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The significance of *Salmonella*, particularly *S.* Infantis, to the Australian egg industry

A report for the Rural Industries Research and Development Corporation

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The significance of *Salmonella*, particularly *Salmonella* Infantis, to the Australian egg industry, with respect to public health

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Foreword

Research investigating the significance of *Salmonella* Enteritidis (SE) in the Australian egg industry was conducted between 1991 and 1993. While several outbreaks of foodborne salmonellosis have been associated with eggs and egg products, there is no evidence linking human salmonellosis with egg consumption, involving SE. However, during the period 1991-1993, most human cases of SE-associated salmonellosis in Australia were seen in Queensland. A proactive study of the incidence of *Salmonella* in Queensland layer flocks was undertaken with the support of the then Egg Industry Research and Development Corporation (now the Egg Program of RIRDC). That research demonstrated an extremely low incidence of SE in layer flocks, yielding SE phage type (PT) 26 in three layer flocks in north Queensland. This type of SE is regularly isolated from a range of sources in Queensland and is considered endemic to that region. It does not appear to be closely associated with poultry, and further investigation of isolates suggested little potential for systemic colonisation of layers, as has been found overseas with certain strains of SE PT4. Although the research found little incidence of SE in Queensland layer flocks, it revealed a variable but overall significant carriage rate in layers of a range of salmonellae. More specifically, it revealed a high incidence of serovar Infantis, particularly among layer flocks of some of the larger egg producers in that region. While none of these serovars are considered to pose the same public health threat via eggs as foreign, virulent strains of SE, they are nevertheless potentially capable of causing human disease. Thus, a further project was initiated, funded by the Egg program of RIRDC, to investigate the significance of serovar Infantis to the Australian egg industry. This research, conducted by Dr Anna Sartor as a RIRDC Junior Research Fellow, is the subject of this report.

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About the authors

Dr Julian Cox is a Senior Lecturer in Food Microbiology in the Department of Food Science and Technology at The University of New South Wales. He has worked in and with the poultry industry, particularly the egg industry, for over a decade. After working in the egg industry between 1989 to 1991, he has worked as a full-time researcher and academic, conducting several projects on behalf of the egg and or chicken meat industries, relating to food safety and the foodborne pathogens *Salmonella* and *Listeria*. He currently acts as a technical advisor to the Australian Egg Industry Association on matters of food safety.

Dr John Woolcock recently retired from his position as Associate Professor in Medical Microbiology in the Department of Microbiology at the University of Queensland. Prior to joining that Department, he worked for many years as a member of the academic staff in the Veterinary School, University of Queensland.

Dr Anna Sartor conducted the reported research in the Department of Microbiology, in fulfillment of the requirements for a PhD from the University of Queensland, under the supervision of Drs Cox and Woolcock. Anna's research was supported by a grant from the Egg Industry program of RIRDC, while she was personally supported throughout her PhD candidature by a RIRDC Junior Research Fellowship.

Abbreviations

cfu	colony forming units
DIG	digoxigenin
HEK	Hektoen Enteric agar
IST	insertion sequence (200) type
LMG	Lysine Mannitol Glycerol agar
PFGE	pulsed field gel electrophoresis
PI	post-infection
PT	phage type
RAPD-PCR	randomly amplified polymorphic DNA-polymerase chain reaction
RT	ribotype
SE	*Salmonella enterica* serovar Enteritidis (= *Salmonella enteritidis*, = *Salmonella* Enteritidis)
SI	*Salmonella enterica* serovar Infantis (= *Salmonella infantis* = *Salmonella* Infantis)
SPF	specific pathogen-free
SS	*Salmonella enterica* serovar Singapore (= *Salmonella singapore*, = *Salmonella* Singapore)
TBE	Tris-borate-EDTA buffer
TBGB	Tetrathionate Brilliant Green Broth

Executive Summary

Salmonella Infantis, associated with the egg and especially the chicken meat industry, has been of significant public health concern in many countries, and remains so in some regions. Due to its previously observed dominance in layer flocks and in raw egg products in south-east Queensland, it was considered to be of potential significance to the Australian egg industry, with regard to egg-borne transmission to humans and the likely public health consequences.

Its significance was first investigated by conducting a longitudinal survey of a selected farm (previously shown to harbour the serovar) over a period of seven months, to establish if the presence of *S.* Infantis in the poultry environment was influenced by specific parameters.

A further survey of raw egg products was conducted to determine the persistence of *S.* Infantis beyond the farm production environment. While the incidence of *S.* Infantis was quite low compared to the incidence of other *Salmonella* serovars, the recovery of *S.* Infantis from egg yolk was considered to be particularly significant. As yolk is produced by separation, and the likelihood of contamination of contents from the shell is considered low, it is possible intact shell eggs may represent a significant vector for this serovar. A high prevalence of *Salmonella* was observed in layer flocks and in raw egg pulp. The frequency of isolation of *S.* Infantis was low compared with a high prevalence of an antigenically closely related serovar *S.* Singapore. The dominance of *S.* Singapore throughout the production environment correlated with the presence of the serovar in poultry feed and raw feed components, particularly meat meal.

Epidemiological tools are required to determine any potential relationships between isolates of *S.* Infantis. A collection of over 150 isolates, including poultry isolates from layer farms in Queensland, environmental isolates, animal isolates and clinical isolates, were analysed using a range of phenotypic and genotypic methods. Due to a lack of variation in plasmid carriage and profile, antibiotic susceptibility, and biochemical activity, phenotypic tests proved to be of little discriminatory value. Among the genotypic tests, pulsed field gel electrophoresis (PFGE) alone proved to be the most discriminatory method for comparing and differentiating *S.* Infantis isolates for epidemiological purposes. Many clonal groups were recognised by combining the results for PFGE, ribotyping and IS*200* typing. Although *S.* Infantis isolates from an outbreak were unavailable, poultry isolates from different geographic origins and numerous human isolates were clearly discriminated. A certain degree of relatedness was found among isolates from a given region at different times and among the poultry isolates from Queensland.

Either transovarian transmission, or the ability of a bacterium to penetrate the eggshell once present on the surface, may lead to contamination of egg contents. The ability of *S.* Infantis, as well as other serovars potentially significant to industry, to penetrate the eggshell under different conditions was investigated. Isolates of *S.* Infantis, *S.* Singapore and *S.* Enteritidis from sources including egg yolk, chicken faeces and poultry feed were used in an eggshell penetration study. Organisms were suspended at a population of 10^8 cfu/g in sterile chicken faeces and inoculated onto the surface of first quality eggs. Penetration of the eggshell was monitored at 4ºC, 25ºC and at 35ºC. At 25ºC, all isolates except one *S.* Enteritidis were able to penetrate the egg shell in one day and in most cases contaminate the egg yolk. At 4ºC and 35ºC, penetration was much slower although cells remained viable at 4ºC.

Commercial layer hens were experimentally infected with a *S.* Infantis isolate to follow the course of infection and to investigate the potential for production of contaminated eggs. Point-of-lay hens were challenged orally with an isolate *S.* Infantis originally obtained from egg yolk at an inoculum size of 10^9 cfu/ml. The bacterium colonised the gastrointestinal tract, leading to persistent shedding of the organism in faeces for up to 6 weeks. Internal organs, including the liver, spleen, caecum, ovary, oviduct and pre-ovulatory follicle were culture-positive for *S.* Infantis at various times post-

inoculation. *S.* Infantis was isolated from eggshell surface washings but not from egg contents, implying that it was more likely that contaminated eggs result from eggshell penetration rather than transovarian transmission of the organism.

Prevalent serovars of *Salmonella* in the Australian egg industry could pose a threat to public health. However, by conducting appropriate surveys to monitor their prevalence, vital information can be acquired to help assess the status of Australian layer flocks. Application of molecular techniques such as PFGE enables good discrimination between *S.* Infantis isolates for epidemiological purposes, providing information on the existing relationships between isolates of this serovar from similar and different environments. *S.* Infantis was able to penetrate the egg shell and grow in some cases within the contents of the egg, thereby stressing the importance of removing faecal material from the surface of the egg and to maintain safe and good food handling practices along the egg production environment. Experimental infection of layer hens demonstrated that transovarian transmission is less likely to occur than egg shell penetration.

Introduction

During the last two decades *Salmonella* infections in humans have increased worldwide and continue to be of major public health concern (Rodrigue et al., 1990). In the United States and in the United Kingdom this increase has been dramatic; *Salmonella* Enteritidis and its association with egg and egg products has had the most significant impact on the incidence of human salmonellosis (Cowden *et al.*, 1989b; Stevens *et al.*, 1989; Luby & Jones, 1993). Many other countries have also experienced outbreaks of human salmonellosis caused by *Salmonella* Enteritidis, identifying the consumption of eggs or foods containing eggs as the major vehicle of transmission (Perales & Audicana, 1989; Rodrigue *et al.*, 1990; van der Giessen *et al.*, 1992; Wong *et al.* 1994; Cox 1995; Sakai & Chalermchaikit 1996).

The dramatic increase in *S.*Enteritidis infections occurring in other countries has not been observed in Australia. In 1995 there were a total of 154 human clinical cases of salmonellosis attributed to *Salmonella* Enteritidis, the majority belonging to phage type (PT) 4 and acquired overseas (Powling, 1995). *S.*Enteritidis ranked eighth among the top ten human cases of *Salmonella* serovars for 1995 and did not rank among the top ten serovars from non-human sources (Powling, 1995). Thus, there is no direct evidence implicating eggs or egg products as a major vehicle for salmonellosis, and certainly not the transmission of specific *Salmonella* serovars such as *S.*Enteritidis.

In 1991, a project began at the University of Queensland, investigating the potential threat posed by *S.*Enteritidis to Australian layer flocks, as well as assessing the prevalence of other *Salmonella* serovars. The project demonstrated that the prevalent serovars in layer flocks in south-east Queensland included *S.*Orion, its variants, and *S.*Infantis (Cox, unpublished). Surveillance, conducted as part of the same project, of raw egg pulp and yolk produced in south-east Queensland during the period 1991-1993, also identified *S.*Infantis as a prevalent serovar. Of particular significance was the presence of *S.*Infantis in raw egg yolk, suggesting that eggs may represent a significant vector of transmission of this serovar, as has been the case with *S.*Enteritidis. Egg-borne salmonellosis has been associated with transovarian transmission or internal carriage of the bacterium in the layer hens (Barnhart *et al.*, 1991).

A number of studies have found *S.*Infantis to be a prevalent serovar within the poultry industry worldwide. A large *S.*Infantis epidemic in broiler chickens associated with high incidence of human salmonellosis in Finland during 1975 implicated contaminated feed as a possible source of the bird infection (Raevuori *et al.*, 1978). *S.*Infantis was also reported as the second most common serovar isolated from layer flocks in Canada in 1991 (Poppe *et al.*, 1991a). In Germany, *S.*Infantis was shown to be a prevalent serovar in layer flocks, second only to *S.*Enteritidis (Hinz *et al.*, 1996).

There have also been dramatic increases in the number of isolations of serovars Livingstone, Berta, Typhimurium and Infantis from cases of human salmonellosis, also acquired from poultry (Olsen *et al.*, 1992a; Pelkonen *et al.*, 1994; Millemann *et al.*, 1995; Crichton *et al.*, 1996). Thus there is a need to investigate both the overall incidence of *Salmonella* in layer flocks, and to determine the significance of prevalent serovars, especially those that have been associated previously with poultry and, as a consequence, human disease.

In order to identify potential relationships between isolates of a particular *Salmonella* serovar, methods for demonstrating relatedness must be established. Such methods may be phenotypic, relying on expressed features of the organism to differentiate strains. Methods commonly used to differentiate salmonellae include phage typing, biotyping and antibiotic resistance. The methods may be genotypic, these based on analysis of nucleic acids of the organism, including chromosomal and plasmid DNA, or RNA molecules, and include plasmid profiling, ribotyping, the randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and pulsed-field gel electrophoresis (PFGE).

Although there are many studies of phenotypic and/or genotypic analysis of a wide range of *Salmonella* serovars, there are few that have focussed on *Salmonella* Infantis. Individual and multiple typing methods can be used to characterise large numbers of isolates (Crichton *et al.*, 1996; Liebisch & Schwarz, 1996b).

Antibiotic susceptibility testing may be considered because of the wide range of antibiotics that have been used within the poultry industry in Australia. Plasmid profiling used together with biotyping or phage typing has been useful in demonstrating epidemiological links between isolates (Stubbs *et al.*, 1994). Few phage typing schemes have been developed for *S.*Infantis (Kasatiya *et al.*, 1978; Laszlo *et al.*, 1988) and at present none are effective. Phage can be derived from human sewage and from lysogenised isolates and can be used for typing epidemiologically related and unrelated isolates.

Molecular techniques that analyse chromosomal DNA are useful for typing several *Salmonella* serovars (Cox & Fleet 1997). Determination of rRNA gene restriction patterns (ribotyping) and fingerprinting the DNA insertion sequence IS*200* in genomic Southern blots (IS*200* typing), where the copy number is sufficiently high, have provided discriminatory profiles of the chromosome in *Salmonella* serovars including *S.* Infantis (Pelkonen *et al.*, 1994). Pulsed field gel electrophoresis has also been used successfully to perform comparative chromosomal DNA analyses of *Salmonella* serovars for epidemiological investigations (Nair *et al.*, 1994; Suzuki *et al.*, 1994). PFGE, ribotyping and IS*200* typing have been applied alone and in combination to isolates of serovars which have proved difficult to discriminate, such as *Salmonella* Enteritidis phage type 4 (Olsen *et al.*, 1994). RAPD PCR and a host of other PCR-based subtyping methods have also been applied to *Salmonella* isolates and demonstrated greater discriminatory power than the techniques previously mentioned (Lin *et al.*, 1994; Nastasi & Mammima, 1995; Hermans *et al.*, 1996; Kerouanton *et al.*, 1996). RAPD PCR however, involves the use of arbitrary primers, oligonucleotide primers that are capable of recognising DNA polymorphisms among isolates, and because there is no way of predicting which primers will be useful, large numbers of primers must be screened.

Eggs and egg dishes are important vehicles of *Salmonella* infection in humans, particularly infection with *S.*Enteritidis. Eggs with contaminated yolks are a potential hazard for the consumer and research in the United Kingdom has shown that approximately 3% of eggs from a hen infected with *S.*Enteritidis will be contaminated internally with that organism (Humphrey et al., 1989c). Contaminated eggs can occur by one of two routes either by eggshell penetration or via transovarian transmission. Transient intestinal infection of laying birds can lead to shells becoming contaminated during and after laying.

Serovars of *Salmonella* found to be dominant in layer flocks and/or implicated in egg-associated outbreaks are cause for public health concern and the ability of these organisms to penetrate and grow within the egg contents should be examined. Methods for studying microbial penetration through the outer structures of the avian egg have been established since the late 1960's (Williams & Whittemore, 1967). These methods provide a means of studying eggshell penetration under natural conditions and are designed for numerous variations and adaptations. Eggshell penetration by salmonellae has been shown to be highly variable among species and dependent on shell quality (Sauter & Petersen, 1974).

Salmonella and other pathogens readily penetrate eggshells at varying temperatures and inoculum sizes (Sauter & Petersen, 1974; Schoeni *et al.*, 1995). Penetration patterns through the outer egg structures by salmonellae pathogenic for poultry and economically important to the hatchery and poultry industry provide useful information. Organisms can be recovered from the inner surface of the shell membranes shortly after the surface of the shell has been challenged (Williams *et al.*, 1968). *S.*Enteritidis, *S.* Typhimurium and *S.* Heidelberg have been shown to penetrate the eggshell within three days when inoculated at high level (10^6 cfu/g faeces) and incubated at 25ºC (Schoeni *et al.*, 1995). Chicken faeces has been shown to facilitate the penetration of an organism into the egg (Clay & Board, 1991), and simulates a natural process. Hence damp, sterilised or fresh chicken faeces is

employed as the carrier of organisms on the egg surface. The course of microbial infection of shell eggs can occur in three stages: penetration of the cuticle and the shell; colonisation of the underlying membrane, and; contamination of the albumen leading eventually to contamination of the egg contents (Board, 1966). Various factors, including inoculum size, the temperature of incubation, and the strain of *Salmonella*, may affect the course of infection. *Salmonella* can migrate from the albumen of the egg into the yolk and in this matrix the population of *Salmonella* readily increases (Berrang *et al.*, 1991; Braun & Fehlhaber, 1995). Temperature and length of storage time play a very important role in controlling *Salmonella* infections and maintaining egg quality. Researchers have shown that *S.* Enteritidis can grow in eggs at temperatures as low as 4ºC (Kim *et al.*, 1989), *S.* Typhimurium at 10ºC (Humphrey, 1990), and *S.heidelberg* also at 4ºC (Schoeni *et al.*, 1995).

Previous research has attempted to explain the process by which laying hens become infected with *Salmonella* and subsequently produce contaminated eggs. Most of this work has been done with the serovar *S.*Enteritidis, as transovarian transmission has been proposed as the mechanism by which *Salmonella* Enteritidis-contaminated eggs are produced (Humphrey *et al.*, 1989a; Gast & Beard, 1992a; Thiagarajan *et al.*, 1994). Ovaries are infected following localisation and colonisation of the large intestine. Contaminated eggs containing relatively small numbers of *Salmonella* Enteritidis were produced after hens were orally inoculated with 10^9 cfu (Gast & Beard, 1992a). Alternatively, egg contamination can occur by penetration of the shell during or after lay, as the egg passes through the cloaca of the chicken and organisms present in chicken faeces can deposit on the outside of the shell (Snoeyenbos *et al.*, 1969).

Eggs contaminated with *S.*Enteritidis have been traced back to flocks of laying hens that were culturally or serologically positive for the organism. Research has indicated that approximately 3% of the eggs from *S.*Enteritidis infected birds will be contaminated with *S.*Enteritidis (Humphrey et al., 1989c). The relationship between the degree of *S.*Enteritidis infection of a flock and the production of contaminated eggs is not clear. In the United States one commercial layer farm estimated the overall prevalence of *S.*Enteritidis contaminated eggs to be 2.28 per 10 000 birds (Kinde *et al.*, 1996). In Canada a low percentage of *S.*Enteritidis was present in the ovaries and oviducts of one flock and *S.*Enteritidis was isolated from the contents of only one egg among 14 040 eggs laid by this flock in one day (Poppe *et al.*, 1992). Effective methods for checking large numbers of eggs for *S.*Enteritidis and sufficiently sensitive to detect small numbers of cells have been developed in order to more accurately identify *S.*Enteritidis infected flocks and eggs (Gast, 1993).

There are a number of factors that may contribute to the shedding of *Salmonella*, such as age, environmental stress, moulting and the breed of the bird. Differences in shedding attributable to age and breed of chicken have been shown (Humphrey *et al.*, 1991b; Protais *et al.*, 1996). More extensive investigations have involved internal examinations of commercial layer hens to determine the localisation of *S.*Enteritidis in organs of infected hens. Experimental models, involving infection of chicks and laying hens with *S.*Enteritidis have been used as an alternative for studying infection in the field, to overcome problems that are encountered in the environment. *S.*Enteritidis can colonise and persist in the gastrointestinal tract, usually being shed in faeces. Laying hens, inoculated orally with 10^9 cells of *S.* Enteritidis, continued to shed the organism for 10 weeks post-inoculation (Gast & Beard, 1990b). Shivaprasad *et al.*, (1990) also demonstrated persistent shedding of *S.*Enteritidis for at least 42 days in some birds when intravenously injected with $2x10^8$ cells of *S.*Enteritidis. In that study the frequency of recovery of *S.*Enteritidis from cloacal swabs declined steadily over the test period, although persistent colonisation in some hens was evident. The duration of excretion of *S.*Enteritidis PT 4 in faeces may be directly related to the size of the dose (Humphrey *et al.*, 1991a).

The frequency and level of *S.*Enteritidis contamination of the contents of fresh eggs laid by experimentally infected hens has been investigated widely. In some investigations experimentally infected hens have not produced eggs with contaminated contents. Barrow and Lovell (1991) found that eggs are more likely to become contaminated during passage through the cloaca than as a result of transovarian infection. Birds were inoculated both orally and intravenously with various inoculum sizes and only on two occasions were egg contents infected. Gast and Beard (1992a) also reported the

level of contamination of the contents of eggs produced by experimental hens with high doses of *S.*Enteritidis to be comparatively low. In contrast, studies by other researchers report that experimentally infected hens can lay substantial numbers of eggs with contaminated contents. Bichler *et al.* (1996) indicated that birds inoculated with *S.*Enteritidis produced *S.*Enteritidis positive eggs at high frequencies (63.9% of eggs in the first week) which decreased over time. Similar results were also reported by Gast and Beard (1990b) where older birds of 62 weeks of age, inoculated orally with *S.*Enteritidis, produced a significantly higher proportion of *S.*Enteritidis positive egg yolks in the first week post-inoculation. Generally very few culture positive egg contents and fewer culture positive egg yolks were laid within the first ten days post-inoculation (Timoney *et al.*, 1989; Shivaprasad *et al.*, 1990; Gast & Beard, 1992b).

Experimental Section 1

Prevalence of *Salmonella* Infantis and other *Salmonella* serovars among layer flocks and raw egg products in southeast Queensland

Objectives

The purpose of this study was to expand upon previous survey work of layer flocks in Queensland by conducting longitudinal and point-in time surveys of *Salmonella* faecal carriage and feed contamination on farms. In addition, the incidence of *Salmonella*, and particularly *S.*Infantis, in raw egg pulp was determined, as there may be a link between *Salmonella* serovars identified from the farm survey and those present in egg pulp.

Methodology

Layer chicken farms

A longitudinal survey was carried out on a single farm (Farm A). This farm was chosen for its proximity to the University of Queensland, from four farms that demonstrated a high incidence of *S.*Infantis in a previous farm surveillance program in 1991 (unpublished data). The selected farm contained three flocks housed in separate sheds approximately 50 metres apart. Samples of faeces and feed were collected (as detailed below) on a monthly basis for six consecutive months and on one other occasion after the farm had closed down. The remaining three farms (B, C, D), also located in south-east Queensland, were involved in a cross-sectional study and were sampled on two occasions, 12 months apart. The flock surveys were conducted between September 1993 and January 1995.

Flocks and sampling plan

For the purpose of this study a flock was defined as a single shed where birds are housed together and are not separated by any structures other than cages. Three of the four farms were considered small farms with up to three flocks housed in ground sheds. The fourth farm, a much larger establishment, consisted of 16 flocks and included ground sheds as well as high-rise sheds. For the three small farms all flocks were examined and on the larger establishment five flocks, chosen at random across the two shed types, were sampled.

