Fowl typhoid: The disease, immunity and control. Ph.D. thesis, University of Nairobi.

CAMERON, C.M. 1976. Immunity to *Salmonella* infections. *Journal of the South African Veterinary Association*, 47:89.

CAMPBELL, P.A. 1976. Immunocompetent cells in resistance to bacterial infections, 40:284.

COLLINS, F.M. 1968. Recall of immunity in mice vaccinated with *Salmonella enteritidis* and *Salmonella typhimurium*. *Journal of Bacteriology*, 95:2014.

COLLINS, F.M. 1971. Mechanisms of antimicrobial immunity. *Journal of Reticuloendothelial Society*, 10:58.

COLLINS, F.M. 1972a. Acquired resistance to mycobacterial infections. *Advances in Tuberculosis Research*, 18:1.

COLLINS, F.M. 1972b. Salmonellosis in orally-infected specific-pathogen-free C57 B1 mice. *Infection and Immunity*, 5:191.

COLLINS, F.M. 1974. Vaccines and cell-mediated immunity. *Bacteriological Reviews*, 38:371.

DAVIES, R. & KOTLARSKI, I. 1976. A role for antibody in the expression of cellular immunity to *Salmonella typhimurium* C5. *Australian Journal of Experimental Biology and Medical Sciences*, 54:207.

EWING, W.H. 1986. *Edward and Ewing's identification of Enterobacteriaceae*, 4th ed. New York: Elsevier Science Publishing.

FALK, R.E. & ZABRISKIE, J.B. 1971. The capillary technique for measurement of inhibition of leucocyte migration—an assessment of cell-mediated immunity, in *In vitro methods in cell-mediated immunity*, edited by B.R. Bloom & P.R. Glade. New York & London: Academic Press: 301–306.

GORDON, R.F. & BUXTON, A. 1945. The isolation of *Salmonella thompson* from outbreaks of disease in chicks. *Journal of Hygiene*, Cambridge, 44:179.

GORDON, R.F. & GARSIDE, J.S. 1944. *Salmonella* infections in ducks and ducklings. *Journal of Comparative Pathology and Therapeutics*, 54:61.

HERBERT, W.J. 1973. Passive haemagglutination with special reference to the tanned cell technique, in *Handbook of experimental immunology*, 1. Scientific Publications, edited by D.M. Weir. Oxford, London, Edinburgh, Melbourne.

HOBSON, D. 1957a. Resistance to reinfection in experimental mouse typhoid. *Journal of Hygiene*, Cambridge, 55:334.

HOBSON, D. 1957b. Chronic bacterial carriage in survivors of experimental mouse typhoid. *Journal of Pathology and Bacteriology*, 73:399.

HORNICK, R.B. & WOODWARD, T.E. 1967. Appraisal of typhoid vaccine in experimentally infected human subjects. *Trans American Clinical Climatological Association*, 78:70.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.C. & RANDALL, R.J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biology and Chemistry*, 193:265.

MACKANESS, G.B. 1967. The relationship of delayed hypersensitivity to acquire cellular resistance. *British Medical Bulletin*, 23:52.

MACKANESS, G.B. 1970. In *Infectious agents and host reactions*, edited by S. Mudd. Philladelphia: W.B. Saunders: 61–75.

MAGWOOD, S.E. & BIGLAND, C.H. 1962. Salmonellosis in turkeys. Evaluation of bacteriological and serological evidence of infections. *Canadian Journal of Comparative Medicine and Veterinary Science*, 26:151.

MARMION, D.E., NAYLOR, G.R.E. & STEWARD, I.P. 1953. Second attacks of typhoid fever. *Journal of Hygiene*, Cambridge, 52:260.

NAKAMURA, M., SATO, S., OHYA, T., SUZUKI, S. & IKEDA, S. 1985. Possible relationship of a 36-megadalton *Salmonella enteritidis* plasmid to virulence in mice. *Infection and Immunity*, 47:831.

NETER, E., BERTRAM, C.F. & ARBESMAN, C.E. 1952. Demonstration of *E. coli* 055 and 0111 antigens by means of hemagglutinaton test. *Proceedings of the Society of Experimental Biology and Medicine*, 79:255.

POMEROY, B.S. 1984. *Diseases of Poultry*, 8th ed., edited by M.S. Hofstad, H.J. Barnes, B.W. Calnek, W.M. Reid & H.W. Yoder Jr. Iowa State University Press: 79–91.

REED, L.J. & MUENCH, H. 1938. A simple method for estimating 50 percent end-points. *American Journal of Hygiene*, 27:493.

REITMAN, M. 1967. Infectivity and antigenicity of streptomycin-dependent *Salmonella typhosa*. *Journal of Infectious Diseases*, 117:101.

ROCKLIN, R.E. 1976. Products of activated lymphocytes, in *Clinical Immunobiology*, 3, edited by F.H. Bach & R.A. Good. New York, San Francisco, London: Academic Press: 195–220.

SNOEYENBOS, G.H., 1984. *Diseases of Poultry*, 8th ed., edited by M.S. Hofstad, H.J.Barnes, B.W. Calnek, W.M. Reid & H.W. Yoder Jr. Iowa State University Press: 79–91.

SOLOTOROVSKY, M. & SODERBERG, S.F. 1972. *Macrophages and Immunity*, edited by A.I. Laskin & H. Lechevalier. CRC Press: 77–123.

TERAKADO, N., SEKIZAKI, T., HASHIMOTO, K. & NAITOH, S. 1983. Correlation between the presence of fifty megadalton plasmid in *Salmonella dublin* and virulence to mice. *Infection and Immunity*, 41:443.

TIMMS, L.M. 1974. Leucocyte migration inhibition as an indicator of cell-mediated immunity in chickens. *Avian Pathology*, 3:177.

TIMMS, L.M. 1979. Correlation of the lymphocyte transformation, leucocyte migration inhibition and the delayed cutaneous hypersensitivity tests in chickens sensitised to *Mycobacterium tuberculosis*. *Research in Veterinary Science*, 27:347.

TIMMS, L.M. & ALEXANDER, D.J. 1977. Cell-mediated immune response of chickens to Newcastle disease vaccines. *Avian Pathology*, 6:51.

WHO Technical Report. 1973. *Cell-mediated immunity and resistance to infection.* Serial No. 519, Geneva WHO: 6–64.

WRAY, C., MORRIS, J.A. & SOJKA, W.J. 1975. A comparison of indirect haemagglutination tests and serum agglutination tests for the serological diagnosis of *Salmonella dublin* infections in cattle. *British Veterinary Journal*, 131:727.

YAMAMOTO, T., HONDA, T., MIWATANI, T., & YOKOTA, T. 1984. A virulence plasmid in *Escherichia coli* enterotoxigenic for humans: Intergeneric transfer and expression. *Journal of Infectious Diseases*, 150:688.