Characterisation of *Salmonella*

(09 11406)

MAF Technical Paper No: 2011/67

Prepared for MAF Biosecurity Operational Research
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ISBN 978-0-478-38701-8 (online)
ISSN 2230-2794 (online)

November 2010

Ministry of Agriculture and Forestry
Te Manatū Ahuwhenua, Ngāherehere

BIOSECURITY NEW ZEALAND

NEW ZEALAND FOOD SAFETY AUTHORITY

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1. Abstract

Objective:
The objectives of this study were to investigate the molecular epidemiology of *Salmonella* Typhimurium DT160 between 1999 and 2009 and examine the relatedness between isolates obtained from avian species, the environment associated with poultry, and humans.

Methods:
Ninety New Zealand (NZ) *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT160 (DT160) isolates obtained in the period between 1999 and 2009 were assessed for colony morphology and serotype, and four isolates were further evaluated with Triple Sugar Agar, biochemical and motility tests. The susceptibility of the isolates to 11 antimicrobials was also determined. In an attempt to discriminate between potentially pathogenic and pathogenic *Salmonella* isolates, virulotyping was performed based on 12 potential virulence genes. Pulsed Field Gel Electrophoresis (PFGE) patterns were determined using restriction enzymes *Xba*I and *Spe*I.

Results:
All 90 isolates were confirmed as *Salmonella* spp. All isolates were susceptible to the antimicrobials used in this study, with the exception of susceptibility to tetracycline and oxytetracycline, for which 26 and 8 isolates, respectively, fell within the intermediate range. However, all isolates were positive for at least 10 of the 12 virulence genes. Two of the six isolates PCR negative for one of the virulence genes (*invA*, *iroN*, *pefA* or *sifA*) were of human, and the remaining four were of sparrow origin. PFGE combined with virulotyping demonstrated that the genotype profile AA1 accounted for 78/90 (86.7%) of the isolates and persisted as the dominant genotype throughout the time period, whilst the second most common profile, AA2, was found in only three isolates (3.3%), comprising two isolates from sparrows and one from a human. The remaining nine profiles were found in single isolates. The AA2 isolates were PCR negative for *sifA.*

Conclusion:
There was no indication of multi-drug resistance within the isolates examined in this study and no obvious correlation was found between intermediate susceptibility to tetracycline or oxytetracycline, and virulotype, PFGE profile, origin of the isolate, or seasonality. There was little overall variation between the isolates with the majority demonstrating a single combined virulence gene and PFGE genotype profile. The similarity between the isolates that originated from avian, human and poultry environments over this time period suggests that a dominant epidemiologically-important genotype of DT160 was transmitted freely between humans, wildlife and farmed animals over an extended period. Further, the genotyping data provide evidence that a relatively small number of variants arose in the population between 1999 and 2009, but these did not become prevalent or replace the dominant genotype.

Key words: *avian, human salmonellosis, S. Typhimurium DT160, PFGE, virulotyping, zoonosis*

2. Introduction

2.1. BACKGROUND

Salmonellosis is a zoonotic bacterial disease of national and international importance. The worldwide distribution of salmonellosis often parallels the patterns of trade of animal products and food, and the migration patterns of humans and animals (Callaghan & Simmons 2001; ESR 2010; Gilbert *et al.* 2010; Penfold *et al.* 1979; Wong *et al.* 2007). In common with other countries New Zealand (NZ) has experienced the repeated introduction and dissemination of a wide range of *Salmonella* serotypes that have become a major concern for both human and animal health. The rate of human salmonellosis notifications in NZ ranged between 26.2 to 64.7 cases per 100,000 population between 2000 and 2009 (Lim et al 2010) and currently salmonellosis is the second most commonly notified enteric disease in NZ, with campylobacteriosis being the most common [Institute of Environmental Science and Research (ESR), NZ, 2010)]

Taxonomically, the genus *Salmonella* which belongs to the *Enterobacteriaceae* family is divided into two species: *Salmonella bongori* and *Salmonella enterica*. Using the Kauffmann-White Scheme, members of this genus are classified into more than 2500 serotypes based on differences between the O (cell wall) and H (flagella) antigens (Luderitz *et al.* 1966; Grimont & Weill 2007). Lipopolysaccharide is a major cell wall component comprising lipid A, core oligosaccharide and O antigenic polysaccharide chain (Luk & Lindberg 1991). While some *Salmonella* serotypes such as *S. enterica* Typhimurium and *S.* Enteritidis can infect humans as well as several animal species, others such as *S.* Cholerasuis, *S.* Dublin, and *S.* Typhi are restricted to fewer host species, primarily infecting pigs, cattle and humans, respectively (Kingsley & Baumler 2000; Parry *et al.* 2002).