Sixty fresh faecal droppings were collected from each flock. This sampling plan ensured a 95% probability of detecting at least one contaminated sample (*Salmonella*-positive) sample, assuming that any given *Salmonella* serovar would be present in at least 5% of birds in a given flock (Cannon & Roe, 1982; Irwin *et al.*, 1994). Faecal droppings were collected (below) and pooled into composites of five sub-samples to give a total of 12 pooled faecal samples per flock. Pooling of samples has been identified as a method that reduces the number of samples to be tested while still ensuring a relatively small standard error for prevalence estimates (Sacks *et al.*, 1989). In addition to the faecal samples, a feed sample was taken from each shed, where possible from the hopper, and individual feed components were obtained for premises producing their own feed.

Sample collection: farm survey

Five faecal samples weighing approximately 5 g each were collected using sterile (autoclaved) teaspoons and all five deposited into one sterile plastic Stomacher bag. This was repeated until 12

pooled samples (60 samples in total) were obtained. Flock feed samples and individual feed components (where available) were also collected directly into sterile bags. Feed samples (25 g) were weighed back at the laboratory. Disposable gloves were worn and changed between sampling of each flock, before taking whole feed samples, and between sampling of each feed component. Samples were stored at <10ºC and tested immediately upon arrival to the laboratory.

Sample collection: egg pulp survey

A single egg processing facility receiving eggs and egg pulp from layer farms located in south-east Queensland was chosen for this survey, as eggs were received and pulp either received or produced from eggs from all four farms described above. A total of 1031 raw egg product samples were collected over 55 weeks between June 1994 and July 1995. Upon collection, the samples were labelled as unpasteurised whole egg, egg pulp, egg yolk and chilled or frozen egg pulp. Collection was performed weekly with the number of samples varying on each occasion. The samples of egg pulp, supplied in 100ml volumes, were kept at 4ºC and labelled, as appropriate, with a farm code and the date of collection. These codes were changed upon arrival at the laboratory in order to maintain anonymity of the farms.

Salmonella *isolation and maintenance*

Faeces, feed and whole egg pulp were processed using the same method and differed only in the first step. Faeces and egg pulp were diluted 1: 4 in Buffered Peptone Water (Merck, Australia) while feed was diluted 1:9. The samples were incubated statically at 37ºC for 18-24 hours. The overnight broth (100 µl) was transferred to 10 ml of Rappaport-Vassiliadis Soya peptone broth (RVS; Oxoid) and incubated in a waterbath at 42ºC for 18-24 hours. Mannitol Selenite Cystine broth was not used, as previous work had demonstrated a lack of recovery of *Salmonella* with this medium. A 10 µl loopful of RVS was then streaked onto Lysine-Mannitol-Glycerol agar (LMG; Cox, 1993) and Brilliant Green Agar (BGA; Oxoid). Both agar plates were incubated at 37ºC overnight. Presumptive *Salmonella* colonies from both plates were selected and streaked onto Tryptone Soy Agar (TSA; Oxoid), so as to obtain isolated colonies. Single colonies from pure cultures were used to stab inoculate slopes of Triple-Sugar Iron agar (TSI; Oxoid). After incubation at 37ºC for 24 h, the TSI slopes were examined for reactions typical of *Salmonella*. Those showing typical reactions were subcultured from the pure TSA culture onto duplicate TSA slopes and stored at room temperature. Isolates were further screened serologically using a *Salmonella* latex agglutination test (Serobact, Oxoid) if TSI reactions were doubtful and those that agglutinated were retained on slopes. A slope of each confirmed *Salmonella* isolate was sent to the Institutes of Medical and Veterinary Science (IMVS), Adelaide to be serovard. Once cultures were confirmed as serovars of *Salmonella* by IMVS, the second, duplicate TSA slope was sealed, catalogued and stored at 4ºC.

Results

Farm survey

The farm survey, including all farms, was initiated in September 1993 and completed in January 1995. During this period, 538 samples, including 63 feed samples, were collected and cultured for *Salmonella*. A sample was *Salmonella* positive if any *Salmonella* serovar was isolated from it.

The overall rate of detection of *Salmonella* on all four farms was 32% (171/538) and 4.1% (7/171) of *Salmonella* isolates were identified as *S.*Infantis. The highest incidence of *Salmonella* positive and *S.*Infantis positive samples occurred on the same farm. *S.*Infantis was not detected on two of the farms. A large number of individual serovars (30) were found among the faecal *Salmonella* isolates including, in prevalence order, Singapore, Cerro, subspecies 1 serovar 4,12:d, Zanzibar, Agona, Senftenberg, Johannesburg, Infantis, subpecies 2 serovar 1,4,12,27, Mbandaka, Orion, Lille,

Lexington Ohio, Anatum Bredeney, Havana, Orion var 15+, 34+, Typhimurium (PT12a) and Virchow. Note that this list includes only those serovars isolated on two or more occasions. Multiple serovars (up to three) were frequently isolated from pooled faecal samples. No serovar was isolated from all four farm locations. *Salmonella* Singapore was the most frequently occurring serovar, found in 40 of 538 (7.4%) samples, and representing 23.4% (40/171) of the *Salmonella* isolates.

*S.*Infantis was not recovered from any feed samples either composite or individual feed components. Of the 63 feed samples, 78% were contaminated with 21 different serovars. The dominant serovars were Singapore and Cerro.

In the longitudinal survey, Farm A was sampled on a monthly basis for six months and a total of 266 samples including both faecal (246) and feed (20) were collected. This survey was shortened from 12 to 7 months due to the unforeseen closure of the farm.

On Farm A, 35% (94/266) of samples were *Salmonella* positive and 1% (3/266) were positive for *S.*Infantis. The dominant serovar on this farm over a six-month period was *S.*Singapore. Of the 94 *Salmonella*-positive samples 39 or 41.5% were contaminated with *S.*Singapore. It is this figure that is primarily responsible for the overall dominance of *S.*Singapore when data from all four farms is analysed. Of the feed samples, both complete and individual components, 18/20 (90%) were *Salmonella*-positive and *S.*Singapore was present in half of these samples. *S.*Singapore was isolated on almost every occasion from both feed and faeces in all three flocks on Farm A. The serovar Zanzibar persisted in Flock 1 over the six month period, although it was not detected on every occasion and was not isolated from any feed components. *S.*Infantis on the other hand was prevalent in Flock 2 during 1992, but during the longitudinal survey was isolated only sporadically from Flocks 2 and 3.

The frequency of isolation of any *Salmonella* serovar from a flock was highly significant ($P< 0.0001$). The isolation of *Salmonella* from faecal samples compared to those isolated from egg pulp samples was not significant ($P> 0.99$). The comparison of the frequencies of isolation of *Salmonella* from all four farms was highly significant ($P<0.0001$) demonstrating that the isolation of *Salmonella* was variable between the farms.

Egg pulp survey

The survey involved the collection of egg pulp samples on a weekly basis from a single egg processing facility over a 14-month period. A total of 1031 samples, including whole egg, egg pulp, egg yolk and individual farm egg pulp were cultured for *Salmonella*. Overall, 326 of 1031 (32%) samples were *Salmonella*-positive and 7 (0.7%) were positive for *S.*Infantis. The highest incidence (2.7%) of *S.*Infantis was found in unpasteurised whole egg, a mixture of albumen and yolk excluding the shell. *S.*Infantis was only rarely detected in egg pulp samples originating from a particular farm or location. In contrast to *S.*Infantis the isolation of any *Salmonella* serovar was, as expected, much higher.

Almost every sample of raw whole egg, 105/110 (95.5%) samples, was contaminated with *Salmonella*, while fewer samples of raw yolk and farm egg pulp were *Salmonella* positive, 31% and 32% respectively. Most of the farm egg pulp samples (657/856) were culture-negative for *Salmonella*. This result was not surprising considering that for whole egg pulp samples (pulp from a number of farms), it may be possible for contaminated egg pulp from one farm to contaminate the rest of the pulp.

Salmonella isolates (399) belonging to 25 different serovars were detected overall, excluding 13 distinct phage types of *Salmonella* Typhimurium which were also found. In a number of cases more than one serovar was isolated from a single sample and up to five different serovars were found, in one whole egg pulp sample. Twenty-three serovars were isolated from raw whole egg samples while 20 serovars were identified from farm egg pulp samples. The 10 most common *Salmonella* serovars

detected across all raw egg products were Singapore, Mbandaka, Cerro, subspecies 1 serovar 16:l,v, Kottbus, Oranienburg, Typhimurium (untypable), Infantis and subspecies 1 serovar 4,12:d.

The dominant serovar found in raw egg pulp was *S.*Singapore which accounted for 25% (81/326) of all *Salmonella* culture-positive samples. *S.* Singapore was closely followed by *S.*Mbandaka (23%) and *S.*Cerro (19%). The majority of *S.*Singapore isolates were detected in raw whole egg which represents egg pulp from a number of farms mixed together while the majority of *S.*Mbandaka-positive samples came from individual farm egg pulp samples. The incidence of *S.*Singapore from raw egg products decreased over the summer months with no isolations of this serovar occurring between December and March. During this time there was an increase in the diversity and number of other *Salmonella* serovars. Although the incidence of *S.*Mbandaka remained frequent there was a dramatic increase in isolations of this organism in the month of January. Another important observation here is that both of these serovars plus seven others, including *S.*Infantis, were isolated from raw egg yolk.

Discussion

In Australia, the range of *Salmonella* serovars isolated and reported has not changed dramatically for many years, unlike in the United Kingdom and the United States where *Salmonella* Enteritidis has emerged as the dominant serovar (Rodrigue *et al.*, 1990). A previous farm surveillance program involving layer flocks in South-East Queensland demonstrated that the prevalent serovars were dominated by *S.* Orion and its variants, as well as *S.*Infantis (unpublished data). *S.*Infantis has been widely isolated in Australia (1987-1992), more frequently from pigs than any other food animal and with high frequency in eggs (Murray, 1994b). It is the high prevalence of this serovar in eggs, particularly in raw egg pulp, found here in Australia and in other countries such as Canada, where *S.*Infantis was found to be the second most common serovar in layer flocks, that has caused concern (Poppe *et al.*, 1991a). The isolation of *S.*Infantis from the ovaries of commercial layer chickens at the time of slaughter (Barnhart *et al.*, 1991) has also caused public health concern with the hypothesis that the organism may be transmitted to the egg via transovarian infection. The significance of *S.*Infantis in the Australian egg industry was investigated by expanding on past survey work and conducting new surveys involving layer flocks and raw egg pulp in South-East Queensland.

The longitudinal survey involved a single layer farm in the greater Brisbane region which housed three flocks with approximately 10 000 layers per flock. In a previous survey carried out in February 1992, 43% of salmonellae from culture-positive samples of faeces, litter and feed were serovar Infantis. The majority of these isolates from pooled faecal samples, were detected in Flock 2. The longitudinal study carried out over six consecutive months from September 1993 to March 1994 demonstrated that of a total of 266 samples tested, 35% were culture positive for *Salmonella* and of these only three (1%) harboured Infantis. The most prevalent serovar was *S.*Singapore, which appeared in October 1993 in Flock 3 and by December was found in all three flocks and in numerous feed components, particularly meatmeal. *S.*Singapore persisted in each flock until the end of the survey. The presence of *S.*Singapore in feed may be one explanation for its prevalence, but may be due to more prolonged or significant colonisation of the caeca of layers. The prevalence of the serovar may also prevent colonisation by other *Salmonella* serovars that are antigenically closely related, such as *S.*Infantis (Barrow *et al.*, 1987).

New birds (pullets) may have become colonised with a number of serovars already present in the layer house, including Singapore, Cerro and Senftenberg in Flocks 1 and 2, and Singapore, Infantis and Bredeny in Flock 3. As the birds aged to approximately 50 weeks they continued to shed Singapore. The most likely source of reinfection or contamination appeared to be the feed, as *S.*Singapore was isolated from a number of feed components.

On a revisit to the farm, 12 months later and after its closure, *S.*Singapore was isolated from a litter sample taken from within the shed that housed Flock 3. *S.*Infantis was not isolated from this litter. In the previous survey a single isolate of *S.*Infantis was cultured from a composite feed sample in Flock

2 and this may have been responsible for the prevalence of this organism at the time. In the current survey *S.*Infantis was not isolated from any feed samples.

The prevalence of Infantis found in the first survey compared to the low incidence of the serovar 18 months later represents a change in the pattern of serovar incidence that is difficult to attribute to any one or even a combination of factors. The fact that the initial survey was a snap shot or 'point-in-time' result compared with the longitudinal survey may suggest from the results that at a certain point in time a particular serovar was dominant for some reason. Over time this serovar may be outcompeted by other serovars such as Singapore which, due to its reintroduction into the flock through feed, persists for a longer period of time. Contaminated feed has been shown previously to be an important factor in the spread of *S.*Infantis in cattle and broiler chickens and *Salmonella*-contaminated feed is a possible means of transmitting infection throughout a flock (Raevuori *et al.*, 1978; Gast & Beard, 1992b). The ability of *Salmonella* to survive for many years in dry environments such as floor dust has also been demonstrated (Robertson, 1972). According to the annual National *Salmonella* Surveillance Scheme (NSSS) reports for years 1993-1995 *S.*Singapore occurred in the top ten *Salmonella* serovars isolated from animal feeds in Australia, isolated exclusively from meatmeal or poultry feeds (Powling, 1992a; Powling *et al.*, 1993; Powling *et al.*, 1994; Powling, 1995).

There have been few longitudinal surveys investigating the prevalence of *Salmonella* in layer flocks. Most are point-in-time surveys to establish the prevalence of *S.*Enteritidis among layer flocks because of its potential threat to the industry (Poppe *et al.*, 1991a, b; Ebel *et al.*, 1992; Irwin *et al.*, 1994; Rusul *et al.*, 1996).

The second part of the farm survey involved two visits approximately 12 months apart to three additional farms where *S.*Infantis was previously found to be dominant. In the initial survey Infantis constituted 7%, 24% and 50% of the *Salmonella* isolates on each of farms B, C and D. The present study found Infantis represented 20% of *Salmonella* isolates on farm D, but was not detected during either visit to farms B and C. The 'random' testing of these three farms shows that there is intermittent shedding of various serovars and that on one particular farm *S.*Infantis persisted and continued to be shed in faecal material. The pattern of serovar incidence on each farm differed greatly with no pattern identified between farms or between farm visits. Only one serovar, Senftenberg, was found on all three additional farms.

*S.*Singapore was found to be the most frequent isolate from both the farm survey and the egg pulp survey. *S.*Singapore was isolated from egg pulp samples during most months except between December and March, 1995. Interestingly, serovar Mbandaka was prevalent during this period, most isolations being recorded in January. Evidence has shown that increased temperature particularly in summer months may explain an increased frequency of *Salmonella*-positive egg samples (Ebel *et al.*, 1993). It has also been shown that colder conditions can cause a resurgence of small numbers of *Salmonella* that are outnumbered by background microflora (Davies & Wray, 1996). Perhaps a diverse group of serovars is continually present in small, undetectable numbers in layer flocks and their resurgence facilitated by an increase or decrease in temperature. A particular serovar may then dominate others.

A longitudinal survey in the United States reported that after monitoring egg pulp for a 52 week period, a seasonal pattern in the frequency of *Salmonella* positive samples was evident (Ebel *et al.*, 1993). Increased temperature was proposed as one factor leading to the increased frequency of *Salmonella* positive egg samples. In our study the seasonal change to summer may have caused an increase in the number and variety of *Salmonella* serovars isolated from egg pulp.

Humphrey *et al.* (1989b) conducted a longitudinal survey examining the contents of eggs from chickens naturally infected with *S.*Enteritidis suggesting that the production of contaminated eggs may be intermittent. In our study the low prevalence of *Salmonella* isolates from both raw egg pulp and farm egg pulp may be explained by intermittent shedding of the organism.

A number of studies have shown that particular serovars such as *S.*Enteritidis and *S.*Typhimurium may cause transovarian infection in hens leading to the production of *Salmonella* contaminated eggs (Snoeyenbos *et al.*, 1969; Hopper & Mawer, 1988; Lister, 1988; Perales & Audicana, 1989; Barnhart *et al.*, 1991). One study in particular has found that some non-host-adapted salmonellae may produce local infections of the ovary and peritoneum of laying chickens with high likelihood of transmission to the egg yolk (Snoeyenbos *et al.*, 1969). *Salmonella* found in the yolk of eggs suggests that transovarian transmission may have occurred. Eight different serovars were found in yolk samples in this survey including *S.*Singapore, *S.* Mbandaka and *S.*Infantis. In a previous survey of egg pulp (unpublished data) *S.*Infantis was isolated on several occasions from raw egg yolk and a study in India found similar results for this serovar (Khurana & Kumar, 1993).

A diversity of *Salmonella* serovars was isolated from the farm survey and the egg pulp survey, 30 and 25 respectively. On many occasions from the same farm, the same serovar was isolated from faeces and egg pulp, perhaps implicating shell contamination as a second mode of egg contamination by *Salmonella*. Faecal material on the surface of the egg shell may harbour *Salmonella* which may be able to penetrate the permeable egg shell under conditions conducive to the growth of microbial contaminants in the contents of the egg (Sauter & Petersen, 1974; Clay & Board, 1992).

Environmental factors such as water activity and temperature, and management practices such as animal density and housing can influence the *Salmonella* status of a flock whether they are *Salmonella* positive or negative (Opara *et al.* 1992; Angen *et al.*, 1996). The presence or absence of detectable *Salmonella* in the present study has demonstrated a change in the pattern of serovar incidence both in the farm and egg pulp survey compared with the initial surveys. Sampling in the initial survey gave a point in time result demonstrating a high prevalence of *S.*Infantis. The longitudinal survey of a selected farm and of egg pulp revealed the high prevalence of an antigenically closely related serovar *S.*Singapore. This serovar may have persisted in the layer flocks simply because of its presence in raw feed components and under those conditions out competed *S.*Infantis or other serovars which may have been dominant at the time. Particular serovars may also be transient organisms, unable to or not colonising the caecum of the bird, therefore passing through the bird intermittently. An organism present in feed may then become prevalent in birds due to the lack of any other serovar being present. The presence of *S.*Infantis in egg pulp from various locations in south-east Queensland may also reflect a wider use of feed contaminated with this serovar and more importantly the possibility that this serovar has the ability to cause transovarian infection in the laying hen.

In summary, the main findings of the farm survey demonstrate a continued high prevalence of *Salmonella* among layer flocks in south-east Queensland, with a particularly high incidence of *Salmonella* in poultry feed. The longitudinal survey of a selected farm found *S.*Singapore to be a dominant serovar. It was also dominant in the egg pulp survey. *S.*Infantis was still detected among layer flocks and also in egg pulp and egg yolk although in very low frequencies.

A national survey of layer flocks in Australia, similar to those carried out in Canada (Poppe *et al.*, 1991a), the United States (Ebel *et al.*, 1992) and Germany (Hinz *et al.*, 1996), would provide an overall estimate of the prevalence of *Salmonella* among commercial layer flocks and identify prevalent serovars. Such a thorough investigation may draw more conclusive relationships between production practices and incidence of salmonellae in layer flocks.

Experimental Section 2

Typing of *Salmonella enterica* subspecies *enterica* serovar Infantis using phenotypic and genotypic techniques

Objectives

The aim of this study was to apply a range of methods to the discrimination of a large and diverse collection of Australian isolates of *S.*Infantis from a wide range of sources. The methods to be used are antibiotic susceptibility testing, biochemical typing, plasmid profiling, phage typing, ribotyping, IS*200* typing, PFGE and RAPD-PCR.

Methodology

Bacterial isolates

The collection of 139 *Salmonella enterica* subspecies *enterica* serovar Infantis (*S.*Infantis) isolates of poultry, human, animal, and environmental origin used in this study originated in Australia between 1991 and 1994. Isolates were obtained from three major sources. Ten isolates were provided by the *Salmonella* Reference Laboratory, in Adelaide, 58 isolates by the Microbiology Diagnostic Unit at Melbourne University and 71 poultry isolates from previous and current survey work conducted at the University of Queensland. Isolates from Adelaide and Melbourne were derived from all sources mentioned. Isolates in the UQ collection came from layer farms and egg pulp in South-East Queensland, derived from layer chicken faeces, litter, feed, feed components and raw egg pulp. Control strains were used for each typing method and included *Escherichia coli* ACM 1803 and 1901 (=ATCC25922; Australian Collection of Microorganisms, Department of Microbiology, University of Queensland), *Salmonella* Enteritidis ACM3696 (PT4), *Salmonella* Orion var. O15+ (local isolate) and *Salmonella* Typhimurium ACM3598. *Salmonella* stock cultures were maintained on Tryptone Soya Agar (TSA; Oxoid, Australia) slopes at room temperature. Working cultures were prepared on TSA slopes. Master cultures were maintained at -20ºC in TSB containing 15% glycerol.

Antibiotic susceptibility

Susceptibility to a range of antibiotics was assessed using two methods, and the control strain *E.coli* ACM1901. The agar disk diffusion method (Barry & Thornsberry, 1980) on Mueller-Hinton agar (Oxoid) was used to assess the susceptibility of 131 isolates using the following antibiotic disks (Oxoid): ampicillin (10 μg); ciprofloxacin (5 μg); tetracycline (30 μg); chloramphenicol (30 μg); gentamicin (10 μg) and sulphamethoxazole-trimethoprim (25 μg). The second method, an agar dilution method (van Duijkeren *et al.*, 1995) was used for the antibiotic furazolidone (Sigma-Aldrich, Australia) with a representative group of 30 *S.*Infantis isolates. For furazolidone, isolates susceptible to 2 mg/ml were considered sensitive, 4 mg/ml intermediate, and >8 mg/ml resistant (Cox *et al.*, 1996). The concentrations of all antibiotics, except furazolidone, were those recommended for disk diffusion by the National Committee for Clinical Laboratory Standards (NCCLS, 1994).

Biotyping

*S.*Infantis (131 isolates) were characterised using 28 previously described tests (Duguid *et al.*, 1975). A primary biotype was assigned to each isolate according to their reaction to five tests (fermentation of D-xylose, *meso*-inositol, L-rhamnose, *d*-tartrate and *meso*-tartrate).

Plasmid profiling

Isolation of plasmid DNA from 131 isolates of *S.*Infantis was attempted using the method of Birnboim and Doly (1979). Plasmid DNA was separated on 0.8% agarose gels in Tris Borate EDTA buffer (TBE; Maniatis *et al.*, 1982). The plasmids of *S.orion var.*O15+ AJM 1, previously sized at 20.0 kb, 1.9 kb and 1.0 kb (unpublished data), and lambda DNA *Hind*III digest (New England Biolabs, MA, USA), served as size standards for the determination of plasmid size in *S.* Infantis isolates. Plasmid DNA for restriction enzyme analysis was isolated using the same method and used as a crude preparation or in purified form. The DNA was purified using low melting point agarose followed by phenol-chloroform extraction and ethanol precipitation. Plasmid DNA was digested to completion with the restriction endonucleases *Hind*III, *Hinf*I, *Sma*I and *Sau*3AI (New England Biolabs). In a 50µl reaction, 10 µl of plasmid DNA was digested with two units of enzyme. Lambda DNA *Hind*III digest (New England Biolabs) was used as the molecular weight standard.