The spread of salmonellosis is associated with a wide range of sources and transmission pathways including: the consumption of contaminated food products from pigs, poultry and ruminants; contaminated drinking water; overseas travel; and direct contact with domestic and wild animal faeces via environmental and occupational exposure (Mullner et al 2009, Wilson and Baker 2009). The spatial and temporal epidemiology of different serotypes of *Salmonella* in humans often reflect the patterns of infection in animal reservoirs, and this is evident in NZ where some serotypes have been predominant in either South or North Island, whereas others have been widespread across the whole country (ESR 2010).

Salmonella enterica subspecies *enterica* serotype Typhimurium Definitive Type DT160 (DT160) was isolated from sparrows in Canada (Tizzard *et al.* 1979) and in England sparrows infected with DT160 contaminated food for human consumption in a hospital that resulted in gastroenteritis in the human patients (Penfold *et al.* 1979). *In vivo* experiments have shown that the ability of *Salmonella* strains to invade and survive within the intestine may correlate with their ability to cause systemic infections (Connolly *et al.* 2006). In 1998, *S.* Typhimurium DT160 was identified as a human pathogen in NZ (Alley *et al.* 2002; ESR 2010) and since then has become widespread, and a common epidemic strain in NZ (ESR 2010). Various transmission pathways have been identified including: the handling of birds, contact with patients with diarrhoea, the consumption of contaminated food and drinking roof collected rainwater (Thornley *et al.* 2003). However, elsewhere in the world DT160 seems to be uncommon and a rare cause of illnesses (Fisher 2004). Although salmonellosis can be a self-limiting disease, the host may become a persistently infected asymptomatic carrier (Todd *et al.* 2008) with intermittent shedding of bacteria. Epidemics affecting birds have caused high mortality mostly in passerines and, to a lesser extent, in psittacines (Alley *et al.* 2002). Since 2000, the incidence as well as the geographical distribution of DT160 in NZ has

increased significantly, progressing from the South to the North Island (Callaghan & Simmons 2001), although the reason for this has not been identified (Alley *et al.* 2002).

Of growing concern is the use of antimicrobials that may enhance the severity of the disease and select for resistance (Poppe *et al.* 1998; WHO 2002 and 2005). Sub-inhibitory treatment with tetracycline was associated with the emergence of multidrug resistant *S.* Typhimurium DT104 (Weir *et al.* 2008). In addition, diminished sensitivity within the clinically susceptible range may evolve to clinically resistant bacteria (Phillips *et al.* 2004). While numerous outbreaks globally have been caused by multi-drug resistant strains of *Salmonella* Typhi (Rowe *et al.* 1997), in Denmark a high correlation was identified between the mortality rate in humans and infections with *S.* Typhimurium resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline (Helms *et al.* 2002).

2.2. PROJECT OBJECTIVES

The objectives of this study were to investigate the molecular epidemiology of *Salmonella* Typhimurium DT160 in New Zealand between 1999 and 2009, and to examine the genetic relatedness of isolates obtained from three sources: avian species, poultry environments and humans over this time period.

3. Materials and methods

3.1. COLONY MORPHOLOGY, SEROLOGY AND ANTIBIOTIC SENSITIVITY TESTING

3.1.1 Bacterial strains

Ninety isolates of *S.* Typhimurium DT160 were obtained on Dorset egg slopes from ESR Ltd, NZ. The sources of these isolates were avian species, poultry-related environmental samples, and human clinical isolates that had been obtained between the years 1999 and 2009. Thirty isolates were of human origin and 60 were isolated either from avian species or from environments related to poultry. These were selected using a stratified random process whereby isolates in the sampling frame were grouped according to year and source, and the appropriate number randomly selected from each group. Freeze-dried *S.* Menston [National Collection of Type Cultures (NCTC7836)] and *Escherichia coli* (*E. coli*) [America Type Cell Cultures (ATCC 25922)] were purchased from the New Zealand Reference Culture Collection, ESR. *Salmonella* Typhimurium F98 phage type 14 (NCTC12190) and *S.* Typhimurium SL1344 with unknown phage type (NCTC 13347) cultures were purchased in freeze-dried form from the Health Protection Agency, United Kingdom. Prior to further testing, all *Salmonella* strains were sub-cultured on Columbia Horse Blood agar (Fort Richard Laboratories Ltd., Auckland, NZ) and nutrient agar (Merck, Germany), incubated at 37°C for 24 hours.

3.1.2 Colony morphology

All *Salmonella* isolates were streaked onto nutrient agar and onto blood agar plates. Their size and morphological characteristics were observed and recorded.

3.1.3 Serotyping

All 90 *S.* Typhimurium DT160 isolates and the *S.* Menston positive control were serotyped by a slide agglutination serological test using a polyclonal mixture of monoclonal mouse antibodies [Enteroclon Anti-*Salmonella* A-67, omnivalent, specific for O and Vi antigens and Enteroclon Anti-*Salmonella* poly-H phase 1 & 2 sera (SIFIN, Berlin, Germany)] as recommended by the manufacturer. Briefly, using a wire loop, a small amount of bacteria was picked from a well-isolated colony, transferred onto a glass slide and mixed with a drop of either poly-H or poly-O antiserum. The homogenous, slightly milky suspension was tilted back and forth for less than 20 times on the glass slide and the reaction read on a dark surface by naked eye within 1 minute (min) from the time of mixing. A drop of sterile physiological saline (0.85% NaCl) instead of the serum was used as a negative control and the test performed simultaneously with the test sample.

The *Salmonella* isolates that were negative for either poly-H or poly-O anti-*Salmonella* serum from SIFIN were retested using *Salmonella* H antiserum polyclonal rabbit a-z and *Salmonella* O antiserum polyA-I and Vi from BD (Becton Dickinson, NZ) as recommended by the manufacturer. The biochemical profiles of the same isolates were also tested with the API20E bacterial identification test kit (Biomerieux, I'Etoile, France), Triple Sugar Iron (TSI) agar and motility confirmed using the motility test. A well-isolated colony was used for inoculation of the TSI agar taking care to ensure that the bottom portion of the agar had also been inoculated. The cap of the tube was placed on loosely, and the tubes were incubated for 18 to 24 hours (hrs) at 37°C. The motility test was conducted by observing the growth of the bacteria in a semi-solid Bacto motility test medium (Becton Dickinson, United States of America, USA). The centre of the medium was inoculated with well-isolated colony and the

medium incubated for 24 hrs at 37°C. The API20E test consisting of 20 separate biochemical reactions and an additional oxidase test were used as per manufacturer's instructions. Briefly, inocula from the isolates were prepared by mixing a single bacterial colony with 5 millilitres (ml) of sterile distilled water until homogenous suspensions were formed. The biochemical reactions were inoculated with 200 µl suspensions, incubated at for 18-24 hrs at 37ºC and the results read based on colour changes for each reaction. The tests were separated into 3 groups and on each positive reaction a specific numerical value was assigned "1" for a positive reaction in the first test of the group "2" for a positive reaction in the second test of the group and "4" for a positive reaction in the third test of the group. A 7 digit number (e.g. 4305542) was obtained after the values corresponding to positive reactions in each group were added and the analytical profile index was analysed using APILab Plus software (Biomerieux, I'Etoile, France).

3.1.4 Antimicrobial sensitivity testing

All 90 *Salmonella* isolates and the control *E. coli* ATCC25922 were tested using the Kirby Bauer Disc Diffusion method following the "M31-A3 Performance Standards For Antimicrobial Disk And Dilution Susceptibility Tests For Bacteria Isolated From Animals; Approved Standards" (Clinical and Laboratory Standards Institute (CLSI), 2008) guidelines. Isolates were tested for susceptibility to the following antibiotics with the respective disc concentrations: 10 µg ampicillin (AMP), 30 µg amikacin (AK), 30 µg amoxicillin-clavulanic acid (AMC), 30 µg chloramphenicol (C), 30 µg cefoxitin (FOX), 10 µg cefpodoxime (CPD), 5 µg ciprofloxacin (CIP), 30 µg nalidixic acid (NA), 25 µg trimethoprim-sulfamethoxazole (SXT), 30 µg oxytetracycline (OXT) and 30 µg tetracycline (TET) antibiotic discs (Oxoid, NZ Ltd.). It should be noted that, although not included in the initial panel of antimicrobials, isolates were later tested against tetracycline as there were no guidelines available for oxytetracycline. Briefly, a sterile, non-toxic swab was used to collect between 4 to 5 well-separated colonies from an overnight culture streaked on a nutrient agar plate. The bacteria were suspended into 4 to 5 ml of sterile, normal saline and the turbidity of the suspension was adjusted with sterile saline to obtain a suspension visually similar to that of a 0.5 McFarland standard. The turbidity was read against a standard card with black lines on a white background. A new swab was dipped into the suspension and, following removal of excess inoculum by pressing the swab gently against the wall of the tube, bacteria were spread evenly on the Mueller Hinton agar plate (Fort Richard Laboratory Ltd., Auckland, NZ). No more than 6 drug-impregnated 6 mm discs were applied to the agar surface. An automatic dispenser was employed to ensure discs were no closer than 24 mm from centre to centre. Each disc was pressed gently with sterile forceps to ensure complete contact with the agar and the inoculated plates were incubated for 16 to 18 hrs at 37°C. The zones of inhibition were measured using a digital calliper and end points determined based on the areas showing no bacterial growth visible to the naked eye. The interpretation of the inhibition zones for AK, AMC, AMP, C, FOX, CPD, SXT, OXT and TET and *E. coli* ATCC25922 was based on M31-A3, CLSI 2008 and for NA and CIP on those described in M100-S20, CLSI 2010.