Phage Typing

Bacterial cultures. From 131 isolates, 40 were selected as a representative subgroup to be used in the development of phage typing. These isolates were chosen as fulfilling one of the following criteria: diversity, isolates from various layer farms; relatedness, isolates from their known history apparently related, and; clones, isolates which appear to be the same from information provided. The final group of 40 comprised three human isolates, four from egg pulp, one animal isolate and 32 poultry-related isolates, including those from feed, faecal and litter samples.

Isolation of phage from human sewage. Sewage samples collected from anaerobic and aerobic ponds were centrifuged at 3500 rpm for 15 min in a B-20 centrifuge. One ml of the supernatant and a loopful of growth from the propagating isolate were added to 20 ml of TSB and incubated for 5 h at 37ºC with vigorous shaking. The culture was then centrifuged and 100 µl of the supernatant mixed with 100 µl of an overnight broth of the propagating isolate. The mixture (10 µl) was spread onto a TSA plate and incubated overnight at 37ºC. Isolated plaques appearing on the bacterial lawn were then selected and purified.

Lysogenic isolates of S.infantis. Individual isolates were tested for lysogeny by growing them in 20 ml of TSB at 37ºC for 5 h with agitation. The culture was centrifuged at 3500 rpm for 15 min and, on occasion, filtered through a 0.45 µm membrane. The supernatant was mixed with overnight growth from a different isolate and spread onto a TSA plate and incubated at 37ºC overnight. Visible single plaques were selected and purified.

Purification of phage and Routine Test Dilution (RTD). Purification and propagation were conducted by serial passage of a well-isolated plaque three times with culture from the propagating isolate according to Anderson and Williams (1956). The overnight culture was centrifuged and then serially diluted to 10^{-6}. The phage lysate was diluted in 0.1% peptone water (peptone, 10 g/l and sodium chloride, 5 g/l pH 7.2). Drops (10 µl) of the phage lysate at each dilution were spread onto TSA plates of the propagating isolate to determine the RTD. The highest dilution of phage that produced confluent lysis on its host strain was considered the RTD (Callow, 1959).

Phage typing technique and phage storage. A RTD was prepared for each phage, then phage typing performed according to Castro *et al.* (1992). Phage activity was recorded according to reactions described by Anderson and Williams (1956). The RTD and the phage lysates were stored at 4ºC in nutrient broth (Oxoid) and the RTD checked again prior to use.

Preparation of genomic DNA and Southern Blot

Whole cellular DNA for ribotyping and IS*200* typing was extracted using the method of Christensen *et al.* (1993). DNA (10 µl) was digested with 10 units of restriction endonuclease, according to supplier's instructions (New England Biolabs). The restriction endonucleases *Eco*R1 and *Ban*I were chosen for ribotyping after preliminary experiments with a range of restriction enzymes (*Hind*III, *Not*I, *Pst*I, *Sma*I and *Xba*I). For IS*200* typing, four restriction enzymes (*Ban*I, *Bgl*II, *Pst*I and *Pvu*II) were trialled and the endonuclease *Ban*I was chosen for further digests. Restriction enzyme digests were electrophoresed on 0.8% horizontal agarose gels at 30 V for 16 h. Lambda DNA *Hind*III digest served as the DNA size standard. The genomic DNA restriction digests were transferred to nylon hybridisation membrane (Hybond-N, Amersham, UK) by vacuum blotting as recommended by the supplier (Pharmacia, LKB, Sweden). The membrane was soaked in 10xSSC for 5 min before vacuum blotting. The conditions for vacuum blotting were depurination at 50 millibars (mb) pressure for 4 min, denaturation at 40 mb for 3 min, neutralisation at 40 mb for 3 min and transfer at 40 mb for 55 min. DNA was cross-linked to the membrane by exposure to UV light for 4 minutes. If not used immediately the membranes were wrapped in foil and stored at 4°C until required for hybridisation.

Ribosomal RNA gene probe

An *E.coli* probe consisting of the DNA encoding ribosomal RNA (rDNA) was generated using a digoxigenin (DIG) DNA labelling and detection kit (Boehringer Mannheim). The concentration of DIG-labelled probe was estimated against control DNA from the kit and used appropriately for hybridisation. A second 16S rRNA gene probe from *E.coli* (ACM 1803) was generated using PCR amplification and the universal primer set 27F and 1492R (Lane, 1991). The amplified product was purified using the Wizard Miniprep Kit (Promega) and labelled with DIG using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Australia) according to the manufacturer's instructions. Hybridisation and the detection of hybrids on membranes of digested electrophoresed DNA was performed using a DIG DNA Detection Kit (Boehringer Mannheim) as recommended by the manufacturer.

*IS*200 *probe*

A probe for the DNA insertion element IS*200* was generated by amplifying a 692 bp product using *Pst*I digested *S.typhimurium* (ACM 3598) chromosomal DNA as the template. Whole cellular DNA was extracted as described previously (Christensen *et al.*, 1993) and 6 µl pre-digested with 20 units of the restriction enzyme *Pst*I. *Pst*I was chosen because there are no recognition sites within the IS*200* element (Stanley *et al.*, 1994). The *Pst*I digested DNA (5 µl) was the template for a PCR to amplify the 692 bp product with the primer set described by Baquar *et al.* (1994). The amplified product was purified with the Promega Magic PCR Preps DNA Purification System. The IS*200* probe was labelled with DIG by the same method described for the 16S rRNA probe. The probe was stored at -20°C until ready for use. The probe was removed from the freezer and denatured in 8 ml of standard hybridisation buffer by immersing into boiling water for 10 min. Standard hybridisation buffer and other buffers were prepared strictly according to the manufacturers instructions from the DIG PCR and Detection Kit (Boehringer Mannheim).

Pulsed field gel electrophoresis (PFGE)

Genomic DNA was prepared and PFGE was performed using a modification of the method described by Christensen *et al.* (1994). Isolates (n=139) were grown overnight on LB agar plates at 37°C. Four colonies were inoculated into LB broth and incubated at 37°C until an OD_{600} of 0.1 was reached. The cells were centrifuged (10,000 x *g*) for 5 min. and the pellet resuspended in 5 ml of ice cold Pett IV buffer (1 M NaCl, 10 mM Tris pH 8.0 and 10 mM EDTA). The bacterial suspension (0.5 ml) was mixed gently with 0.5 ml of 1% chromosomal grade agarose (Bio-Rad, CA, USA) and dispensed into 1 ml sterile syringes. The syringes were kept at 4°C for 10 minutes to allow the agarose to set and

then the agarose was sliced into plugs. Plugs were treated as described by Christensen *et al.* (1994). Two restriction enzymes, *Not*I and *Xba*I, were used for restriction digests. An agarose plug was placed into an eppendorf with 250 µl of the relevant enzyme buffer and incubated at room temperature for one hour. The buffer was removed and replaced with 50 µl of fresh buffer and 20 units of the enzyme. Reactions were incubated overnight to allow complete digestion. The resulting DNA fragments were separated by PFGE in a CHEF-DRII system (Bio-Rad). The pulse times for *Xba*I were 0.5 to 60 seconds over 26 h and for *Not*I, 5 to 30 seconds over 22 h. Polymerised phage lambda DNA (New England Biolabs) served as the size standard. Agarose gels were stained with ethidium bromide (0.5 µg/ml) and were photographed under UV transillumination.

Randomly amplified polymorphic DNA (RAPD)-PCR

Bacterial Isolates. A total of 32 isolates of *S.*Infantis from various sources were used. The isolates selected were both epidemiologically related and unrelated so as to include isolates from almost every source within the collection. A isolate of *Salmonella* Sofia was included to evaluate interserovar differences.

DNA preparation. A loopful of growth for each isolate was picked from an agar plate and resuspended in 400 µl of saline EDTA (Sigma). Lysozyme (15 µl of 0.1 mg/ml; Sigma) was added and the solution incubated at 37°C for 1 h. Proteinase K (7.5 µl of a 2% solution) and 15 µl of 25% SDS was added and the solution mixed then incubated at 60°C for 1 h. Phenol (400 µl) was added and the preparation vortexed. The supernatant was collected in a fresh tube after spinning for 10 min in a microcentrifuge. Phenol/chloroform/isoamyl alcohol (25:24:1; 400 µl) was added, the solution mixed and centrifuged at 3500 rpm for 10 min. The top layer was retained and 0.25 volume of 3 M sodium acetate added, then 2.5 volumes of 100% ethanol. The solution was precipitated at -70°C for at least 1 h or at -20°C overnight and then centrifuged and the ethanol decanted. The pellet was washed with 500 µl of 70% ethanol and after centrifugation, the ethanol was again decanted. The pellet was allowed to dry and then resuspended in 100 µl of sterile distilled water.

Primers. Primers were chosen on the basis of their previous success with RAPD PCR using other bacteria, as well as their availability. The majority of primers were synthesised by the Centre for Molecular Biotechnology at the University of Queensland, or purchased from Operon Technology Inc. (USA).

RAPD amplification. PCR reactions were overlayed with 40 µl of mineral oil. A final volume of 25 µl contained 25 ng/µl of *Salmonella* DNA, 1.5 mM $MgCl_2$, 10 picomoles/µl of primer, 1 unit per reaction of *Taq* DNA polymerase (Promega) and 200 mM (each) dATP, dCTP, dGTP and dTTP (Promega) in 20 mM Tris.HCl (pH 8.4) - 50 mM KCl. A Perkin Elmer TC900 was used for amplification. Amplifications were carried out for one cycle of 94°C for 5 min; 6 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min; 28 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min; and one cycle of 72°C for 5 min.

Analysis of PCR products. The amplification products were electrophoresed on 2% agarose gels using 1xTBE buffer. The RAPD profile was visualised by staining the gel with ethidium bromide and photographed under UV transillumination.

Results

Antibiotic resistance

Of 131 isolates, only five (4%) were found to be resistant to one or more antimicrobial agents. Only one, pork isolate 29, demonstrated multiple resistance to three antibiotics, ampicillin, sulphamethoxazole-trimethoprim and tetracycline. All poultry isolates were susceptible to the panel of antibiotics and all isolates were susceptible to ciprofloxacin, chloramphenicol, gentamicin, and furazolidone.

Biotyping

Five primary biotypes (Duguid *et al.*, 1975) were found among 131 isolates. Further subtyping was not possible as all isolates gave the same result for six out of the eight designated secondary tests. No subtypes within primary biotypes were therefore determined using these results. The largest group of 110 isolates corresponded to biotype 12 of Duguid *et al.* (1975) and 96% of isolates fell into one of two types, discriminated by their ability to ferment D-xylose in Bitter's medium. For 14 out of the 28 differentiating criteria all *S.*Infantis isolates gave an identical reaction. There was a distinct cluster of poultry isolates and a single human isolate comprising biotype 28.

Plasmid profiling

Of the 131 isolates, 93 (71%) carried a single plasmid of 35 kb, and additional plasmids of higher molecular weight were found in 13 other isolates. The molecular sizes ranged from 35 to 95kb and no small plasmids were detected (<20 kb). Five different plasmid profiles were demonstrated. A low level of diversity was observed in the plasmid profiles of isolates isolated from layer flocks and egg pulp in Queensland, of which 75% belonged to one profile. Fingerprints for isolates from humans showed the greatest degree of diversity with isolates being represented in each profile. All but one animal isolate and 67% of environmental isolates harboured the 35kb plasmid. There appeared to be no relationship between antibiotic resistance and possession of plasmids, which was not unexpected given the lack of antibiotic resistance among *S.*Infantis isolates. However four out of the five isolates found to be resistant to one or more antimicrobials harboured the 35 kb plasmid. Plasmid DNA restriction digests confirmed the size of plasmids for the control strain *S.orion var.* O15+ AJM 1. However, no plasmid DNA from any *S.*Infantis isolates could be cut with the restriction enzymes used.

Phage typing

A total of 15 phage were isolated including five from sewage. These five phage were kept at 4°C for three months and tested again on ten isolates of *S.*Infantis. The phage did not remain stable at 4°C during this time and they were unable to be used for further phage typing studies. The remaining ten phage were obtained by direct isolation from lysogenic isolates of *S.*Infantis. The ten phage were stored at 4°C and were used to type 40 isolates on five separate occasions. The phage were stable over this period of time and the RTD was adjusted when necessary on each occasion.

The results of phage typing were determined by various patterns of lysis that reflected the degree of susceptibility of an isolate to the ten typing phage used. Reactions were reported in terms of those phage that produced varying degrees of lysis. Using this set of ten typing phage, 40 isolates of *S.*Infantis representing various sources, geographical locations and times of isolation were classified into six distinct patterns and hence phage types. A significant number (20%) of isolates were resistant to all the typing phage. The most common phage type (PT) was PT 4.

Isolates from chicken faeces and chicken litter sampled from the same farm in most cases belonged to the same phage type, as did four egg pulp isolates. A single porcine isolate of *S.*Infantis represented PT 6 and the three human isolates grouped into PT 1 or PT 2 together with chicken faeces isolates.

Ribotyping

Initially an *E.coli* rDNA probe was prepared using reverse transcriptase and DIG labelling, but the efficiency of labelling was low and the probe could not be produced easily in a quantity sufficient for the large number of isolates to be examined. The second method for probe generation involved using the PCR DIG Probe Synthesis Kit, which yielded high volume, high concentration preparations of labelled probe.

Genomic DNA digested with the following restriction enzymes: *Ban*I; *EcoR*I; *Hind*III; *Not*I; *Pst*I; *Sma*I; and *Xba*I produced several hundred bands, evident by agarose gel electrophoresis. Strain discrimination by restriction fragment length polymorphism (RFLP) of chromosomal DNA alone, based on these results, was not attempted. Using *EcoR*I, 16 different ribotypes (RTs) were observed among 139 isolates of *S.*Infantis. Using a subset of 28 isolates, *Ban*I failed to further differentiate ribotypes produced using *EcoR*I. The number of fragments hybridising with the 16S rDNA probe ranged from 8 to 18.

The majority (81%) of isolates fell into four distinct profiles, with a further 12 ribotypes identified among the remaining 26 isolates. The poultry isolates grouped into 15 of the 16 ribotypes and poultry isolates alone dominated seven of these ribotypes. These seven ribotypes included those poultry isolates originating from south-east Queensland layer farms. The remaining ribotypes included human, animal and environmental isolates. The diversity found among the poultry isolates was not observed in the other sources. All human isolates grouped with poultry isolates and, with a single environmental isolate (human sewage isolate), represented Ribotype 2. RTs 8 and 9 were clearly distinguishable from all other patterns, but between them differed by only a single band at the 2.3 kb marker.

*IS*200 *typing*

Using the single restriction endonuclease *Ban*I, ten unique IS*200* profiles were identified among 139 isolates. Two isolates could not be typed or did not contain an insertion element, and this was not considered a profile. The majority of isolates (79%) fell into one IS*200* pattern or type, IST1. This IS*200* type included isolates of poultry, human, animal and environmental origin. The poultry isolates grouped into six IS*200* types, while human/animal, and environmental isolates grouped into five and three IS*200* types respectively. A single environmental isolate from sewage, with a unique ribotype (RT2) was also found to have a unique IS*200* profile. *Salmonella* Infantis isolates contained up to 13 IS*200*-bearing fragments, in a range of 4-13 (with the exception of isolates devoid of IS*200*). The four fragments were found in every isolate of *S.*Infantis. A second restriction enzyme, *Pvu*II, also demonstrated a high degree of discrimination among isolates of *S.*Infantis representing different sources, geographical locations and times of isolation. Some *Ban*I ISTs could be further subdivided by using the restriction enzyme *Pvu*II.

Pulsed field gel electrophoresis

PFGE of whole cell DNA digested with the rare cutting restriction endonuclease *Xba*I yielded 36 unique restriction fragment length polymorphisms (pulsotypes) among 139 isolates, with 20 patterns each being represented by a single isolate. The most common pattern was demonstrated in 49 (35%) isolates representative of all sources. The diversity of poultry isolates was lower using PFGE compared to that using ribotyping and IS*200* typing, only half the PFGE patterns including poultry isolates. For isolates of human origin the diversity increased substantially, with 15 PFGE patterns identified for 24 isolates. In 20 instances, a single human isolate represented one of the 36 single-isolate PFGE patterns. The environmental (sewage) isolate with the unique RT and IST also yielded a unique PFGE pattern. The DNA from one poultry isolate could not be cut with *Xba*I and was consequently not typable. The number of fragments observed with PFGE varied from 10- 15.

*Grouping based on ribotyping, IS*200 *typing and PFGE*

By comparing the *Eco*R1 RTs, the *Ban*I ISTs and the *Xba*I pulsotypes, 64 genotypes were defined. The largest of these clonal groups, comprising 30 isolates, yielded the same RT, IST and pulsotype. The majority of these isolates were poultry isolates (53%) from two layer farms in South-East Queensland. A second clonally distinct group of five isolates all came from the same layer flock. Single isolates represented 44 genotypes, many being human clinical isolates. Two pork isolates,

representing a distinct genotype, were identical in all results. Another cluster of 12 poultry isolates, originating from three locations in Queensland, subdivided into four closely related genotypes.

RAPD-PCR

A total of 12 primers were screened for use in RAPD-PCR. The first six primers OPE -03, 07, 10, 14, 19 and OPAG-06 were used with two unrelated isolates of *S.*Infantis to establish a suitable primer. OPAG-06 was retained for subsequent studies for its ability to produce clear and distinct patterns. OPAG-06 was used with 30 isolates of *S.*Infantis, yielding five RAPD profiles. Four isolates, all epidemiologically unrelated, produced four different profiles, while the remaining 24 isolates, mainly poultry isolates represented RAPD Profile 5. Reproducibility of the RAPD fingerprinting patterns was confirmed using duplicate runs at different times. Differences between RAPD Profiles 1-4 were based on the presence or absence of one or two bands. RAPD Profiles 1-4 yielded much higher numbers of fragments than Profile 5. Preliminary studies with a second group of six primers (11a, 97ar, M13, ERIC-2, M1 and LI) was carried out on four isolates, three of *S.*Infantis and one of *S.*Sofia. Based on the RAPD patterns produced it appears that M1 would be a suitable primer for future studies. The three isolates of *S.*Infantis were indistinguishable by their RAPD profile while the profile for *S.sofia* was different. The primer allowed discrimination between serovars but not within the serovar *S.*Infantis. However only a small number of isolates were examined.

Discussion

*S.*Infantis has been widely isolated in Australia, more frequently from pigs than any other food animals and with increasing frequency from eggs (Murray, 1994b). The isolation of this serovar from humans was sixfold less than that from eggs. In Australia, between 1987 and 1992 *S.*Infantis was the third most common serovar isolated from eggs, behind *S.* Typhimurium and *S.* Bovismorbificans (Murray, 1994b). A study in Canada (Poppe *et al.*, 1991b) also found *S.*Infantis to be the second most common serovar isolated from layer flocks and the most common serovar isolated from raw egg pulp. The significance of the isolation of the bacterium from eggs is of public health concern. The possible carriage and subsequent transmission of the organism to the ovary of the chicken has been supported by a number of studies (Barnhart *et al.*, 1991; Dreesen *et al.*, 1992).

Typing methods for the differentiation of isolates within a given *Salmonella* serovar enable associations to be made between human cases of salmonellosis as well as between cases and food vehicles, food animals or geographical areas. The collection of isolates used in the present study originated from a diversity of sources, locations within Australia, and dates between 1991 and 1994. The present study aimed to assess or develop typing tools for isolates of *S.*Infantis that might be applicable in epidemiological investigations. Individual bacterial isolates that exhibit identity in a number of typing methods can be grouped together in clusters of relatedness. This relatedness can be described as clonal line or clonality, a high probability that two isolates are related to each other (Christensen *et al.*, 1994).

The use of antibiotics in intensive animal production, such as the poultry industry, has been a controversial issue in Australia (NHMRC, 1986). Their use, particularly in stockfeed has prompted concern that resistant bacteria may spread between animals and man (Frost *et al.*, 1996). In the present study, 131 isolates of *S.* Infantis were typed according to their susceptibility to antibiotics representing the penicillins, tetracyclines, aminoglycosides, quinolones, sulphonamides, and nitrofurans (30 isolates only), as well as trimethoprim and chloramphenicol. Although the use of many antibiotics has now been banned within the poultry industry, this was not enforced until 1993 and the majority of isolates were isolated prior to this time. Nevertheless, resistance among *S.*Infantis isolates to one or more antimicrobials was rare. Only one isolate, showing multiple resistance, originated from pork. Previous extensive use of antibiotics in the pig industry may have led to an environmental pool of antibiotic resistance. There was no incidence of drug resistance among the poultry isolates; all isolates

were susceptible to ciprofloxacin, chloramphenicol, gentamicin and furazolidone. These results agree with previous observations from Canada suggesting that poultry isolates are usually sensitive to commonly used antimicrobials (Poppe & Gyles, 1987), although studies from the United Kingdom have identified significant increases in resistance to antimicrobials among poultry isolates (Threlfall *et al.*, 1993b).

Biotyping demonstrates phenotypic similarities or dissimilarities between isolates of a given species. The biotyping scheme developed for *S.* Typhimurium (Duguid *et al.*, 1975) was applied to *S.*Infantis isolates. This method was reliable and reproducible, two important variables for assessing a typing method. However, *S.*Infantis isolates were differentiated into only five primary biotypes, 84% of isolates belonging to one biotype, with little further discrimination using secondary tests. Although the biotyping scheme was originally devised for *S.* Typhimurium and has been found useful with other serovars such as Montevideo (Old *et al.*, 1985; Reilly *et al.*, 1985), and Paratyphi B (Old & Barker, 1989), it afforded little differentiation of Infantis. The relatively few variant biotypes of Infantis, as found for *S.* Agona (Baker *et al.*, 1995), suggests that this serovar could be of recent origin.

Many investigators have reported the utility of plasmid analysis in the study of epidemiology of *Salmonella* serovars (Olsen *et al.*, 1992a; Singer *et al.*, 1992; Usera *et al.*, 1994). In the present study, we were unable to establish significant separation of the isolates by means of their plasmid profiles. A report from England also found a low degree of diversity of plasmid profiles in isolates of *S.*Enteritidis from common sources (Threlfall *et al.*, 1989). Five plasmid profiles were recognised among 131 isolates of *S.*Infantis and 71% of these, representing isolates from all sources, carried a single plasmid of 35 kb. Plasmid profiling of other *Salmonella* serovars has been used to compare isolates from humans with isolates from poultry (Olsen *et al.*, 1992b), and has been regarded as a tool with high typability and good discriminatory power. Although plasmid profiling provided little discrimination within our isolates, it should not be dismissed as a potential subtyping method. Typing of *S.*Infantis isolates based on plasmid profiling has been reported on numerous occasions with multiple profiles being demonstrated in isolates of human, environmental and poultry origin (Nolan *et al.*, 1991; Borrego *et al.*, 1992; Olsen *et al.*, 1994; Hasenson *et al.*, 1995). High and low molecular weight plasmids have been identified ranging from 3 kb to 146 kb and the number of plasmids from one (high or low molecular weight plasmid) to four. Profiles have been associated with and without antimicrobial resistance (Olsen *et al.*, 1994). This raises questions about the stability of plasmids found in *S.*Infantis isolates. Plasmids represent mobile extrachromosomal elements that do not code for essential properties and therefore may be lost from or acquired by *S.*Infantis cells. Thus, additional typing methods based on chromosomal DNA should be employed and may be combined with plasmid profiling when these limitations are recognised.

Phage typing can only be of epidemiological value if it is able to differentiate isolates of the same serovar into types or groups that are easily recognisable with epidemiological significance. In this study a bacteriophage typing system was described for *S.* Infantis, based on a method by Anderson and Williams (1956) and Ward *et al.* (1987). Ten phage were selected, all of which were obtained by lysogeny. Many attempts were made to try and isolate phage from sewage but most were unsuccessful. In the first instance the initial phage group were all isolated from sewage without difficulty and it is not known why difficulties were encountered after this time. One explanation may be the conditions for which the phage were stored. It is not vital that the final phage set be composed of phage from sewage. Indeed Ward *et al.* (1987) used only one typing phage isolated from sewage in their final phage scheme.

There are several types of phage typing schemes that have been developed for *Salmonella*. They differ in the principles employed for the development of the scheme as well as how successfully they are applied. The typing scheme here consisted solely of lysogenic phage which were isolated from *Salmonella* isolates belonging to the serovar for which the scheme was evolved. Several terms are used for such phage, such as prophage, natural phage or temperate phage. An example of this typing scheme was created for *S.dublin* described by Smith (1951). The lysogenic character is generally

stable although spontaneous losses of lysogeny have been reported in some bacterial isolates (Adams, 1959).

Six distinct phage type patterns were recognised among 40 isolates of *S.*Infantis. The typing phage remained stable over a three-month period and the method was repeated on five occasions. Phage reactions for each isolate were duplicated on some occasions however the scheme was not reproducible. Sixteen isolates produced the same lysis pattern on every occasion, including one human isolate and 15 poultry isolates. Many isolates originating from the layer farm were grouped together to represent distinct phage types in their own right. Unrelated single isolates often grouped as a single phage type.

Reproducibility of phage patterns can be demonstrated by retyping cultures and obtaining the same results or by showing that epidemiologically related isolates belong to the same phage type. Rigorous standardisation of the method is necessary to achieve strong reproducibility. In a number of cases lack of reproducibility can be explained in terms of segregation or acquisition of plasmids or lysogenic phage (Adams, 1959). Some plasmids may have phage-inhibiting properties and cultures stored for months at a time and then retyped could subsequently exhibit different patterns. Variations of these phage types were due to lysogenic phage. If the rate at which lysogenic phage lysogenise sensitive isolates is high, it may be difficult to detect the lysis because of heavy secondary over-growth by lysogenised cells. It is possible that within this typing scheme the storage of isolates or the loss or acquisition of plasmids may have caused slight changes in the phage reaction of particular isolates of *S.*Infantis producing different patterns on different occasions.

The data indicates that the scheme needs further development and that perhaps the isolation and use of phage from sewage may enhance the reproducibility of the method. Phage from sewage usually yield virulent phage and these phage are the most convenient for phage typing because they produce clear lysis which makes the results easy to read (Adams, 1959). In the past, phage typing of *S.*Infantis has been shown to be a useful tool for establishing relationships during an outbreak and for tracing the origin of infection (Kasatiya *et al.*, 1978; Laszlo *et al.*, 1988). The standardisation of a phage set to obtain serovar-specific phage is needed to develop a phage typing scheme useful as an epidemiological tool for discriminating between isolates of *S.*Infantis.

In population genetic studies, the purpose of which is to identify clonal lines of the serovar being investigated, promising results have been achieved with techniques such as ribotyping, IS*200* typing and PFGE (Olsen & Skov, 1994; Olsen *et al.*, 1994). Sixteen unique rDNA restriction patterns were demonstrated with the restriction enzyme *Eco*RI among 139 isolates. This distribution is comparable to results of ribotyping studies with other serovars (Esteban *et al.*, 1993; Olsen *et al.*, 1994). A similar study with approximately the same number of *S.*Infantis isolates did find fewer patterns (Pelkonen *et al.*, 1994). The four most common ribotypes; one, seven, eight, and nine constituted 81% of isolates. Profiles eight and nine differed by a single band while one and seven essentially contained up to six other bands. These ribotypes were found to be particularly common among poultry isolates most notably in isolates from a particular farm. This behaviour of some clones confirms that they are to some extent host adapted, only sporadically involving other animal species and humans in their ecological cycle. For those sources where a number of isolates were examined the high number of ribotypes (n=11) involving only a single or a few isolates of *S.*Infantis could reflect one or more of the following situations (i) clones newly introduced into Australia either through travel as seen in human isolates or (ii) isolates originating from the importation of poultry feed components such as fishmeal or cottonmeal isolates or (iii) mutations in isolates already established in Australia.

IS*200* typing classified the isolates into ten unique profiles using the restriction enzyme *Ban*I. This enzyme, used previously in a study with *S.*Infantis isolates produced eleven profiles with a similar number of isolates (Pelkonen *et al.*, 1994). The majority of isolates carried four fragments of the IS*200* element and two isolates were found to be free of any IS*200* sequences. The number of fragments ranged from four to 13, using *Ban*I. These four hybridisation bands were found in all isolates, suggesting that these four fragments are serovar specific. Serovar specific copies of IS*200*

have also been reported among other *Salmonella* serovars such as *S.heidelberg* (Stanley *et al.*, 1992a). IS*200* insertions in plasmids rather than on the chromosome could be discounted due to the fact that exact profiles were seen for isolates with and without the same plasmid profile. Eighty percent of *S.*Infantis fell into one IS*200* profile indicating that IS*200* alone is not a very powerful discriminatory method. Ribotypes may be used to subtype within IS*200* types, particularly within IS*200* type one which contains 11 different ribotypes. Such profiles would not be useful for the identification of clonal lines of *S.*Infantis when used alone.

The use of hybridisation with an IS*200* probe has been shown to be a less sensitive method than ribotyping for discrimination of isolates of *S.*Infantis, because of the lack of diversity of IS*200* insertion sequences among them. On the other hand the opposite has been shown for different serovars such as *S.* Abortusovis where IS*200* was the more sensitive method (Schiaffino *et al.*, 1996). Its' discriminatory power, however, strongly depends on the choice of the restriction enzyme used for cleavage of the whole cellular DNA. The enzyme *Pvu*II appeared to exhibit a higher discriminatory power than *Ban*I and should be applied to a larger group of *S.*Infantis isolates.

PFGE analysis has been shown to be highly effective in assessing the extent of molecular diversity within a species (Prevost *et al.*, 1992; Kelly *et al.*, 1993). This technique has been applied to many *Salmonella* serovars and has been shown to be the most discriminatory method when used with other molecular typing techniques (Christensen *et al.*, 1994; Olsen *et al.*, 1994; Liebisch & Schwarz, 1996a). Our data confirmed the same observation and 36 PFGE patterns were recognised. A single band difference was assessed as a unique pattern. Only DNA fragments above the 97 kb marker were considered to exclude the possibility of restriction polymorphism due to plasmid DNA. PFGE using two restriction enzymes *Xba*I and *Not*I did not enhance the discriminatory value of this technique as *Not*I was unable to further differentiate patterns exhibited by *Xba*I. PFGE was able to be used to subclassify isolates of *S.typhi* from sporadic cases into a number of pulsed field types and group together isolates associated with four separate *S.typhi* outbreaks (Suzuki *et al.*, 1994). In this study, although not dealing specifically with *S.*Infantis isolates from an outbreak, PFGE clearly discriminated among poultry isolates from different geographic origins and always showed a certain degree of relatedness among isolates obtained from a given area. This was also the case with the human isolates of *S.*Infantis; isolates from the same location, recovered at different times. It is clear from the data that considerable heterogeneity exists at the DNA level among *S.*Infantis isolates from different locations within Australia. Thus, PFGE pattern seems to provide a more reliable molecular marker for epidemiological studies than ribotyping or IS*200* typing.

RAPD PCR is still a fairly new technique, which is a rapid and simple procedure that can be used for typing of bacteria. Although a number of phenotypic and genotypic methods were applied to the large collection of *S.*Infantis isolates RAPD PCR was applied on a small scale. RAPD PCR does not require any specific knowledge of the DNA sequences of the target organism. The most difficult aspect of this protocol is the selection of suitable primers. Six primers were examined using two isolates that were epidemiologically unrelated. One primer was chosen for RAPD analysis of the remaining 30 isolates. The primer OPAG-06 produced clear and distinct banding patterns for *S.*Infantis using RAPD PCR fingerprinting. Thirty isolates were differentiated into five distinct profiles. Five isolates (16.7%) gave no result indicating that there may have been a problem with the template DNA in the PCR. Four unrelated isolates demonstrated unique profiles differing by one or two bands. The remaining isolates, mainly from poultry, belonged to profile five.

By comparison with the other molecular methods for the same isolates, RAPD PCR was not as discriminatory as ribotyping and PFGE. A number of studies have compared each of these molecular methods. In a study with *S.dublin* RAPD PCR was not as discriminatory as ribotyping but when combined they enhanced the discrimination among a genotypically homogenous group (Kerouanton *et al.*, 1996). By comparison with methods such as ERIC-PCR, RAPD PCR demonstrated a higher discriminatory value although its usefulness alone for the typing of *S.typhi* and *S.*Enteritidis was questionable (Hermans *et al.*, 1996; Millemann *et al.*, 1996). Lin *et al.*, (1996) reported that *S.*Enteritidis PT 8 isolates that could not be discriminated by other typing methods such as phage

typing, ribotyping and PFGE were resolved into subtypes using RAPD analysis. In our study only a small number of primers were able to be screened whereas a more extensive study involved 65 primers with *S.*Enteritidis (Lin *et al.*, 1996).

In RAPD PCR, apart from temperature and concentration of Mg^{2+} which can significantly affect the amplification of DNA, the primer and template concentration are also critical because they affect the quality of the product (Ellsworth *et al.*, 1993; Park & Kohel, 1994). There is also a degree of artefact within the profiles generated by RAPD PCR resulting in variations in banding patterns between experiments. High and rigorous standardisation of DNA concentrations is needed for consistency between separate amplifications (Ellsworth *et al.*, 1993). The results should be reproducible on separate occasions, between individual thermocyclers and in different thermocycler models (He *et al.*, 1994). The reproducibility of RAPD fingerprinting has been excellent in this regard when the thermocycler is equipped with the best temperature regulation (Meunier & Grimont, 1993). The RAPD banding patterns produced by the 30 isolates here were shown to be reproducible on three separate occasions however the same thermocycler was used each time.

A second group of six primers was also screened using three isolates of *S.*Infantis and one isolate of *S.sofia*. RAPD PCR fingerprinting was able to discriminate between the serovars *S.*Infantis and *S.sofia* but not within the serovar *S.*Infantis. All three isolates gave the same profile and a much larger isolate number would have increased the ability to assess these primers. Under defined conditions RAPD PCR is able to differentiate within and between serovars of *Salmonella* from various sources reliably, economically, rapidly and with high reproducibility. More stringent testing would be required for testing our collection of isolates including the use of an increased range of arbitrary primers, amplification in different thermocyclers and optimisation of primer concentration, DNA template and PCR conditions. These factors were considered here but not optimised for *S.*Infantis. This method, with further development would be a useful tool for typing *S.*Infantis isolates for epidemiological purposes. Variations of this method such as REP PCR, ERIC PCR and PCR ribotyping may also be useful for differentiating isolates of *S.*Infantis as one study has already shown (Lagatolla *et al.*, 1996).

A lack of intraserovar subtyping methods (Stanley *et al.*, 1992a) is evident for a number of *Salmonella* serovars such as *S.*Infantis. The application of antibiotic resistance, biotyping and plasmid profiling for typing of *S.*Infantis isolates has not been well documented. Used alone or in combination these methods have been extremely useful in their discriminatory power with other *Salmonella* serovars. In this study, due to the lack of variant phenotypic characteristics and plasmids, either alone or in combination these methods do not satisfactorily discriminate within Australian *S.*Infantis isolates. The techniques, ribotyping, IS*200* typing and PFGE have different discriminatory values for isolates of different *Salmonella* serovars. In studies involving serovars such as *S.*Enteritidis and *S.dublin* identical patterns were seen among a group of isolates using IS*200* and ribotyping (Liebisch & Schwarz, 1996a, b). However these methods have shown great discriminatory power with the serovar *S.* Typhi (Nastasi *et al.*, 1991; Thong *et al.*, 1994).

The combination of ribotyping, IS*200* typing and PFGE was applied to the isolates in the current study to establish any clonal lineages or genotypes. Many clonal groups were recognised including two major groups of 30 and 12 isolates respectively. The first group contained poultry isolates (53%) and also included Queensland porcine isolates. The second group also demonstrated a high degree of homogeneity. These isolates originated from poultry sources from three different locations in south-east Queensland and may represent our only true clonal line of *S.*Infantis. Our data is in agreement with the conclusion drawn from recent studies of microbial populations that substantial genetic diversity exists within species or serovars. This suggests that isolates are distributed among many genetically diverging lineages (Thong *et al.*, 1996). Multilocus enzyme electrophoresis could be used to further study the genetic relationships among *S.*Infantis isolates. This method has been used previously to study the genetic diversity and relationships among strains of different *Salmonella* serovars although not for any one specific serovar (Beltran *et al.*, 1988).

A summary of the genotyping results shows that over 60 genotypes were recognised based on results for ribotyping, IS*200* typing and PFGE. The majority of the human isolates and the more unusual isolates such as those from a crocodile, separated to represent a unique type, while isolates related in the field clearly grouped together to form their own genotype. Not surprisingly most of the Queensland layer farm isolates grouped together. Isolates from the same farm sampled two years apart also gave identical results suggesting the ability of the organism to persist in the layer flock or survive within the layer shed. Few genotypes involved strains that were unrelated by virtue of their site of isolation or habitat. An example of this is genotype 42, which comprised of a river isolate, human faecal strains and liquid egg white isolates. Presumably a link could be made between these isolates although this would be impossible to validate.

Mutations within a given population of isolates, representing a single serovar, can occur and this may cause slight variations in the patterns or profiles seen for genotypic methods such as ribotyping, IS*200* typing and PFGE. The majority of poultry isolates isolated from layer farms in Queensland represented few ribotypes, IS*200* types and PFGE profiles. Differences between poultry isolates, as demonstrated by different profiles, were minor and in most cases the patterns differed by the presence or absence of a single band. Ribotypes eight and nine differed by a single band and represented 71% of Queensland layer farm isolates. These isolates are closely related by virtue of their habitat and by the typing patterns produced by different genotypic and phenotypic techniques. A mutation in a single clone though, may result in several subtypes when analysed by various molecular methods.

It was found that phenotypic characteristics were more susceptible to change on subsequent retesting after initial typing results demonstrated little discrimination amongst the core group of isolates. For example plasmid profiles for some isolates changed after prolonged storage at room temperature and the loss of a single plasmid was common.

In general the profiles demonstrated by the more discriminatory techniques, such as ribotyping and PFGE, are consistent with the epidemiological findings. In addition to supporting relationships that exist within a group of isolates, originating from the same source, geographical location or time of isolation, the results also define new relationships. Examples of these non-apparent or new relationships include: (1) Queensland porcine isolates and Queensland layer farm isolates gave identical results; (2) human isolates, diverse epidemiologically, were easily discriminated between using PFGE; (3) a human isolate and a single pork isolate produced identical PFGE types, plasmid profiles, antibiotic resistance to ampicillin and similar ribotypes, suggesting that these two organism may represent the same isolate of *S.*Infantis; (4) generally smaller groups of related isolates were grouped together by their ribotyping and PFGE results and (5) single, one off isolates originating from an unusual or uncommon source demonstrated unique profiles with PFGE.

The importance of *S.*Infantis in the Australian pig industry was highlighted by the fact that this serovar was isolated more frequently from pork than any other food source between 1987 and 1992 (Murray, 1994b). It is epidemiologically significant therefore that the three Queensland porcine isolates in our collection gave identical results for each method, including RAPD PCR, as most of the Queensland layer farm isolates. This suggests that there may be transmission of *S.*Infantis from pork to poultry through inclusion of porcine meat and bone meal in poultry feed. Although *S.*Infantis has not been directly associated with the Australian pig industry this serovar is prevalent in pig herds in Denmark (Baggesen *et al.*, 1996; Berends *et al.*, 1996; Wegener & Baggesen, 1996). Cross contamination in meat processing facilities, farm to farm transmission, and contaminated feed represent documented forms of transmission of *Salmonella* between animal and animal, and man and animal. Slaughterhouse by-products, including poultry by-products have been used as raw material in foodstuffs for pigs as a source of protein (Urlings *et al.*, 1993). In Australia feed for pigs may include poultry by-products, contaminated feed thus representing a potential source of infection between the two industries.

The presumption that two isolates are related should be confirmed using more than one typing method. However, isolates belonging to the same unique PFGE profile may be related (Wegener &

Baggesen, 1996). It is of interest that three isolates, two from human sources and one from a crocodile gave an identical and unique PFGE profile. In Australia, culled hens from layer farms are fed to crocodiles and crocodile farming (meat and skin) in Australia is considered to be a tropical intensive animal industry (Bache, 1994). The transmission of *Salmonella* from animals to man and between animals has been well documented. It is conceivable therefore that prevalent *Salmonella* serovars in the layer industry may be transmitted to crocodiles and transmission to man may occur through the consumption of contaminated meat.

In conclusion, methods such as biotyping, antibiotic resistance and plasmid profiling may be used to type *S.*Infantis, and in some situations they may discriminate between isolates. However, due to the low discriminatory power of these methods, the demonstration of the same type does not necessarily indicate that the isolates are related. From preliminary work with phage typing and RAPD PCR and successful application to many other *Salmonella* serovars, it is clear that these techniques could be used for typing of *S.*Infantis. The data from the present study confirmed that ribotyping, IS*200* typing and PFGE are the most suitable methods for typing of *S.*Infantis, particularly PFGE, which was the most discriminatory of the molecular methods applied to the serovar.

Experimental Section 3

Comparative study of egg shell penetration by *Salmonella* serovars Infantis, Enteritidis and Singapore

Objectives

The aim of this study was to determine the ability of isolates of three *Salmonella* serovars, *S.*Infantis, *S.*Singapore, and *S.* Enteritidis to penetrate first quality shell eggs under different conditions of contamination and storage. Serovars Infantis and Singapore were chosen due to their prevalence in Queensland laying flocks, while Enteritidis was included due to its ongoing association with eggs in many other world regions.

Methodology

Bacterial cultures

A group of eight *Salmonella* isolates were selected for eggshell penetration studies; two of *S.*Enteritidis (SE), and three each of *S.*Infantis (SI) and *S.*Singapore (SS). The SE isolates included C6B (PT4), a virulent isolate which represented a positive control (previous work demonstrated that C6B can penetrate and grow in egg contents at various temperatures (Humphrey *et al.*, 1989a)). The second Enteritidis isolate, SE30 (PT4), is a human clinical isolate and served as a good comparison with C6B as it has been shown to be avirulent in chicks.

Three isolates of *S.*Infantis originating from three different sources, yolk (SI159), chicken faeces (SI A15) and poultry feed (SI100), were examined. The high prevalence of *S.*Singapore on layer farms and in raw egg pulp as shown by farm and egg pulp surveys prompted its inclusion in the present study. Three *S.*Singapore isolates, also from yolk (SS262), chicken faeces (SS F1) and poultry feed (SS A29) were selected.

Inoculum preparation

Isolates were grown in 10 ml of TSB for 16 h at 37°C. The cultures were sedimented by centrifugation (2500g for 20 min) and resuspended in 10 ml of 0.1% peptone (0.1 g of bacteriological peptone (Difco) dissolved in 100 ml of distilled water and autoclaved). This was equivalent to approximately 10^9 cfu/ml. Cultures were adjusted to the appropriate level either, 10^4 or 10^8 cfu/ml by dilution in 0.1% peptone. Cell counts of the suspensions were determined by plating appropriate dilutions onto duplicate Hektoen enteric (Oxoid) agar and incubating the plates at 37°C for 48 h.

Preparation of faeces

The method was based on the procedures of Williams and Whittemore (1967) and Schoeni *et al.* (1995). Chicken faeces (100 g) from layer hens kept in the Animal Holding facility within the Department of Microbiology at the University of Queensland were collected into a sterile Stomacher bag. The faecal matter was pressed into three glass Petri dishes and dried in an oven at 120°C for 3 h (uncovered). The dry faeces were then placed into a blender and mixed on low speed for 10 s to form a fine powder. This powder was sterilised by autoclaving. Faeces powder (23 g) was weighed and rehydrated in 37 ml of sterile 0.85% saline, producing paste of a consistency comparable to that of

normal chicken faeces. Faecal paste (18 g) was weighed aseptically and pressed evenly into a sterile Petri dish. Sterile aluminium cylinders, 13 mm internal diameter x 10 mm height, were pressed firmly into the faecal paste, the Petri dishes sealed with parafilm and the lid replaced, then stored at -20°C, until required.

Source and preparation of eggs

Eggs were purchased from a local farm that had been identified previously as free from the presence of the serovars under study. Eggs were supplied clean (free of faecal material on the surface of the shell), free of cracks, and fresh (laid within the previous four hours). On arrival at the laboratory, eggs were examined under strong light in a dark room to confirm the absence of cracks and to locate the position of the air sac. Eggs were surface sterilised by submerging completely in 70% ethanol for 30 seconds. Eggs were removed from the beaker of ethanol with a 70% ethanol soaked towel and placed into cartons that had been rinsed with 70 % ethanol and allowed to dry. The site of inoculation was located a short distance away from the pointed end of the egg and away from the air sac. The weight of each egg was recorded.

Analysis of control eggs

For each of two control eggs per experiment, the yolk and the albumen were separated by cracking the egg on the edge of a sterile beaker and passing the egg contents between the shell halves. The inner membrane was removed with sterile forceps from the shell. Each part of the egg was tested for the presence of *Salmonella*. Yolk and albumen samples were diluted 1:4 w/v with TSB, mixed, and a swab of this mixture spread onto Hektoen Enteric agar (HEK) plates. Plates were prepared in duplicate and incubated at 37°C for 48 h. The inner membrane and shell were placed separately into 5 ml of TSB and a swab of this mixture spread onto HEK plates and incubated. The TSB homogenates for all egg parts were incubated at 37°C for 24 h and samples of these swabbed onto HEK plates.

Eggshell penetration

The test eggs were candled and the air sac marked. The test eggs were equilibrated to the test temperatures; 4°C, 25°C, or 35°C. The faeces was removed from the freezer and the aluminium rings dislodged one at a time. The test egg was held in the palm of one hand (gloves worn) and an aluminium ring containing the faeces placed directly under the air sac with the other hand. The ring was held in place with the thumb and forefinger of one hand while molten paraffin was swabbed around the edge of the ring. The paraffin hardened instantly and the rings were attached firmly. Eggs were held for 30 min at the test temperature. Approximately 80 µl of the appropriate *Salmonella* culture was added to the faeces in each ring (Williams & Whittemore, 1967). Eggs were placed into cartons and held for an additional 30 min at the inoculation temperature, either 4ºC, 25ºC or 35ºC, and then transferred to either 4ºC or 25ºC. Conditions, chosen to reflect different practices at points within the egg production chain, were as follows:

- inoculation of ~10^8cfu/g faeces at 4ºC, incubation at 4ºC for 1 and 3 days;
- inoculation of ~10^8 cfu/g faeces at 25ºC, incubation at 25ºC for 1 and 3 days;
- inoculation of ~10^8 cfu/g faeces at 35ºC, incubation at 4ºC for 1, 3, 7, and 14 days.

While the concentration of *Salmonella* in the inoculum may not necessarily reflect that present in faeces (although such high populations of salmonellae may be encountered), the time and temperature parameters were selected to reflect conditions under which eggs may be held at different stages of production. The inoculum size was standardised both for each isolate and across isolates, so that the rate of egg contamination by each serovar under similar time-temperature conditions could be assessed accurately and precisely.

A sample of uninoculated faeces was tested for *Salmonella* at Day 0 for each test event. Two eggs were assayed for each treatment on each day of sampling. The yolk, albumen, inner shell and inner membrane of each egg were sampled. The faecal paste was removed from the ring, weighed, and diluted 1:9 w/v with 0.1% peptone. The faecal suspension was serially diluted and 100 µl aliquots of the appropriate serial dilutions plated in duplicate onto HEK plates. The plates were incubated as before and colony counts carried out. Once the faeces and aluminium ring were removed the egg was swabbed with 70% ethanol and cracked on the side of a sterile beaker. The yolk and the albumen were separated using the shell halves as before, and each weighed into a sterile bag. The contents were diluted with TSB 1:4 w/v and agitated. Homogenate (1 ml) was serially diluted in 0.01M (1×) phosphate buffered saline (10 × stock; 12.6 g Na_2HPO_4, 1.8 g $NaH_2PO_4 \cdot H_2O$, 85 g NaCl and distilled water up to 1000 ml) to 10^{-4} and 100 µl aliquots of the appropriate dilutions were plated onto HEK plates. After overnight incubation at 37°C, presumptive *Salmonella* colonies were counted. One colony on each plate was picked and presumptively identified using a Serobact latex agglutination kit and *Salmonella* somatic agglutinating sera (Murex Diagnostics, England) for specific *Salmonella* groups. The TSB homogenate was incubated at 37°C for 24 h to enrich for *Salmonella* at low population that may have been undetectable by direct plating. When no growth was evident by direct plating, the incubated TSB enrichment for that sample was streaked onto HEK and incubated. The plates were examined for the presence or absence of *Salmonella* and a colony was picked from each plate where possible and presumptively identified using a Serobact latex agglutination kit.

The egg inner membrane, under the *Salmonella* inoculation site, was removed with sterile forceps and placed into 5 ml TSB and mixed. A swab of the mixture was plated onto HEK. The remaining TSB was incubated at 37°C for 24 h, then plated on HEK. The inner shell under the inoculation site, after removal of the membrane, was swabbed with a TSB soaked cotton swab, which was then placed into 5 ml of TSB. A swab of this mixture and of the enrichment was spread onto HEK. The presence or absence of *Salmonella* was recorded. Presumptive colonies were counted and one isolate from each plate tested with a Serobact kit and serogroup agglutinating serum. Penetration of the eggshell and contamination of egg contents was confirmed by the presence of *Salmonella* in the sample and the number of organisms calculated per ml or gram of sample where possible. The $\log_{10}$ increase in the number of organisms was then determined by comparing the population at sampling to the original inoculum. Although it is impossible to know how many cells entered the egg an increase in number over time was taken to indicate growth of the organism.

Each penetration experiment was performed in duplicate for each *Salmonella* isolate.

Serotyping

A single colony from each of a number of plates was tested with a Serobact latex agglutination kit and the appropriate agglutinating serum for that serovar. Representative cultures from positive yolk, albumen, membrane and shell samples for each trial and serovar were prepared as stab cultures in TSA and sent to the Institute for Medical and Veterinary Science (IMVS) in Adelaide for identification. The identification of typical *Salmonella* colonies in our lab was confirmed to the serogroup level by using *Salmonella* agglutinating sera and this identification was confirmed by the IMVS results.

Results

Salmonella *in inoculated faeces*

Faeces inoculated with between 10^7 and 10^8 cfu/g (Day 0) was taken from test eggs, sampled, and the number of organisms estimated. With the exception of SE30, populations of *Salmonella* in faeces remained stable or increased slightly over three days when inoculated and incubated at 4°C. When inoculated and incubated at 25°C, faecal populations of most isolates increased by 1, or occasionally 2

logs over three days. When inoculated at 35°C and incubated at 4°C, populations fluctuated slightly between enumerations at Days 1 and 3 and Days 7 and 14, but remained essentially stable.

Egg penetration

Salmonella was not recovered from the egg contents of any of the control eggs. Contents from four of the 12 control eggs yielded bacterial growth on HEK although not characteristic of *Salmonella*. Heavy growth of orange/salmon pink colonies on HEK plates was considered indicative of coliforms, present in high numbers. The remaining control eggs were free of contamination.

At 25°C, all isolates except SI A15 were able to penetrate to the yolk by Day 1. Of 128 direct plate counts across Trials 1 and 2 for samples of yolk, albumen, inner membrane and inner shell, *Salmonella* was detected in 37 samples. For example SS F1, in Trial 2 at 25°C, reached populations of 2.5×10^3 cfu/g yolk and 5.3×10^3 cfu/g albumen by Day 3. The highest population (6.08×10^3 cfu/g) was found in an inner membrane sample at 25°C after 3 days using SI159, which was originally isolated from yolk. The remaining samples of inner membrane, inner shell, albumen and yolk from this egg were positive by enrichment. All samples were positive for *Salmonella* after enrichment, indicating penetration of the egg.

When eggs were inoculated and incubated at 4°C, all isolates except SE 30 and SI100 were detected in yolk after one day of incubation. SE C6B was detected in high numbers and the remaining isolates in low numbers. At 4°C, SI100 was able to penetrate by Day 3, while SE30 was not detected in any samples, suggesting inability to penetrate the eggshell at this temperature.

After holding at 35°C for 30 minutes and storage at 4°C five of the test isolates, including all SS isolates, SI159 and SE C6B, were able to reach the yolk by Day 1. Two of the three remaining isolates were detected in the inner shell and egg contents after 3 days at 4°C. The ability of organisms to penetrate at 25°C and 35°C differed slightly with respect to time except for SI A15 and SE 30. At Days 7 and 14 under the above conditions *Salmonella* was no longer detected in the yolk and appeared in inner membrane and inner shell samples only. In some cases yolk samples were culture negative after 3 days and all egg parts culture-negative by Day 14. The prolonged storage of eggs at 4°C, although exposed to 10^7-10^8 cfu/g, inhibited the growth and survival of *Salmonella*. It was only at Day 14 during storage at 4°C that yolk and other parts of the egg were culture positive for one isolate, SS262. *Salmonella*-positive cultures at Days 7 and 14 were obtained only after enrichment.

After Day 1 at 4°C culture positive yolk was observed for six of the eight organisms and in some cases at Day 3 the yolk was culture negative while inner membrane and inner shell samples remained positive. Inoculated and incubated at 25°C, *S.*Enteritidis, *S.*Infantis and *S.*Singapore were present in the egg contents, membrane and on the inner shell. The greatest number of positive samples recovered for any one isolate was observed on day 3 at 25°C for *S.*Singapore (faeces). Egg contents, membrane and inner shell were positive for eight out of eight samples and seven of these were observed by direct plating.

Differences between serovars in their ability to penetrate the eggshell were seen with SE30 and SI100. SI100 was slower to penetrate at each temperature and SE30 was unable to penetrate the eggshell at 4°C. Intraserovar differences therefore occurred within SE and SI. SI100 did not penetrate the eggshell as extensively as SI A15 and SI159. All SS isolates, from yolk, feed and faeces, behaved similarly under all conditions. SS262 was slower to penetrate at 35°C and was culture positive only on Day 14 while SI159 was able to penetrate by Day 1 at this temperature. The origin of the *Salmonella* isolates may contribute to their ability or inability to penetrate and survive in egg contents. Penetration of the eggshell by SS F1 and A29 at 4°C was rapid while other serovars and SS262 took much longer or were unable to reach the yolk at all, penetrating only to the membrane at this temperature.

Penetration into different areas of the egg

The numbers of *Salmonella* detected in yolk remained stable or decreased slightly at 25°C while at 4°C, egg yolk samples positive on Day 1 were culture negative by Day 3. At 4°C penetration into the yolk of the egg, detected on Days 1 and 3, was observed for the two yolk isolates, SI159 and SS262. All other isolates were detected in yolk at either Day 1 or 3 but not at both. A greater number of egg parts were culture-positive at 25°C compared with 4°C and 35°C. The counts of SI A15 and SE30 revealed moderate numbers of *Salmonella* in albumen at Day 1 at 25°C, as detected by direct plating, which decreased by Day 3, when growth was obtained only after enrichment. In a number of trials, populations of *Salmonella* found in egg yolk and albumen, membrane and inner shell on one day were not necessarily detected on the next day of sampling.

Reproducibility

The results for Trials 1 and 2 using eight different isolates demonstrated that the majority of tests were reproducible.

Serotyping

All suspect *Salmonella* colonies on HEK plates tested against the appropriate *Salmonella* somatic agglutinating serum were positive for that serogroup. The representative isolates (belonging to all three serovars) sent to the IMVS were not confirmed in each case. A total of five isolates did not match their original identifications. Three *S.*Enteritidis PT4 isolates were phage typed as 9 by the IMVS. One reaction in the phage typing tests did not conform to a PT4 therefore it was designated PT9. The difference between the two phage types is minor and the isolates identified as PT9 are likely the inoculant PT4 isolates. A *S.*Singapore isolate was serotyped as *Salmonella* subsp. 1 serovar rough:*k*:*e,n,x*. While Singapore belongs to serogroup C1, there was a faint reaction only with antibodies to O antigen 6,7 and consequently it could not be typed as *S.*Singapore. It is possible that this *S.*Singapore isolate mutated to a rough form. A second isolate from a Singapore experiment was serotyped as Infantis and on subsequent retyping by the IMVS was confirmed as Infantis.

Discussion

It is generally accepted that *Salmonella* can contaminate avian eggs via transovarian transmission or through the ability of the organism to penetrate the eggshell and grow within the contents of the egg. Under normal conditions of handling and storage few if any of the organisms on the shell multiply because of the low level of available water, and many die due to desiccation if not protected by faecal matter, soil or house litter. Bacteria on the surface can penetrate the shell via water drawn into the pores by capillary action and the bacteria present in the cool external environment are subsequently sucked into the warm egg as it cools. This may occur if the egg is exposed to moist conditions. Such conditions may be encountered naturally, for example when an egg is laid on a moist surface, or occur artificially, for example, when the temperature of wash water is too low. Of course, the egg provides its own defence mechanisms, both physical and chemical. Physically the shell and its membranes provide a barrier to entry of particulate matter, while albumen is chemically and biochemically unsuitable for microbial growth, as it has an alkaline pH and includes several antimicrobial systems such as the enzyme, lysozyme. However, under conditions favourable to microbial growth, such as higher temperatures, the presence of faecal material and high numbers of the organism, bacteria such as *Salmonella* can penetrate the egg shell and grow within the egg contents (Sauter & Petersen, 1974; Stephenson *et al.*, 1991; Schoeni *et al.*, 1995).

A number of organisms including *Pseudomonas fluorescens* and *Salmonella* serovars such as *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg have been shown to penetrate the egg shell (Hartung & Stadelman, 1963; Schoeni *et al.*, 1995). In the case of the salmonellae, penetration occurred in eggs

inoculated and stored at 4°C and growth occurred sporadically. Isolates of serovars Infantis and Singapore, prevalent in the Queensland layer industry, were investigated in order to assess their ability to penetrate the shell of the egg, and grow and contaminate the egg contents. Isolates of *S.* Enteritidis were included for comparison. The three serovars were inoculated onto the surface of the egg in a medium of sterile faeces. The penetration of eggs by these organisms was investigated using a modification of the method described by Williams and Whittemore (1967).

Seven of the eight isolates, representing the three serovars, were able to penetrate the eggshell and reach the yolk by Day 1 at 25°C. Similarly, most isolates were able to penetrate at 4°C by Day 3 and at 35°C by Day 1. There are few penetration studies with which to compare these results. Schoeni *et al.* (1995) found similar results with Typhimurium, Enteritidis and Heidelberg at an inoculum of 10^6 cfu/g faeces. In the present study, the movement of five out of eight isolates so rapidly into the yolk of the egg at each temperature by Day 1 could be a consequence of the extremely high inoculum, 10^8 cfu/g faeces, coupled with the fact that the inoculum was carried in faeces. Faecal extract has the potential to promote the growth of contaminants (microbes) in the shell membrane and is an environment conducive to the growth of *Salmonella* (Clay & Board, 1991). It has been shown that if the shell is contaminated with faeces and wetted, as in this study (faecal extract is wetted by the addition of the inoculum in peptone water) the faeces may well be drawn through the shell and nutrients deposited on the shell membrane. Such a circumstance may lead to extensive microbial contamination of the egg contents (Clay & Board, 1992). Iron has been cited as an element in faeces that enhances the establishment of contamination in the egg (Bruce & Drysdale, 1991). It has also been demonstrated that when eggs are stored in the oviposition (air sac incubated upwards), as in the current study, the yolk is more dense than the albumen. However the densities of the albumen and yolk are reversed as a consequence of the decay of the internal egg structure with storage (Clay & Board, 1991). The distance for contaminants to travel from the site of contamination to the surface of the yolk therefore progressively diminishes (Clay & Board, 1992). Although the albumen of the egg may be inhibitory toward microbes, the movement of Enteritidis cells from albumen to the yolk has been shown to occur within two days, using an inoculum of only 50 cells/ml of albumen and at a temperature of 8°C (Braun & Fehlhaber, 1995). The yolk provides a nutrient-rich environment for bacterial growth to occur.

At 25°C penetration of the shell occurred more rapidly. The majority of yolk, albumen, inner membrane and inner shell samples at this temperature were culture-positive for *Salmonella* and most of these were determined through direct plating rather than enrichment. By Day 3, all sampling sites within the egg were culture-positive, which was not observed at 4°C or 35°C. The observation that yolk samples were positive for particular isolates and temperatures and not for any other site on a particular day suggests the rapid movement of a few cells through the shell, the membranes and albumen to the yolk. Lack of aseptic technique when the egg was cracked, and the contents separated, may have given rise to a false positive yolk sample. However the high incidence of eggs demonstrating *Salmonella* solely in the yolk or solely in another part of the egg suggests that false positives did not occur. The variation in yolk-positive samples across all experimental conditions suggests that differences exist between serovars in their ability to penetrate and move within the egg.

Storage at 4°C for greater than three days prevented the growth of *Salmonella* in faeces and in egg contents and by Day 7 and Day 14 numbers had clearly declined. Under simulated hatchery conditions, four isolates of *Salmonella* were able to penetrate and were detected in the yolk of the egg by Day 1. These isolates include SE C6B, SI159, SS A29 and SS F1. The *S.*Singapore isolates were detected in every site within the egg and not solely in the yolk. The remaining isolates were slower to penetrate at 4°C reaching the yolk by Day 3 or Day 7 at the latest. Once the organism was cultured from the yolk, storage at 4°C prevented its growth and the organism was not detected at this site by culture again. The recovery of these organisms after this period was from the inner shell or inner membrane. However cells viable at 4°C and then stored at temperatures conducive to growth are able to contaminate yolk and albumen following the same trend as that of inoculated eggs stored at 25°C (Clay & Board, 1991).

At 25°C the numbers of salmonellae present in faecal material on the egg increased by 1 to 2 logs and for SE C6B at an initial inoculum size of 10^4 cfu/g faeces, the increase was greater than 7 logs. The increase at the contamination site may represent a constant challenge to antimicrobial systems (physical, chemical and/or biological) of the egg and may explain variation in cultural results between sampling events. Future work should involve removal of the faeces after a certain time thereby allowing observation of only those cells able to penetrate at a set time. In the current study contamination of the egg contents may at times have involved new cells penetrating the shell. The lower inoculum size in the first trial may have permitted microbial growth while the higher inoculum of 10^8 cfu/g may have rapidly reached the stationary phase of growth. Under storage at 4°C the increase in numbers in faeces was only slight at Day 1 and at Day 3. Our results show that by Day 7 the number of cells in faeces decreased, the decrease being mostly slight or occasionally as high as 1 or 2 logs. For SI159 the number in faeces remained the same over 14 days of storage.

Intraserovar differences in the ability to penetrate shell eggs were observed in this study. SE C6B penetrated the eggshell far more easily than SE30. SE30 was unable to penetrate at 4°C and at 35°C penetrated only to the inner membrane by Day 3. SE30 has been shown to be avirulent in chicks and subsequently demonstrated the poorest ability to penetrate. In comparison, C6B penetrated at all temperatures and was detected in the yolk of the egg. C6B is extremely invasive in layers and causes significant mortality in day-old chicks. Thus there may be properties of salmonellae in common to the two processes of egg penetration and host invasion. In both trials SI A15 and SI159 penetrated the egg and yolk at 4°C and 25°C more rapidly than SI100, suggesting that an SI isolate originally isolated from yolk or faeces may have an increased ability to penetrate.

There were only slight differences in the ability of all three isolates of Singapore to penetrate eggs. SS262 was not isolated from yolk at 4°C until Day 14 compared with Day 1 for SS A29 and SS F1. One explanation for this was that the quality of the eggs used for SS (yolk) at this temperature may have had a greater resistance to penetration compared with those eggs used for the other two isolates. It is possible that the integrity of defence mechanisms varies from egg to egg. This may be dependent on the age of the bird, which could give rise to a weaker shell, and external influences such as handling of the egg. At 25°C, SS A29 was present in the four sampled areas of the egg at Day 3 compared with Day 1 for SS262 and SS F1. Overall, no single isolate of Singapore demonstrated a greater ability to penetrate the eggshell. SS isolates performed better than SI isolates and SE isolates. Thus serovar Singapore is of concern to public health due not only to prevalence of the serovar on layer farms and in egg pulp but the ability of the serovar to penetrate the eggshell. In fact the prevalence of Singapore may be a consequence of eggshell penetration and growth within the egg contents. Similar studies have demonstrated the ability of Enteritidis to penetrate and survive within egg yolk at 4°C with bacteriostatic substances in the albumen unable to destroy Enteritidis at a population of 1.4×10^4 cells/ml (Braun & Fehlhaber, 1995).

Variation among SS isolates coupled with differences in egg quality may have accounted for differences within and between trials. On many occasions results for particular isolates under certain conditions were reproducible, although differences were clearly seen between individual trials for single isolates. The lack of consistency between individual trials may be attributable to variation in the physicochemical properties of eggs. Factors such as specific gravity, the presence or absence of a cuticle and the shell rarely containing only one type of morphological variation at the level of the mamillary layer will all affect the resistance of an egg to bacterial penetration (Board & Halls, 1973; Sauter & Petersen, 1974; Nascimento *et al.*, 1992). Uniformity of shell structure and function does not exist and the inherent defence mechanisms of the shell can alter by the time an egg arrives in the hands of the consumer (Nascimento *et al.*, 1992). Egg quality is an important factor and may affect the ability of an organism to penetrate the eggshell (Sauter & Petersen, 1974). Although clean, uncracked, fresh eggs were used in this study, shell quality may differ with each egg.

Differences in the serotyping results for five isolates may relate to serovar conversion. It has been shown that the transfer of a specific plasmid into a smooth isolate of SE PT4 will produce a smooth isolate of SE PT23 and transfer into a isolate of SE PT8 will result in the formation of SE PT24 (Threlfall *et al.*, 1993a). Plasmid incompatibility studies have also shown isolates of *S.*Enteritidis belonging to PT1 and PT4 can become PT6a, and PT8 can become PT13 (Ridley *et al.*, 1996). Although there is no direct evidence implicating the conversion of PT4 to PT9 in our situation, it is likely that this conversion is possible through the loss or acquisition of a specific characteristic or plasmid. An example of one such loss is lack of Phase 2 flagellar antigen expression, believed to correlate with the emergence of a new variant *Salmonella* serovar 9,12:ll,v:- (Burnens *et al.*, 1996). Similarly the transition of Singapore from a smooth to rough isolate resulting in a different identification may occur, brought about by unidentified factors. These isolates may cause confusion in serotyping and unless the possibility of such occurrences is realised, epidemiological connections between isolates may not be recognised.

The findings of this study indicate the ease with which three serovars were able to penetrate the eggshell and remain or grow (in some cases in high numbers) in egg contents. The data suggests there may be a need to sanitise eggs immediately after they are laid and for the consumer to maintain good food handling practices. Fast penetration rates and high percentages of culture positive yolk at 25°C suggests that conditions under which eggs are stored should be strictly maintained. Faecal contamination of the egg enhances penetration therefore faecal material must be removed from eggs to decrease the potential for penetration by *Salmonella* into egg contents particularly by specific serovars which may be prevalent in the poultry environment (eg. *S.*Singapore which is prevalent in layer flocks and egg pulp in south-east Queensland).

Experimental Section 4

Infectivity of *Salmonella* Infantis in commercial layer hens

Objectives

As isolates of *Salmonella* Infantis were found in egg yolk during the survey of raw liquid egg products (Experimental Section 1) and also found to penetrate intact shell eggs (Section 3), the aim of this study was to determine the infectivity of isolate SI159 in layer hens. More specifically, the aims were to examine the ability of the bacterium to infect layers, and monitor localisation within internal organs, faecal shedding, and egg contamination.

Methodology

Preparation of S.*Infantis culture*

The isolate used in this experiment, *S.*Infantis SI159, was originally isolated from unpasteurised egg yolk in July, 1994. The isolate was maintained on a Tryptone Soy Agar (TSA) slope at 4°C. A loopful of growth was emulsified into 10 ml Tryptone Soy Broth (TSB) and incubated for 18 h at 37°C. After incubation, the broth was centrifuged for 20 min at 2500 *g*. The supernatant was carefully discarded and the pellet resuspended in 0.5 ml of sterile TSB, to a cell concentration of approximately 10^{10} cfu/ml. Plate counts were performed by serial dilution in 0.1% peptone water and plating onto HEK.

Layer hens

Thirty CSIRO Cross Breed (CB; New Hampshire x Australorp) laying hens, representative of the major layer hen used in Queensland, were obtained locally from a commercial source. The birds had been vaccinated against Marek's disease as day-old chicks and were free of any apparent disease throughout the growing and laying periods. Two separate groups of birds were used. In Experiment 1, 20 birds were purchased at 21 weeks of age. They were monitored for carriage of *Salmonella* for ten weeks prior to inoculation to ensure all birds were *Salmonella*-free, in good health, and to allow all birds to reach point-of-lay and to increase their daily mean egg production. In Experiment 2, ten birds were purchased at 28 weeks of age and were already at point-of-lay. The birds were monitored in the same way for five weeks before infection.

Housing

All birds were housed in single-bird wire bottom cages in a clean, disease-free room. Feed and water were provided *ad libitum*. The pelleted feed was essentially a commercial product although it was formulated without any antibiotics or growth promotants. Eggs were collected from the egg rollout and in this way remained clean or free of faecal material on the surface of the shell. Faecal droppings were collected onto trays covered with aluminium foil positioned directly underneath each cage. Once a faecal sample was collected the foil was discarded and replaced with a new piece. Birds were exposed to 14 h of light per day and the temperature of the room kept constant at 22°C.

Experiment 1

After monitoring for 10 weeks, 17 of 20 birds were inoculated orally with 100 µl of a suspension containing 10^9 cfu of serovar Infantis isolate SI159. Control birds were inoculated with sterile distilled water. Feed and water were not withheld from the birds prior to inoculation. An automatic pipetter

and long sterile tip were used to administer the dose into the crop. Control birds were housed under the same conditions and in the same room as the inoculated birds, but in separate cages. Eggs were collected daily and faeces were sampled every two to three days post-inoculation. Birds were monitored for clinical signs of disease and decreased egg production.

Experiment 2

After monitoring for 5 weeks, 10 birds were inoculated with SI159 as described previously. Eggs and faeces were collected every day post-inoculation until Day 10 when all birds were killed. Egg production and clinical signs of disease were monitored over this time.

Bacteriological examination

Culture of samples for *Salmonella*

For Experiment 1, overnight enrichment of samples was performed, as required, using 10 ml Rappaport Vassiliadis Broth (RVB) at 42°C. For Experiment 2, 10 ml Tetrathionate Brilliant Green Broth (TBGB) incubated overnight at 37°C, then at 22°C for at least 24 h, was used as the selective enrichment instead of RVB (Bichler *et al.*, 1996). Salmonellae possess the enzyme tetrathionate reductase and thus are able to grow in the medium. *Proteus* spp. also possess this enzyme and there may be overgrowth of this organism. However the presence of the dye brilliant green improves selectivity and suppresses the growth of *Proteus*. Unless otherwise specified, each enrichment broth was streak-plated to Lysine-Mannitol-Glycerol agar (LMG; Cox, 1993) and Brilliant Green agar (Oxoid), which were incubated overnight at 37°C. Each selected presumptive *Salmonella* colony was confirmed serologically using a Serobact *Salmonella* latex agglutination kit (MedVet Science, Adelaide) and/or serogroup C1 agglutinating serum. If positive, the purity of each colony was confirmed on TSA plates, then isolates were grown on TSA slopes, which were stored at 4°C until sent to the IMVS for final confirmation by serotyping.

Faeces

For both experiments, faeces (approximately 2 g) were collected with a sterile spoon and cultured as outlined above. For Experiment 2, a loopful of faeces was streaked directly onto the two plate media.

Eggs

Eggs were collected daily into sterile bags with a change of gloves between the collection of each egg. The eggs were kept at room temperature for four days before sampling. The surface of the egg shell was dipped into a 2% tincture of iodine for 1 min, removed and allowed to air dry to prevent contamination of egg contents by *Salmonella* on the shell (Gast & Beard, 1990a). The egg was cracked open on the edge of a sterile beaker. For the first week of sampling the yolk and albumen were sampled separately; after this time the yolk and albumen were not separated and were sampled together. In the first instance, 3 ml of yolk and 3 ml of albumen were extracted using a sterile 3ml syringe and placed separately into 5ml free standing vials (sterile) and stored at -20°C. If the yolk or the albumen was culture positive for *Salmonella* the frozen samples were thawed at 4°C then diluted in 0.1% peptone and streaked onto HEK plates. *Salmonella* present in the sample were enumerated by counting the number of colonies for each dilution. After the first week of sampling, the egg contents of two eggs were placed into a sterile bag, TSB was added 1:4 w/v, agitated and the mixture incubated at 37°C for 18 h. An aliquot (100 µl) of this overnight broth was enriched in RVB, plated, and suspect colonies confirmed, purified and stored as described above.

For Experiment 2, eggs were collected as in Experiment 1, but the yolk, albumen, eggshell and eggshell surface were examined individually for each egg. To each sterile bag containing one egg, 50 ml of TBGB was added and the eggshell massaged gently for 3 min. The egg was removed and placed into 2% iodine for one minute and allowed to air dry. While maintaining aseptic conditions, the eggshell was cracked and the yolk and albumen separated into sterile bags. The eggshell was crushed gently in another sterile bag. To each bag 50 ml of TBGB was added and the bag incubated overnight at 37°C. Each sample was streaked onto LMG and BG agar. All samples were further incubated at

22°C for 10 days, then streaked onto LMG and BG plates. After incubation overnight at 37°C, typical *Salmonella* colonies were confirmed serologically, purified and stored, as described above.

Internal organs
In Experiment 1 birds were sacrificed at 5, 9, 10, 21 and 39 days post-inoculation. Birds were euthanased by injecting into a wing vein 1.5 ml of Lethabarb (Virbac, Australia). Organs were removed aseptically. Complete organs including the liver, spleen, caeca, and oviduct were taken and a part of the ovary and one pre-ovulatory follicle were removed. Organs were placed into sterile bags and 20 ml of RVB added. Culture and confirmation after RVB enrichment were performed as described above. Experiment 2 differed only in the use of TBGB enrichment, plating after overnight incubation 37°C, and after incubation for 10 days at 22°C.

Feed
A 25 g sample of the pelleted feed was placed into a sterile bag and pre-enriched overnight in Buffered Peptone Water (Merck). RVB (10 ml) was inocuated with 0.1 ml of preenrichment, then incubated overnight at 42°C. After incubation, RVB was streaked onto LMG and BG. Presumptive *Salmonella* colonies were confirmed as described previously.

Statistical Analysis

The Chi-square test and Fisher's Exact test were used to compare the distribution of infected and non-infected organs in Experiments 1 and 2 (Hogg & Tanis, 1977). The two-sample *t* test, testing for the difference between two means was used to compare differences in egg production for control hens and inoculated hens (Hogg & Tanis, 1977)**.**

Results

Experiment 1

Faecal carriage of *Salmonella*

Prior to inoculation birds were monitored for carriage of *Salmonella* for ten weeks. *Proteus* spp. was isolated from the faeces of nine birds and *Salmonella* from four birds on Day 7 of sampling. *Salmonella* from these birds were tested serologically against C1 *Salmonella* somatic agglutinating serum. The isolates did not belong to group C1, to which *S.*Infantis belongs. These isolates were not identified to the serovar level. Faecal shedding of *Proteus spp.* in nine birds persisted for five weeks and one other bird shed *Salmonella* for five weeks.

Post-inoculation (PI), *S.*Infantis was recovered from the faeces of 93% of inoculated birds at 6d PI, while at 17d PI, 40% were positive for *S.*Infantis, and after 38d PI only one bird (20%) was positive. Faecal samples from control hens were culture negative for *Salmonella.* Culture of faeces from hens in this experiment indicated intestinal colonisation following oral inoculation of hens with *S.*Infantis SI159. Recovery of SI159 from faeces declined significantly over three weeks. At 10d PI and onwards, shedding of *Salmonella* was sporadic and intermittent in most hens. Only one hen consistently shed *Salmonella* up to 22d PI.

Recovery of S.Infantis from eggs

Pooled contents from two eggs were sampled for each bird after storage at room temperature for four days. Yolk and albumen were separated and tested up to 3d PI and after consistent culture-negative results, pooling of yolk and albumen was instituted to reduce labour. *Salmonella* was not isolated from any of 200 eggs chosen from 836 laid in the ten weeks prior to inoculation. *S.*Infantis was not isolated from the contents of any of the 474 eggs produced by infected hens up to 39d PI. All eggs laid by control hens during the test period of six weeks were culture-negative for *Salmonella*.

Egg production

Mean egg production for both *S.*Infantis-inoculated and uninoculated control hens was 0.88 eggs/hen/day. Prior to inoculation the mean egg production increased weekly from 0.36 over the first three weeks, to 0.88 eggs/hen/day during Week 10. Most birds at 21 weeks of age had reached point-of-lay and transfer to a new environment, new housing and feed may have caused some birds to stop laying. At 31 weeks (Week 10) the birds had reached a mean egg production level satisfactory for regular sampling.

Mean egg production in control hens over the course of the six weeks post-inoculation was slightly higher than that for inoculated hens over the same period of time' although the difference was not statistically significant ($P<0.001$), suggesting egg production was not depressed due to *S.*Infantis infection. There was no difference in total mean egg production observed with the infected group between Week 1 PI and Week 6 PI. Similarly over the 6-week study period, post-inoculation egg production for control birds was maintained at the same level.

Clinical response to S.*Infantis 159*

There was no sign or evidence of clinical disease in birds inoculated with *S.*Infantis. Some of the infected birds suffered mild diarrhoea in the few days immediately following oral challenge. Control birds remained in good health and horizontal transmission by aerosol contamination from inoculated birds to uninoculated birds did not occur. Post mortem revealed no signs normally associated with invasive *Salmonella* infection such as peritonitis or caseous granulomas. All birds appeared normal clinically and organs were lesion free. The ovary of each chicken also appeared normal and healthy (as confirmed by Hasan Nili, Veterinary Pathology Department, University of Queensland).

Isolation of S.*Infantis from the internal organs of infected hens*

Birds were euthanased in sets of five inoculated hens plus one control hen at 10, 21 and 39d PI. Two remaining hens orally challenged with 10^9 cfu of *S.*Infantis 159 were euthanased at 5 and 9d PI. Internal organs from control hens killed on the same days were culture negative for *Salmonella.* Hens inoculated with *S.*Infantis and sacrificed at 5, 9, 10 and 21d PI had culture-positive ovaries, liver, spleen and caeca. Overall, 12% had a culture-positive liver, spleen and caeca, 12% a culture-positive spleen and caeca, 12% a culture-positive caeca only and 6% a culture-positive ovary only. In 35% of hens, *Salmonella* was recovered from at least one organ throughout the PI test period. Internal organs in 65% of the infected hens were culture-negative.

At 5 and 10d PI, 50% of organs were culture-positive and by 39d PI, *Salmonella* was not found in any organ. A culture-positive ovary was recovered from one bird at 9d PI while all other organs were culture negative. The caecum was the most commonly infected organ with 30% of caeca culture-positive followed by the spleen (18%) and the liver (12%). The oviduct and the pre-ovulatory follicle were culture-negative for all birds. *S.*Infantis was recovered from 43% of spleens, 29% of livers and 57% of caecal samples from inoculated hens killed within the first 10d PI, while after 10d PI, *S.*Infantis was detected at low frequency, from only one organ.

Experiment 2

Based on the findings of Experiment 1 it was decided to repeat the initial experiment with a smaller number of birds with the same inoculum size and route of infection. Hens were killed at 10 days post-inoculation to confirm the recovery of *S.*Infantis from the majority of organs including the ovary. Conditions were maintained throughout as in Experiment 1 except for the age of the birds, purchased at 28 weeks rather than 21, and the enrichment broth RVB was replaced with tetrathionate-brilliant green broth.

Faecal carriage of *Salmonella*

During the five-week monitoring period prior to inoculation, all ten birds were positive on every sampling day for the carriage of organisms other than *Salmonella* that are capable of growing on enteric media. The high frequency (111/120 or 93% of samples) of isolation of these non-salmonellae

was not seen in Experiment 1, suggesting this second group of birds may have had exposure to a wider range of bacteria encountered in their previous environment. Eggshell samples and eggshell washings from eggs laid by these birds were culture-positive for the same types of enteric bacteria. The birds were internally colonised with other enteric organisms, but were free of *Salmonella* prior to oral challenge.

Faeces for each bird was cultured daily for nine days post-inoculation. *S.*Infantis was shed in faeces by 70% of hens on each of the nine days and the remaining hens shed *Salmonella* for eight out the nine days with *Salmonella*-negative cultures occurring on either Day 8 or 9. By comparison, in Experiment 1, 93% of birds were shedding *Salmonella* in their faeces at Day 6 PI, which dropped to 60% by Day 10. In Experiment 2, the rate of recovery of *Salmonella* remained at almost 100% for 9 days PI. Enteric organisms recovered from faeces prior to inoculation were also isolated from Day 1 to Day 9 PI.

Recovery of S.Infantis from eggs

Egg contents, eggshell and the outer eggshell surface were sampled individually by direct plating and by enrichment. *Salmonella* was not isolated from any of the eggs tested. However, 3% of eggs had culture-positive contents for enteric organisms other than *Salmonella*. Of the eggshell surfaces, 41% were culture-positive and 10% of eggshells were culture-positive for enteric bacteria other than *Salmonella*. Post-inoculation, *S.*Infantis was isolated at very low frequency, from five eggshell surfaces. Four egg surfaces were positive for *Salmonella* on Day 1 PI and the fifth egg surface was culture-positive on Day 5 PI. Of the five positive cultures, three were positive after incubation at 37°C for 1 day and the other two were positive after incubation for 10 days at room temperature. A different bird laid each egg yielding a culture-positive shell surface.

*S.*Infantis was not isolated from yolk, albumen or shell for 86 eggs collected over the nine days PI. Enteric bacteria other than *Salmonella* were isolated after enrichment for 10 days at 22°C from 52 of 86 (60%) yolk samples. Albumen and whole eggshell samples were culture-negative for other enteric organisms. However four eggshell washings from three different birds were culture positive for these organisms.

Egg production

Ten cross breed laying hens were purchased at 28 weeks of age, older than in Experiment 1, in order to more quickly obtain a higher mean egg production. The mean egg production increased from 0.6 eggs/hen/day in Week 1 to 0.96 in Week 5, prior to inoculation. The egg production for this second group of birds was slightly higher than for birds in Experiment 1. Mean egg production for Infantis-inoculated birds and the control bird was 0.98. Over the course of the post-inoculation period egg production for all ten birds did not significantly change. On Day 1 PI, ten eggs were laid and this dropped to eight eggs on one occasion at Day 9 PI. Using the two-sample *t* test, testing for difference between two means the result was not significant ($P<0.001$).

Clinical response to S.Infantis 159

In Experiment 2 there were no signs or evidence of clinical disease in birds inoculated with *S.*Infantis 159. There were no signs of depression and egg production was not reduced. Mild diarrhoea was observed in two birds over the 9 days PI. Overall the birds appeared healthy.

Isolation of S.*Infantis from internal organs of infected hens*

Post-mortem results revealed normal internal organs with no visible lesions. The ten layer chickens were euthanased at 10 days PI. In that time 97% of faecal samples were culture-positive for *Salmonella* although *Salmonella* was not isolated from egg contents or eggshell. Infected hens yielded culture-positive liver, spleen, caeca, ovaries, oviducts and pre-ovulatory follicles. *Salmonella* was recovered from all spleens and caeca, from 70% of livers and ovaries, 50% of oviducts and 20% of

pre-ovulatory follicles. All birds had at least two organs infected with *Salmonella* and one bird had a complete set of culture-positive organs.

Significant differences were observed between Experiment 1 and 2 but there were some similarities. The highest rates of recovery of *Salmonella* were from the spleen and the caecum in both experiments with slightly lower recovery rates from the liver and ovarian tissues. *S.*Infantis was not recovered from the oviducts or pre-ovulatory follicles in Experiment 1 while low rates of isolation from these organs were observed in Experiment 2.

All birds in Experiment 2 were killed at ten days PI compared to five birds in Experiment 1. In Experiment 1, 20% of organ samples were culture positive for *Salmonella* compared to 68% in Experiment 2. The low incidence of culture-positive organs in Experiment 1 was attributable to the majority of reproductive organs (ovary, oviduct and pre-ovulatory follicle) being culture-negative.

Salmonella was not recovered from egg contents in either experiment. *S.*Infantis was isolated from ovaries, oviducts and from the yolk of pre-ovulatory follicles but not from eggs removed from the oviduct. Organs were plated directly and enriched by incubating at 37°C overnight and at room temperature for a further 24h. A significant proportion (20%) of culture-positive organs derived from direct plate counts, although populations were low, with less than ten colonies on any one plate.

Enteric bacteria other than *Salmonella* were isolated from organs post-inoculation. These bacteria were isolated from 48% of organs including those that were *Salmonella*-positive. It is interesting to note that 60% of egg yolks PI were culture-positive for enteric bacteria suggesting that these organisms were able either to penetrate the egg shell or be transmitted via the ovary to the egg yolk.

The distribution of infected and non-infected organs in Experiments 1 and 2 were compared. The Chi-square value was 11.94 with one degree of freedom and the P value was 0.0005, which is considered extremely significant. The Fisher's Exact test gives a more accurate result, the P value of 0.0004 indicating that there is a significant difference between the number of infected and non-infected organs in Experiments 1 and 2.

Serotyping

Colonies considered to represent *Salmonella* were tested against C1 *Salmonella* somatic agglutinating serum. Culture-positive samples gave positive agglutination reactions with the Serobact latex agglutination kit for *Salmonella* and with the C1 agglutinating serum. A total of 43 isolates from faeces, organs and egg shell (pre and post-inoculation) were sent to the Institute for Medical and Veterinary Science. All 43 isolates were typed as *Salmonella* Infantis.

Feed

Feed samples taken in Experiment 1 and 2 prior to inoculation were culture negative for *Salmonella*.

Control birds

Inoculated and uninoculated birds were housed in the same room. *Salmonella* Infantis was isolated from the faeces, eggshell surface and from the caecum and ovary of the control bird in Experiment 2. The control bird was housed adjacent to an inoculated bird.

Discussion

Due to the public health significance of egg-associated salmonellosis involving *Salmonella* Enteritidis, significant attention has been paid to the mode by which this bacterium contaminates eggs. A number of studies suggest eggs become contaminated via transovarian infection (rather than via eggshell penetration) and that recovery of the organism from internal organs can be used to indicate that the isolate has enhanced invasiveness, capable of causing transovarian production of

contaminated eggs (Gast & Beard, 1990a; Gast & Beard, 1990b). Other studies suggest that contamination of egg contents derives from contamination of the eggshell during passage through the cloaca, which is more likely to occur than transovarian transmission (Barrow & Lovell, 1991; Bichler *et al.*, 1996). Little is known regarding egg contamination by other serovars of industry relevance and public health significance, including Infantis.

*S.*Infantis has been frequently isolated from raw egg pulp and occasionally from yolk in Queensland (Cox *et al.*, unpublished data). As has been demonstrated for SE, possible routes of egg contamination by SI include transovarian transmission and eggshell penetration. Thus, the present study involved experimental infection of commercial laying hens to investigate whether contaminated eggs could be produced after oral inoculation. Birds were challenged with a high dose of *S.*Infantis 159, an isolate that had been recovered from yolk. A study of individual epidemics and hospitalisation rates suggested that there was a dose-severity relationship for *S.*Infantis in humans (Glynn & Bradley, 1992) and so a high dose was used for this study.

CSIRO Cross Breed (CB) birds represent the major type of layer hen in the Queensland poultry industry. Interestingly, this breed was found to be more resistant to fulminant infection by *Salmonella* Enteritidis than other commercial layer breeds (Cox *et al.*, unpublished). This may mean that other *Salmonella* serovars are better able to colonise layer breeds other than CB, although other serovars may not colonise layers systemically in the same way in which isolates of SE are able.

Prior to inoculation the birds were not starved for 24 h as recommended by some researchers, this decision based on a study which found that starvation had no impact on the invasion of tissues by salmonellae compared with fed birds (Humphrey *et al.*, 1993).

*S.*Infantis was not isolated from contents, including separate yolk and albumen samples, of eggs produced by infected hens in either experiment. Gast and Beard (1990a) demonstrated that yolk samples free of yolk membrane and albumen from 423 eggs were free of *S.*Enteritidis (SE) after inoculation. In the present study *S.*Infantis was isolated from the eggshell washings of five eggs laid by five different birds in Experiment 2. It is most likely that as the eggs passed through the cloaca of the chicken faecal material attached to the egg. There was no recovery of *Salmonella* taken from surface-sterilised whole eggshells of all eggs laid PI. Many researchers have reported the production of *S.*Enteritidis-contaminated egg contents after experimental infection of layers with high doses of that serovar (Gast, 1993; Keller *et al.*, 1995; Bichler *et al.*, 1996) although relatively low frequencies of contamination were found in eggs laid shortly after inoculation. Gast and Beard (1992a) found 3% of freshly laid eggs contaminated with SE PT13a after oral inoculation with 10^9 cells. High rates of SE-positive eggs have been reported in other experimental infections. Keller *et al.* (1995) and Bichler *et al.* (1996) reported recovery rates of 63.9% and 29.5% respectively from eggs laid within one week PI and from forming eggs taken from the oviduct. The absence of *S.*Infantis-contaminated eggs observed in this study clearly demonstrates that SI159, or perhaps serovar Infantis in general, differs from SE in its ability to contaminate eggs via infected ovaries. The invasive nature of SE PT4 and PT8 has been shown previously. Timoney *et al.* (1989) showed that a particular isolate of *S.*Enteritidis was very invasive for the adult hen causing bacteraemia with infection of many body sites including the peritoneum, ovules and oviduct. *S.*Enteritidis can also colonise pre-ovulatory follicles by interacting with ovarian granulosa cells, a precursor to transovarian transmission (Thiagarajan *et al.*, 1994). *S.*Infantis 159, used in this study, was able to infect many body sites including the pre-ovulatory follicle, suggesting this isolate is invasive. A general mechanism of resistance may apply to all serovars of *Salmonella* in chickens. Several lines of chicken have been shown to be resistant to several serovars of *Salmonella* including *S.* Enteritidis (Bumstead & Barrow, 1993). The line of bird used in this study may provide resistance to *S.*Infantis; day-old chicks of the CB isolate were found to be more resistant to infection with SE than chicks of two other commercial layer isolates (Cox *et al.*, unpublished).

An *in vitro* model for studying bacterial interactions in the avian caecum found the growth of caecal bacteria from a healthy adult chicken was in fact inhibitory to SI (Nuotio & Mead, 1993). Birds in the

current study were presumably in good health, and caecal bacteria may have prevented effective colonisation by SI159. The colonisation of one hen in Experiment 1 may have been similarly influenced by the existence of a non-Infantis *Salmonella* in that hen.

In Experiments 1 and 2 there was continual shedding of *S.*Infantis in the faeces of most birds at 10 days PI. After that recovery of SI from faeces was intermittent for each bird, although one layer hen continually shed the organism for 22 days PI. SI159 appeared to have colonised part or the whole of the alimentary tract, the organism shed in faeces for up to 38 days PI. This has also been demonstrated in previous work where high excretion rates of SI were observed for several weeks after inoculation with approximately 10^9 cfu/ml (Barrow *et al.*, 1988). Lack of excretion after inoculation with a mutant (non-motile, non-haemagglutinating) isolate suggested that an unidentified microbial factor is essential for colonisation (Barrow *et al.*, 1988). Future work could involve preparation of SI159 mutants to observe different factors that may be associated with colonisation.

Colonisation of the caecum requires the physical attachment of *Salmonella* to the epithelium. It has been suggested that Type 1 pili or fimbriae promote colonisation of the murine intestine by *S.* Typhimurium and were subsequently investigated for their role in attachment in layer hens (Barrow *et al.*, 1988). It was found that none of flagellar antigens, somatic antigens, mannose-sensitive haemaglutinins nor the possession of a virulence plasmid were essential for colonisation. Based on these results adhesion to the epithelium of the caecum by SI needs to be investigated.

There were slight differences in the recovery rates of *S.*Infantis from internal organs in Experiments 1 and 2. At 10d PI there appeared to be a peak in the number and type of organs that were infected. At 39d PI no internal organs were culture-positive for *Salmonella*, although caeca remained colonised. Caeca are external organs, open to environmental contamination; so this result is hardly surprising. In Experiment 1 only one bird had a culture-positive ovary at 9d PI, while in Experiment 2, 70% or seven birds had infected ovaries. It has been shown previously that infection of the ovary consistently occurs at approximately 10d PI. Contaminated eggs are also usually produced at the same time. There may be a generalised mechanism of infection with *Salmonella* leading to this phenomenon. *S.*Infantis was able to infect the ovaries as well as the oviduct and pre-ovulatory follicle in lower frequencies. Although a high percentage of the reproductive organs were culture-positive for *Salmonella* no contaminated eggs were produced. Barrow and Lovell (1991) found similar results, where organs such as the oviduct and ovary were culture-positive for SE, while eggs removed from the oviduct were culture-negative. It may be that eggs are in fact contaminated during production, but low numbers of SI may not survive after lay.

The patterns of isolation of SI observed in this study are similar to those seen in experimental infection with SE. In the latter case high recovery rates from organs occur in the first week post-inoculation and then decline steadily in the following weeks while infection of the ovary in some hens persisted for several weeks (Barrow & Lovell, 1991). Specific organs are more frequently and more easily colonised than other organs. Patterns of organ colonisation in this study demonstrate that the spleen and caecum were more rapidly infected followed by the liver and ovary, oviduct and pre-ovulatory follicle. Keller et al. (1995) observed a 100% recovery rate from the ovary and intestinal pools within 2 days PI. High rates of SE isolations from liver (51%), spleen (47%) and caeca (58%) and lower rates from ovaries (17%) and oviduct (17%) were reported by Gast and Beard (1990) with *S.*Enteritidis persisting in all internal organs at low frequency for as long as 22 weeks after exposure. Following oral inoculation of 10^6 cells of *S.* Enteritidis, Timoney *et al.* (1989) isolated *S.*Enteritidis from the caeca, peritoneum and liver of some birds for up to 42 days PI.

In the present study, slight differences in recovery rates between the two experiments were observed. While such differences may be attributed to the inherent variability in animal experimentation, changes in cultural technique may have also contributed. Tetrathionate-brilliant green broth was used in Experiment 2 and this may have been more sensitive in detecting small numbers of *Salmonella* than RVB in Experiment 1.

After orally inoculating the birds with 10^9 cells of *S.*Infantis they were monitored for changes in egg production as well as for any clinical signs of disease or changes in their health in general. There was no reduction in the number of eggs produced by either group prior to or after inoculation. In Experiment 1 the mean egg production in the week before inoculation was the same as at 6 weeks PI. Like other non-typhoid salmonellae, infection with SE has not been associated with severe clinical responses in mature chickens. Timoney *et al.* (1989) found that SE infection of hens produced only minimal clinical effects and, although ovules and oviducts were infected total egg production was not affected. Likewise Bichler *et al.* (1996) observed no significant differences between the mean egg production for inoculated and uninoculated hens when SE was administered at a dose of 10^{10} cfu. Oral inoculation of up to 10^6 cells of SE PT4 produced no signs of illness or lesions (Humphrey *et al.*, 1989a). In this study, some birds suffered mild diarrhoea in the first few days PI. However there were no other signs of clinical disease or changes in the health of the birds. Examination at post-mortem also failed to demonstrate abnormalities or the presence of lesions in any organ. In other studies significant differences have been observed in egg production and clinical signs of disease have been clearly evident. Shivaprasad *et al.* (1990) found that infection with a isolate of SE isolated from yolk resulted in anorexia, diarrhoea and a drop in egg production, while an isolate of SE isolated from a human caused depression, anorexia, diarrhoea, reduced egg production and some mortality. Decreases in egg production of around 10% (Gast & Beard, 1990b) and 26% (Barrow & Lovell, 1991) have been reported, with egg production not returning to pre-inoculation levels in some hens. No eggs laid by inoculated birds were contaminated with SI159, indicating a lack of transovarian transmission in either Experiment 1 or 2, even though SI159 was able to colonise the alimentary tract and invade internal organs including the spleen, caecum, liver, ovary, oviduct and pre-ovulatory follicle.

In Experiment 1, all control birds remained free of SI, while in Experiment 2 the single control bird did yield several positive cultures from faeces, eggs and organs. While experimental error was considered possible, a likely explanation for the *Salmonella*-positive control hen may involve airborne transmission or contact transmission via the infected bird in the adjacent cage. Baskerville *et al.* (1992) exposed hens to small-particle aerosols of an strain of SE isolated from an egg and observed that even at low doses (2×10^2 cells) the organism caused a generalised infection and prolonged faecal excretion, with diarrhoea and mortality at an inoculum of 10^5 cells. Likewise, exposure to a small inoculum of SE in an aerosol or via conjunctiva caused an infection in layer hens (Chart *et al.*, 1992). More importantly Humphrey *et al.* (1991) found that control birds kept in the same room as challenged birds became infected. In another study Shivaprasad *et al.* (1990) reported a small number of control birds became infected as evidenced by culture of SE from organs and by serology. Experimental hens were in contact with control birds and the most probable mode of infection was by aerolisation of faeces. This suggests that the organism in question, whether SE, or SI, as in the current study, was highly contagious under the experimental conditions. This has significance and implications for the egg industry, given the intensity of housing.

A number of factors such as the strain of bird and the age of the bird may have affected the response to *S.*Infantis. More extensive work with such variables and with airborne and contact transmission of the organism is required. The use of a isolate of *S.*Infantis isolated from a human infection and others from egg yolk in an experimental model such as that used here might also provide valuable information and perhaps support the theory that some strains belonging to the same serovar may be more invasive than others. Detection of the organism by immunohistochemical staining using monoclonal antibodies specific for SI, or the use of *lux*$^+$ strains would also allow the course of invasion and colonisation to be more easily and more closely examined. Limitations of the current study may also include the fact that hens were purchased at point-of-lay and were not bought as chicks. Older birds may have been exposed to bacteria in the poultry environment, which may have interfered with infection by SI. The use of SPF birds raised in the laboratory from day olds or hatch would eliminate problems with other bacteria and the experiment would have been more controlled. However the use of specific pathogen free birds has its own disadvantages in not representing birds in the field.

Implications

Investigation of the incidence of salmonellae among selected layer flocks, including a comprehensive longitudinal study of one farm, revealed a high carriage rate of *Salmonella* in layers. All serovars found, including the prevalent Singapore, can be considered potentially hazardous to public health. It appears, as many previous studies have shown, that carriage in layers correlates with contamination of feed. Unless the *Salmonella* status of feed is improved, it is almost certain that the carriage rate of the organism in layers will remain high.

A range of techniques was assessed for efficacy in differentiating isolates of serovar Infantis. Of these, pulsed-field gel electrophoresis (PFGE) was found to be the most discriminatory method. Although phage typing is used routinely to discriminate between isolates of serovars for which phage sets exist, molecular techniques are more likely to be used in the future, as they become more reproducible and, through automation, more objective with respect to interpretation. It is important that typing techniques are developed to enable tracking of serovars, and their management throughout the production chain.

Studies showed that *Salmonella* serovars Infantis and Singapore, prevalent in the egg industry in South-East Queensland, could penetrate the shell of eggs under simulated conditions of production and storage, although the fate of these salmonellae varied with temperature of storage and location in the egg. It appears that long-term refrigerated storage (>14 days) is a suitable means of preservation, leading to growth inhibition or death of the isolates tested. Clearly, eggs must be handled with utmost care throughout the prodution chain to minimise contamination with salmonellae and to minimise growth if contamination has occured.

Infectivity experiments with a isolate of SI demonstrated the ability of the bacterium to colonise layers, and the localisation of the bacterium in a number of internal organs, including reproductive tissue. While no contaminated eggs were detected, only a small number of birds were used. In commercial production, infection of large flocks with SI (or any other serovar capable of causing human infection) might yield a small yet significant number of contaminated eggs, as has been the case with SE. Production of contaminated eggs may in fact then be responsible for hitherto undefined cases of salmonellosis, so management of *Salmonella* throughout egg production is crucial.

Recommendations

The carriage rate of salmonellae in the layer flocks investigated in this study was quite high, and correlated with incidence in feed, and specifically the animal protein components of feed. In order to minimise carriage of *Salmonella* in layers, every effort should be made to use *Salmonella*-free feed. Previous studies in Queensland have shown a significantly lower carriage rate in layers fed heat-treated, pelleted feed rather than mash.

With respect to typing of *S.* Infantis (and other industry-associated serovars), it is suggested that genotypic rather than phenotypic methods be adopted as the approach of choice for discrimination of isolates, where the latter (*e.g.* phage typing) do not exist. Genotypic methods such as PFGE are being developed at various sites, including the *Salmonella* reference laboratories, and should be developed for all salmonellae prevalent in the poultry industry.

Prevalent *Salmonella* serovars are able to penetrate shell eggs if present on the surface, and then grow if held under poor conditions of storage. It is recommended that faecal matter be removed as quickly as possible from the surface of shell eggs to minimise risk of initial contamination of eggs with

salmonellae. Additionally, eggs should be maintained at refrigeration temperatures throughout the production chain to minimise growth or potentially eliminate any salmonellae that may have entered eggs. Together, these measures should minimise the threat to public health posed by salmonellae that contaminate eggs only by horizontal transmission.

As it appears that various salmonellae are capable of establishing an infection of reproductive tissue of layers, which in turn potentially produce contaminated eggs, it is recommended that further efforts be made in managing the incidence and prevalence of salmonellae throughout the egg production chain.

Intellectual Property

As the research reported in this study resides in the public good domain, there is little if any content that is considered commercialisable.

Bibliography/References

Adams, M.H. (1959). Bacteriophages. New York: Interscience Publishers. pp.1-11, 365-380.

Anderson, E.S. and Williams, R.E.O. (1956). Bacteriophage typing of enteric pathogens and staphylococci and its use in epidemiology. Journal of Clinical Pathology. **9**:94-127.

Angen, O., Skov, M.N., Chriel, M., Agger, J.F. and Bisgaard, M. (1996). A retrospective study on *Salmonella* infection in Danish broiler flocks. Preventive Veterinary Medicine. **26**:223-237.

Bache, J. (1994). Crocs and chooks complement each other. Milne's Poultry Digest. **June/July**:20.

Baggesen, D.L., Wegener, H.C., Bager, F., Stege, H. and Christensen, J. (1996). Herd prevalence of *Salmonella enterica* infections in Danish slaughter pigs determined by microbiological testing. Preventive Veterinary Medicine. **26**:201-213.

Baker, J.R., Hall, A., Hiby, L., Munro, R., Robinson, I., Ross, H.M. and Watkins, J.F. (1995). Isolation of salmonellae from seals from UK waters. The Veterinary Record. **136**:471-472.

Baquar, N., Threlfall, E.J., Rowe, B. and Stanley, J. (1994). Phage type 193 of *Salmonella typhimurium* contains different chromosomal genotypes and multiple IS*200* profiles. FEMS Microbiology Letters. **115**:291-296.

Barnhart, H.M., Dreesen, D.W., Bastien, R. and Pancorbo, O.C. (1991). Prevalence of *Salmonella enteriditis* and other serovars in ovaries of layer hens at time of slaughter. Journal of Food Protection. **54**:488-491.

Barrow, P.A. and Lovell, M.A. (1991). Experimental infection of egg-laying hens with *Salmonella* Enteritidis phage type 4. Avian Pathology. **20**:335-348.

Barrow, P.A., Simpson, J.M. and Lovell, M.A. (1988). Intestinal colonisation in the chicken by food-poisoning *Salmonella* serovars; microbial characteristics associated with faecal excretion. Avian Pathology. **17**:571-588.

Barrow, P.A., Tucker, J.F. and Simpson, J.M. (1987). Inhibition colonisation of the chicken alimentary tract with *Salmonella typhimurium* gram-negative facultatively anaerobic bacteria. Epidemiology and Infection. **98**:311-322.

Barry, A.L. and Thornsberry, C. (1980). Manual of Clinical Microbiology, 3rd edn. Washington, D.C.: American Society for Microbiology. pp.463-474.

Baskerville, A., Humphrey, T.J., Fitzgeorge, R.B., Cook, R.W., Chart, H., Rowe, B. and Whitehead, A. (1992). Airborne infection of laying hens with *Salmonella* Enteritidis phage type 4. The Veterinary Record. **130**:395-398.

Beltran, P., Musser, J.M., Helmuth, R., Farmer, J.J., Frercihs, W.M., Wachsmuth, I.K., Ferris, K., McWhorter, A.C., Wells, J.G., Cravioto, A. and Selander, R.K. (1988). Toward a population genetic analysis of *Salmonella* : Genetic diversity and relationships among strains of serovars *S. choleraesuis, S. derby, S. dublin, S. enteriditis, S. heidelberg, S.* Infantis, *S. newport*, and *S. typhimurium*. Proceedings of the National Academy of Sciences, USA. **85**:7753-7757.

Berends, B.R., Urlings, H.A.P., Snijders, J.M.A. and Van Knapen, F. (1996). Identification and quantification of risk factors in animal management and transport regarding *Salmonella* spp. in pigs. International Journal of Food Microbiology. **30**:37-53.

Berrang, M.E., Cox, N.A., Bailey, J.S. and Blankenship, L.C. (1991). Methods for inoculation and recovery of *Salmonella* from chicken eggs. Poultry Science. **70**:2267-2270.

Bichler, L.A., Nagaraja, K.V. and Halvorson, D.A. (1996). *Salmonella* Enteritidis in eggs, cloacal swab specimens, and internal organs of experimentally infected White Leghorn chickens. American Journal of Veterinary Research. **57**:489-495.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. **7**:1513-1522.

Board, R.G. (1966). The course of microbial infection of the hens' egg. Journal of Applied Bacteriology. **29**:319-341.

Board, R.G. and Halls, N.A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. British Poultry Science. **14**:69-97.

Borrego, J.J., Castro, D., Jimenez-Notario, M., Luque, A., Martinez-Manzanares, E., Rodriguez-Avial, C. and Picazo, J.J. (1992). Comparison of epidemiological markers of *Salmonella* strains isolated from different sources in Spain. Journal of Clinical Microbiology. **30**:3058-3064.

Braun, P. and Fehlhaber, K. (1995). Migration of *Salmonella* Enteritidis from the albumen into the egg yolk. International Journal of Food Microbiology. **25**:95-99.

Bumstead, N. and Barrow, P. (1993). Resistance to *Salmonella gallinarum*, *S.pullorum*, and *S.*Enteritidis in Inbred Lines of Chickens. Avian Disease. **37**:189-193.

Burnens, A.P., Stanley, J., Sechter, I. and Nicolet, J. (1996). Evolutionary origin of a monophasic *Salmonella* serovar, 9,12:1,v:- , revealed by IS*200* profiles and restriction fragment polymorphisms of the *fljB* gene. Journal of Clinical Microbiology. **34**:1641-1645.

Callow, B.R. (1959). A new phage-typing scheme for *Salmonella typhi-murium*. Journal of Hygiene. 7:346-359.

Cannon, R.M. and Roe, R.T. (1982). Livestock disease surveys a field manual for veterinarians. Canberra: Australian Government Publishing Service. pp.1-9.

Castro, D., Morinigo, M.A., Martinez-Manzanares, E., Cornax, R., Balebona, C., Luque, A. and Borrego, J.J. (1992). Development and application of a new scheme of phages for typing and differentiating *Salmonella* strains from different sources. Journal of Clinical Microbiology. **30**:1418-1423.

Chart, H., Baskerville, A., Humphrey, T.J. and Rowe, B. (1992). Serological responses of chickens experimentally infected with *Salmonella* Enteritidis PT4 by different routes. Epidemiology and Infection. **109**:297-302.

Christensen, J.P., Olsen, J.E. and Bisgaard, M. (1993). Ribotypes of *Salmonella enterica* serovar *Gallinarum* biovars *gallinarum* and *pullorum*. Avian Pathology. **22**:725-738.

Christensen, J.P., Skov, M.N., Hinz, K.H. and Bisgaard, M. (1994). *Salmonella enterica* serovar Gallinarum biovar gallinarum in layers: epidemiological investigations of a recent outbreak in Denmark. Avian Pathology. **23**:489-501.

Clay, C.E. and Board, R.G. (1991). Growth of *Salmonella* Enteritidis in artificially contaminated hens' shell eggs. Epidemiology and Infection. **106**:271-281.

Clay, C.E. and Board, R.G. (1992). Effect of faecal extract on the growth of *Salmonella* Enteritidis in artificially contaminated hens' eggs. British Poultry Science. **33**:755-760.

Cowden, J.M., Lynch, D., Joseph, C.A., O'Mahony, M., Mawer, S.L., Rowe, B. and Bartlett, C.L.R. (1989b). Case-control study of infections with *Salmonella* Enteritidis phage type 4 in England. British Medical Journal. **299**:771-773.

Cox, J.M. (1993). Lysine-mannitol-glycerol agar, a medium for the isolation of *Salmonella* spp., including *S.typhi* and atypical strains. Applied and Environmental Microbiology. **59**:2602-2606.

Cox, J.M. (1995). *Salmonella* Enteritidis: the egg and I. Australian Veterinary Journal. 72:108-115.
Cox, J.M., Brook, M.D. and Woolcock, J.B. (1996). Sensitivity of Australian isolates of *Salmonella* Enteritidis to nitrofurantoin and furazolidone. Veterinary Microbiology. **49**:305-308.

Cox, J.M. and Fleet, G.H. (1997). Chapter 4. New Directions in the microbiological analysis of foods. In *Foodborne Microorganisms of Public Health Significance*, Hocking, A.D., Arnold, G., Jenson, I., Newton, K., Sutherland, P. (eds) pp. 71-138. AIFST (NSW) Food Microbiology Group, Sydney.

Crichton, P.B., Old, D.C., Taylor, A. and Rankin, S.C. (1996). Characterisation of strains of *Salmonella* serovar Livingstone by multiple typing. Journal of Medical Microbiology. **44**:325-331.

Dreesen, D.W., Barnhart, H.M., Burke, J.L., Chen, T. and Johnson, D.C. (1992). Frequency of *Salmonella* Enteritidis and other salmonellae in the caeca of spent hens at time of slaughter. Avian Diseases. **36**:247-250.

Duguid, J.P., Anderson, E.S., Alfredsson, G.A., Barker, R. and Old, D.C. (1975). A new biotyping scheme for *Salmonella typhimurium* and its phylogenetic significance. Journal of Medical Microbiology. **8**:149-166.

Ebel, E.D., David, M.J. and Mason, J. (1992). Occurrence of *Salmonella* Enteritidis in the U.S. commercial egg industry: report on a national spent hen survey. Avian Diseases. **36**:646-654.

Ebel, E.D., Mason, J., Thomas, L.E., Ferris, K.E., Beckman, M.G., Cummins, D.R., Scroeder-Tucker, L., Sutherlin, W.D., Glasshoff, R.L. and Smithhisler, N.M. (1993). Occurrence of *Salmonella enteriditis* in unpasteurized liquid egg in the United States. Avian Diseases. **37**:135-142.

Ellsworth, D.L., Rittenhouse, D. and Honeycutt, R.L. (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques. **14**:214-217.

Esteban, E., Snipes, K., Hird, D., Kasten, R. and Kinde, H. (1993). Use of ribotyping for characterisation of *Salmonella* serovars. Journal of Clinical Microbiology. **31**:233-237.

Frost, J.A., Kelleher, A. and Rowe, B. (1996). Increasing ciprofloxacin resistance in salmonellas in England and Wales 1991-1994. Journal of Antimicrobial Chemotherapy. **37**:85-91.

Gast, R.K. (1993). Detection of *Salmonella* Enteritidis in experimentally infected laying hens by culturing pools of egg contents. Poultry Science. **72**:267-274.

Gast, R.K. (1994). Understanding *Salmonella* Enteritidis in laying chickens: the contributions of experimental infections. International Journal of Food Microbiology. **21**:107-116.

Gast, R.K. and Beard, C.W. (1990a). Isolation of *Salmonella* Enteritidis from internal organs of experimentally infected hens. Avian Diseases. **34**:991-993.

Gast, R.K. and Beard, C.W. (1990b). Production of *Salmonella* Enteritidis-contaminated eggs by experimentally infected hens. Avian Diseases. **34**:438-446.

Gast, R.K. and Beard, C.W. (1992a). Detection and enumeration of *Salmonella* Enteritidis in fresh and stored eggs laid by experimentally infected hens. Journal of Food Protection. **55**:152-156.

Gast, R.K. and Beard, C.W. (1992b). Evaluation of a chick mortality model for predicting the consequences of *Salmonella* Enteritidis infections in laying hens. Poultry Science. **71**:281-287.

Glynn, J.R. and Bradley, D.J. (1992). The relationship between infecting dose and severity of disease in reported outbreaks of salmonella infections. Epidemiology and Infection. **109**:371-388.

Hartung, T.E. and Stadelman, W.J. (1963). *Pseudomonas fluorescens* penetration of egg shell membranes as influenced by shell porosity, age of the egg and degree of bacterial challenge. Poultry Science. **42**:147-150.

Hasenson, L., Gericke, B., Liesegang, A., Claus, H., Poplawskaja, J., Tscherkess, N. and Rabsch, W. (1995). Epidemiological and microbiological investigations on salmonellosis in Russia. Zentralblatt Hygiene. **198**:97-116.

He, Q., Viljanen, M.K. and Mertsola, J. (1994). Effects of thermocyclers and primers on the reproducibility of banding patterns in randomly amplified polymorphic DNA analysis. Molecular and Cellular Probes. **8**:155-160.

Hermans, P.W.M., Saha, S.K., van Leeuwen, W.J., Verbrugh, H.A., van Belkum, A. and Goessens, W.H.F. (1996). Molecular typing of *Salmonella typhi* strains from Dhaka (Bangladesh) and development of DNA probes identifying plasmid-encoded multidrug-resistant isolates. Journal of Clinical Microbiology. **34**:1373-1379.

Hinz, K.H., Legutko, P., Schroeter, A., Lehmacher, W. and Hartung, M. (1996). Prevalence of motile salmonellae in egg-laying hens at the end of the laying period. Journal of Veterinary Medicine. **43**:23-33.

Hogg, R.V. and Tanis, E.A. (1977). Probability and statistical inference, 2nd ed. New York: Macmillan Publishing Co. pp.195.

Hopper, S.A. and Mawer, S. (1988). *Salmonella* Enteritidis in a commercial layer flock. The Veterinary Record. **123**:351.

Humphrey, T.J. (1990). Growth of salmonellas in intact shell eggs: influence of storage temperature. Veterinary Record. **126**:292.

Humphrey, T.J., Baskerville, A., Chart, H. and Rowe, B. (1989a). Infection of egg-laying hens with *Salmonella* Enteritidis PT4 by oral inoculation. The Veterinary Record. **125**:531-532.

Humphrey, T.J., Baskerville, A., Chart, H., Rowe, B. and Whitehead, A. (1991a). *Salmonella* Enteritidis PT4 infection in specific pathogen free hens: influence of infecting dose. The Veterinary Record. **129**:482-485.

Humphrey, T.J., Baskerville, A., Mawer, S., Rowe, B. and Hopper, S. (1989b). *Salmonella enteriditis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiology and Infection. **103**:415-423.

Humphrey, T.J., Baskerville, A., Whitehead, A., Rowe, B. and Henley, A. (1993). Influence of feeding patterns on the artificial infection of laying hens with *Salmonella enteriditis* phage type 4. Veterinary Record. **132**:407-409.

Humphrey, T.J., Chart, H., Baskerville, A. and Rowe, B. (1991b). The influence of age on the response of SPF hens to infection with *Salmonella* Enteritidis PT4. Epidemiology and Infection. **106**:33-43.

Humphrey, T.J., Greenwood, M., Gilbert, R.J., Rowe, B. and Chapman, P.A. (1989c). The survival of salmonellas in shell eggs cooked under simulated domestic conditions. Epidemiology and Infection. **103**:35-45.

Irwin, R.J., Poppe, C., Messier, S., Finley, G.G. and Oggel, J. (1994). A national survey to estimate the prevalence of *Salmonella* species among Canadian registered commercial turkey flocks. Canadian Journal of Veterinary Research. **58**:263-267.

Kasatiya, S., Caprioli, T. and Champoux, S. (1978). Bacteriophage typing scheme for *Salmonella* Infantis. Journal of Clinical Microbiology. **10**:637-640.

Keller, L.H., Benson, C.E., Krotec, K. and Eckroade, R.J. (1995). *Salmonella* Enteritidis colonisation of the reproductive tract and forming and freshly laid eggs of chickens. Infection and Immunity. **63**:2443-2449.

Kelly, W.J., Huang, C.M. and Asmundson, R.V. (1993). Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. Applied and Environmental Microbiology. **59**:3969-3972.

Kerouanton, A., Brisabois, A., Grout, J. and Picard, B. (1996). Molecular epidemiological tools for *Salmonella* Dublin typing. FEMS Immunology and Medical Microbiology. **14**:25-29.

Khurana, R. and Kumar, A. (1993). Occurrence of *Salmonella* in eggs and meat. Journal of Food Science and Technology. **30**:447-448.

Kim, C.J., Emery, D.A., Rinke, H., Nagaraja, K.V. and Halvorson, D.A. (1989). Effect of time and temperature on growth of *Salmonella* Enteritidis in experimentally inoculated eggs. Avian Diseases. **33**:735-742.

Kinde, H., Read, D.H., Chin, R.P., Bickford, A.A., Walker, R.L., Ardans, A., Breitmeyer, R.E., Willoughby, D., Little, H.E., Kerr, D. and Gardner, I.A. (1996). *Salmonella* Enteritidis, phage type 4 infection in a commercial layer flock in southern California: bacteriologic and epidemiologic findings. Avian Diseases. **40**:665-671.

Lagatolla, C., Dolzani, L., Tonin, E., Lavenia, A., Di Michele, M., Tommasini, T. and Monti-Bragadin, C. (1996). PCR ribotyping for characterising *Salmonella* isolates of different serovars. Journal of Clinical Microbiology. **34**:2440-2443.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In Nucleic acid techniques in bacterial systematics., pp. Edited by E. Stackebrandt & M. Goodfellow. New York: John Wiley & Sons.

Laszlo, V.G., Csak, K. and Csorian, E.S. (1988). A phage typing system for *Salmonella* Infantis. Acta Microbiologica Hungarica. **35**:55-69.

Liebisch, B. and Schwarz, S. (1996). Evaluation and comparison of molecular techniques for epidemiological typing of *Salmonella enterica* subsp. *enterica* serovar Dublin. Journal of Clinical Microbiology. **34**:641-646.

Liebisch, B. and Schwarz, S. (1996b). Molecular typing of *Salmonella enterica* subsp. *enterica* seovar Enteritidis isolates. Journal of Medical Microbiology. **44**:52-59.

Lin, A.W., Usera, M.A., Barrett, T.J. and Goldsby, R.A. (1996). Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella* Enteritidis. Journal of Clinical Microbiology. **34**:870-876.

Lin, T.L., Wu, C.C., Rosenberger, J.K. and Saif, Y.M. (1994). Rapid differentiation of infectious bursal disease virus serovars by polymerase chain reaction. Journal of Veterinary Diagnostic Investigation. **6**:100-102.

Lister, S.A. (1988). *Salmonella* Enteritidis infection in broilers and broiler breeders. The Veterinary Record. **123**:350.

Luby, S. and Jones, J. (1993). Outbreak of gastroenteritis due to *Salmonella* Enteritidis from locally produced grade A eggs, South Carolina. Southern Medical Journal. **86**:1350-1353.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory. pp.368-369.

Meunier, J.R. and Grimont, P.A.D. (1993). Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Research in Microbiology. **144**:373-379.

Millemann, Y., Lesage, M., Chaslus-Dancla, E. and Lafont, J. (1995). Value of plasmid profiling, ribotyping, and detection of IS*200* for tracing avian isolates of *Salmonella typhimurium* and *S.*Enteritidis. Journal of Clinical Microbiology. **33**:173-179.

Millemann, Y., Lesage-Descauses, M., Lafont, J. and Chaslus-Dancla, E. (1996). Comparison of random amplified polymorphic DNA analysis and enterobacterial repetitive intergenic consensus-PCR for epidemiological studies of *Salmonella*. FEMS Immunology and Medical Microbiology. **14**:129-134.

Murray, C.J. (1994). Salmonella serovars and phage types in humans and animals in Australia 1987-1992. Australian Veterinary Journal. **71**:78-81.

Nair, S., Poh, C.L., Lim, Y.S., Tay, L. and Goh, K.T. (1994). Genome fingerprinting of *Salmonella typhi* by pulsed-field gel electrophoresis for subtyping common phage types. Epidemiology and Infection. **113**:391-402.

Nascimento, V.P., Cranstoun, S. and Solomon, S.E. (1992). Relationship between shell structure and movement of *Salmonella* Enteritidis across the eggshell wall. British Poultry Science. **33**:37-48.

Nastasi, A. and Mammina, C. (1995). Epidemiological evaluation by PCR ribotyping of sporadic and outbreak-associated strains of *Salmonella enterica* serovar Typhimurium. Research Microbiology. **146**:99-106.

Nastasi, A., Mammina, C. and Villafrate, M.R. (1991). rRNA fingerprinting as a tool in epidemiological analysis of *Salmonella typhi* infections. Epidemiology and Infection. **107**:565-576.

NCCLS (1994). Performance standards for antimicrobial disk susceptibility tests. M2A4. Villanova, Pa.: National Committee for Clinical Laboratory Standards. pp.20-21.

NHMRC (1986). Antibiotics in stockfeeds. Canberra: Australian Government Publishing Service. pp.2-4.

Nolan, L.K., Wooley, R.E., Brown, J. and Payeur, J.B. (1991). Comparison of phenotypic characteristics of *Salmonella* spp isolated from healthy and ill (infected) chickens. American Journal of Veterinary Research. **52**:1512-1517.

Nuotio, L. and Mead, G.C. (1993). An *in vitro* model for studies on bacterial interactions in the avian caecum. Letters in Applied Microbiology. **17**:65-67.

Old, D.C. and Barker, R.M. (1989). Numerical index of the discriminatory ability of biotyping for strains of *Salmonella typhimurium* and *Salmonella paratyphi* B. Epidemiology and Infection. **103**:435-443.

Old, D.C., Munro, D.S., Reilly, W.J. and Sharp, J.C.M. (1985). Biotype discrimination of *Salmonella montevideo*. Letters in Applied Microbiology. **1**:67-69.

Olsen, J.E., Brown, D.J., Baggesen, D.L. and Bisgaard, M. (1992a). Biochemical and molecular characterisation of *Salmonella enterica* serovar *berta*, and comparison of methods for typing. Epidemiology and Infection. **108**:243-260.

Olsen, J.E., Brown, D.J., Baggesen, D.L. and Bisgaard, M. (1994). Stability of plasmids in five strains of *Salmonella* maintained in stab culture at different temperatures. Journal of Applied Bacteriology. **77**:155-159.

Olsen, J.E. and Skov, M. (1994). Genomic lineage of *Salmonella enterica* serovar Dublin. Veterinary Microbiology. **40**:271-282.

Olsen, J.E., Skov, M.N., Threlfall, E.J. and Brown, D.J. (1994). Clonal lines of *Salmonella enterica* serovar Enteritidis documented by IS*200*-, ribo-, pulsed-field gel electrophoresis and RFLP typing. Journal of Medical Microbiology. **40**:15-22.

Olsen, J.E., Sorensen, M., Brown, D.J., Gaarslev, K. and Bisgaard, M. (1992b). Plasmid profiles as an epidemiological marker in *Salmonella enterica* serovar *berta* infections. APMIS. **100**:221-228.

Opara, O.O., Carr, L.E., Russek-Cohen, C.R., Tate, C.R., Mallison, E.T., Miller, R.G., Stewart, L.E., Johnston, R.W. and Joseph, S.W. (1992). Correlation of water activity and other environmental conditions with repeated detection of salmonella contamination on poultry farms. Avian Diseases. **36**:664-671.

Park, Y. and Kohel, R. (1994). Effect of concentration of $MgCl_2$ on random-amplified DNA polymorphism. Biotechniques. **16**:652-656.

Pelkonen, S., Romppanen, E., Siitonen, A. and Pelkonen, J. (1994). Differentiation of *Salmonella* serovar Infantis isolates from human and animal sources by fingerprinting IS*200* and 16S *rrn* loci. Journal of Clinical Microbiology. **32**:2128-2133.

Perales, I. and Audicana, A. (1989). The role of hens' eggs in outbreaks of salmonellosis in north Spain. International Journal of Food Microbiology. **8**:175-180.

Poppe, C. and Gyles, C.L. (1987). Relation of plasmids to virulence and other properties of salmonellae from avian sources. Avian Diseases. **31**:844-854.

Poppe, C., Irwin, R.J., Forsberg, C.M., Clarke, R.C. and Oggel, J. (1991a). The prevalence of *Salmonella enteriditis* and other *Salmonella* spp. among Canadian registered commercial layer flocks. Epidemiology and Infection. **106**:259-270.

Poppe, C., Irwin, R.J., Messier, S., Finley, G.G. and Oggel, J. (1991b). The prevalence of *Salmonella enteriditis* and other *Salmonella* spp. among Canadian registered commercial chicken broiler flocks. Epidemiology and Infection. **107**:201-211.

Poppe, C., Johnson, R.P., Forsberg, C.M. and Irwin, R.J. (1992). *Salmonella* Enteritidis and other *Salmonella* in laying hens and eggs from flocks with *Salmonella* in their environment. Canadian Journal of Veterinary Research. **56**:226-232.

Powling, J. (1992). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 5/93.

Powling, J. (1993). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 4/93.

Powling, J. (1995). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 7/95.

Powling, J. (1996). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 5/96.

Powling, J., Huang, Q., Lightfoot, D. and Adams, P. (1995). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 7/95.

Powling, J., Truong, B., Lightfoot, D. and Adams, P. (1994). National Salmonella Surveillance Scheme. Microbiological Diagnostic Unit, University of Melbourne. 6/94.

Prevost, G., Jaulhac, B. and Piemont, Y. (1992). DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. Journal of Clinical Microbiology. **30**:967-973.

Protais, J., Colin, P., Beaumont, C., Guillot, J.F., Lantier, F., Pardon, P. and Bennejean, G. (1996). Line differences in resistance to Salmonella enteritidis PT4 infection. British Poultry Science. **37**:329-339.

Raevuori, M., Seuna, E. and Nurmi, E. (1978). An epidemic of *Salmonella* Infantis infection in Finnish broiler chickens in 1975-76. Acta Veterinara Scandinavia. **19**:317-330.

Reilly, W.J., Old, D.C., Munro, D.S. and Sharp, J.C.M. (1985). An epidemiological study of *Salmonella montevideo* by biotyping. Journal of Hygiene. **95**:23-28.

Ridley, A.M., Punia, P., Ward, L.R., Rowe, B. and Threlfall, E.J. (1996). Plasmid characterisation and pulsed-field electrophoretic analysis demonstrate that ampicillin-resistant strains of *Salmonella* Enteritidis phage type 6a are derived from *Salm.* Enteritidis phage type 4. Journal of Applied Bacteriology. **81**:613-618.

Robertson, M.H. (1972). Survival of *Salmonella typhi-murium* in floor dust - a possible reservoir of infections in institutions. Public Health London. **87**:39-45.

Rodrigue, D.C., Tauxe, R.V. and Rowe, B. (1990). International increase of *S.*Enteritidis: A new pandemic? Epidemiology and Infection. **105**:21-27.

Rusul, G., Khair, J., Radu, S., Cheah, C.T. and Yassin, R. (1996). Prevalence of *Salmonella* in broilers at retail outlets, processing plants and farms in Malaysia. International Journal of Food Microbiology. **33**:183-194.

Sacks, J.M., Bolin, S.R. and Crowder, S.V. (1989). Prevalence estimation from pooled samples. American Journal of Veterinary Research. **50**:205-206.

Sakai, T. and Chalermchaikit, T. (1996). The major sources of *Salmonella* Enteritidis in Thailand. International Journal of Food Microbiology. **31**:173-180.

Sauter, E.A. and Petersen, C.F. (1974). The effect of egg shell quality on penetration by various salmonellae. Poultry Science. **53**:2159-2162.

Schiaffino, A., Beuzon, C.R., Uzzau, S., Leori, G., Cappucinelli, P., Casadesus, J. and Rubino, S. (1996). Strain typing with IS*200* fingerprints in *Salmonella abortusovis*. Applied and Environmental Microbiology. **62**:2375-2380.

Schoeni, J.L., Glass, K.A., McDermott, J.L. and Wong, A.C.L. (1995). Growth and penetration of *Salmonella* Enteritidis, *Salmonella heidelberg* and *Salmonella typhimurium* in eggs. International Journal of Food Microbiology. **24**:385-396.

Shivaprasad, H.L., Timoney, J.F., Morales, S., Lucio, B. and Baker, R.C. (1990). Pathogenesis of *Salmonella enteriditis* infection in laying chickens. I. Studies on egg transmission, clinical signs, faecal shedding, and serological responses. Avian Diseases. **34**:548-557.

Singer, J.T., Opitz, M., Gershman, M., Hall, M.M., Muniz, I.G. and Rao, S.V. (1992). Molecular characterisation of *Salmonella* Enteritidis isolates from Maine poultry and poultry farm environments. Avian Diseases. **36**:324-333.

Snoeyenbos, G.H., Smyser, C.F. and Van Roekel, H. (1969). *Salmonella* infections of the ovary and peritoneum of chickens. Avian Diseases. **13**:668-670.

Stanley, J., Burnens, A., Powell, N., Chowdry, N. and Jones, C. (1992a). The insertion sequence IS*200* fingerprints chromosomal genotypes and epidemiological relationships in *Salmonella heidelberg*. Journal of General Microbiology. **138**:2329-2336.

Stanley, J., Powell, N., Jones, C. and Burnens, A.P. (1994). A framework for IS*200*, 16S rRNA gene and plasmid-profile analysis in *Salmonella* serogroup D1. Journal of Medical Microbiology. **41**:112-119.

Stephenson, P., Satchell, F.B., Allen, G. and Andrews, W.H. (1991). Recovery of *Salmonella* from shell eggs. Journal of the Association of Official Analytical Chemists. **74**:821-826.

Stevens, A., Joseph, C., Bruce, J., Fenton, D., O'Mahony, M., Cunningham, D., O'Connor, B. and Rowe, B. (1989). A large outbreak of *Salmonella* Enteritidis phage type 4 associated with eggs from overseas. Epidemiology and Infection. **103**:425-433.

Stubbs, A.D., Hickman-Brenner, F.W., Cameron, D.N. and Farmer, J.J. (1994). Differentiation of *Salmonella* Enteritidis phage type 8 strains: evaluation of three additional phage typing systems, plasmid profiles, antibiotic susceptibility patterns, and biotyping. Journal of Clinical Microbiology. **32**:199-201.

Suzuki, S., Komase, K., Matsui, H., Abe, A., Kawahara, K., Tamura, Y., Kijima, M., Danbara, H., Nakamura, M. and Sato, S. (1994). Virulence region of plasmid pNL2001 of *Salmonella* Enteritidis. Microbiology. **140**:1307-1318.

Suzuki, Y., Ishihara, M., Matsumoto, M., Arakawa, S., Saito, M., Ishikawa, N. and Yokochi, T. (1995). Molecular epidemiology of *Salmonella* Enteritidis. An outbreak and sporadic cases studied by means of pulsed-field gel electrophoresis. Journal of Infection. **31**:211-217.

Thiagarajan, D., Saeed, A.M. and Asem, E.K. (1994). Mechanism of transovarian transmission of *Salmonella* Enteritidis in laying hens. Poultry Science. **73**:89-98.

Thong, K., Cheong, Y., Puthcheary, S., Koh, C. and Pang, T. (1994). Epidemiologic analysis of sporadic *Salmonella typhi* isolates and those from outbreaks by pulsed-field gel electrophoresis. Journal of Clinical Microbiology. **32**:1135-1141.

Thong, K., Cordano, A., Yassin, R.M. and Pang, T. (1996). Molecular analysis of environmental and human isolates of *Salmonella typhi*. Applied and Environmental Microbiology. **62**:271-274.

Threlfall, E.J., Chart, H., Ward, L.R., de Sa, J.D.H. and Rowe, B. (1993a). Interrelationships between strains of *Salmonella enteriditis* belonging to phage types 4,7,7a,8,13,13a,23,24 and 30. Journal of Applied Bacteriology. **75**:43-48.

Threlfall, E.J., Rowe, B. and Ward, L.R. (1989). Subdivision of *Salmonella enteriditis* phage types by plasmid profile typing. Epidemiology and Infection. **102**:459-465.

Threlfall, E.J., Rowe, B. and Ward, L.R. (1993b). A comparison of multiple drug resistance in salmonellas from humans and food animals in England and Wales, 1981 and 1990. Epidemiology and Infection. **111**:189-197.

Timoney, J.F., Shivaprasad, H.L. and Baker, R.C. (1989). Egg transmission after infection of hens with *Salmonella* Enteritidis phage type 4. The Veterinary Record. **125**:600-601.

Urlings, H.A.P., Mul, A.J., van 't Klooster, A.T., Bijker, P.G.H., van Logtestijn, J.G. and van Gils, L.G.M. (1993). Microbial and nutritional aspects of feeding fermented feed (poultry by-products) to pigs. The Veterinary Quarterly. **15**: 146-151.

Usera, M.A., Popovic, T., Bopp, C.A. and Strockbine, N.A. (1994). Molecular subtyping of *Salmonella* Enteritidis phage type 8 strains from the United States. Journal of Clinical Microbiology. **32**:194-198.

van de Giessen, A.W., Dufrenne, J.B., Ritmeester, W.S., Berkers, P.A.T.A., van Leeuwen, W.J. and Notermans, S.H.W. (1992). The identification of *Salmonella enteritidis*-infected poultry flocks associated with an outbreak of human salmonellosis. Epidemiology and Infection. **109**:405-411.

van Duijkeren, E., van Klingeren, B., Vulto, A.G., Sloet van Oldruitenborgh-Oosterbaan, M., Breukink, H.J. and van Miert, A.S.J.P.A.M. (1995). In vitro susceptibility to antimicrobial drugs of 62 Salmonella strains isolated from horses in The Netherlands. Veterinary Microbiology. **45**:19-26.

Ward, L.R., de Sa, J.D.H. and Rowe, B. (1987). A phage-typing scheme for *Salmonella* Enteritidis. Epidemiology and Infection. **99**:291-294.

Wegener, H.C. and Baggesen, D.L. (1996). Investigation of an outbreak of human salmonellosis caused by *Salmonella enterica* ssp. *enterica* serovar Infantis by use of pulsed field gel electrophoresis. International Journal of Food Microbiology. **32**:125-131.

Williams, J.E., Dillard, L.H. and Hall, G.O. (1968). The penetration patterns of *Salmonella typhimurium* through the outer structure of chicken eggs. Avian Diseases. **12**:445-466.

Williams, J.E. and Whittemore, A.D. (1967). A method for studying microbial penetration through the outer structures of the avian egg. Avian Diseases. 467-489.

Wong, S.S.Y., Yuen, K.Y., Yam, W.C., Lee, T.Y. and Chau, P.Y. (1994). Changing epidemiology of human salmonellosis in Hong Kong. Epidemiology and Infection. **113**:425-434.