3.1.5 Statistical analysis

To assess concordance, an inter-rater agreement Kappa quadratic weighted analysis was performed for repeat antimicrobial susceptibility using MedCalc Software bvba version 11.4.20 (www.medcalc.be/manual/kappa.php, Belgium). Kappa analysis evaluates the agreement between two classifications on ordinal or nominal scales (Fleiss *et al.* 2003).

3.2. VIRULOTYPING

3.2.1 DNA extraction

DNA of *S.* Typhimurium was extracted by boiling bacteria in water as previously described by Hughes *et al.,* 2008, with some modifications. Briefly, genomic DNA was extracted from an overnight culture grown on a nutrient agar plate. Using a disposable wire loop, three to four colonies were picked up and suspended in 1 ml of 2% Chelex (BioRad Laboratories, USA) and mixed by vortexing. The negatively-charged Chelex resin binds divalent ions in the samples and may improve the efficiency of the PCR (Walsh *et al.* 1991). The mixture was heated at 95°C for 10 min using a heating block and the tube was cooled at room temperature and centrifuged at 12,470 *g* for 3 min. Approximately 800 µl of the supernatant containing extracted DNA was aliquoted and stored at -20°C until further used.

3.2.2 Polymerase chain reaction (PCR)

The PCR reaction was synthesised with primers, the sequences of which were previously published (Table 1), in a 25 µl mixture containing 5 µl crude DNA, 1X PCR reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.2 µM of each of the primers and, 0.5 U Platinum *Taq* polymerase (Invitrogen NZ Ltd., NZ). The PCR cycle parameters were as follows: 96°C for 2 min followed by 32 cycles at 94°C for 30 sec for denaturation, 55°C for 30 sec (annealing) and at 72°C for 30 sec (extension) applied for the *orfL, pefA, pipD, sopB, spiC,* and *sifA* genes and 1 min (extention) for the *invA, iroN, misL, prgH, sitC, and sopE* genes. A final extension at 72°C for 2 min was used for all genes.

3.2.3 Gel electrophoresis and documentation

Three microlitres of PCR products from *misL, orfL, pipD, pefA, sopB, spiC, and sifA* were electrophoresed on a 1.3% agarose gel (AppliChem GmbH, Germany) and run at a constant 70 V for 105 min. The PCR products for the remaining genes were electrophoresed on a 1.8% agarose gel and run at constant 70 V for 120 min. A 1 kb plus DNA ladder (Invitrogen NZ Ltd., NZ) was used as the molecular marker. The PCR products were visualised with ethidium bromide as per the manufacturer's recommendations and documented using Gel Doc with Quantity One 4.6.2 (Bio-Rad, Segrate, Milan, Italy) software. (Figure 1)

3.2.4 PCR sequence analysis

To confirm that the PCR products were the targeted sequences, PCR products obtained from the two control strains, *S.* Typhimurium SL1344 and F98, using the primers described in Table 1 were sequenced. The PCR products were purified using an ethanol precipitation technique and products quantified using a NanoDrop Spectrophotometer ND-1000 (Biolab, USA). Separate stocks were prepared for forward and reverse primers at a concentration of 3.2 pmol/15 µl. The templates were prepared to a concentration of 2 ng/100 bp/15 µl then premixed with the forward primer in a total reaction volume of 15 µl by adding sterile distilled water. The same method was applied for the reverse primer mixture. Sequencing was performed by capillary separation on an ABI3730 DNA Analyzer at the Allan Wilson Centre Genome Services, Massey University, Palmerston North, NZ.

The nucleotide sequences from all targeted genes were aligned with the previously published sequences of *S.* Typhimurium SL1344 (Wellcome Trust Sanger Institute, UK) using the BLAST nucleotide algorithm at the National Center for Biotechnology Information, United States National Library of Medicine.

3.3. PULSED FIELD GEL ELECTROPHORESIS (PFGE)

3.3.1 Preparation of plugs

All 90 DT160 isolates and the 3 control strains, *S.* Braenderup H9812, *S.* Typhimurium SL1344 and F98, were placed on Columbia Horse Blood agar (Fort Richard) and incubated for 24 hrs at 37ºC. Two millilitres of a cell suspension buffer (CSB) (100 mM Tris, 100 mM EDTA, pH 8.0) was transferred into a 12 mm x 75 mm Falcon tube and a small portion of a bacterial colony from an agar plate was transferred to the tube with a cotton swab moistened with CSB and then mixed until an even suspension was formed. The concentration of the cells was adjusted to between 0.40 and 0.45 optical density using a Dade Microscan Turbidity Meter and read at 610 nanometres. Four hundred microlitres of the adjusted suspension was transferred into a 1.5 ml microcentrifuge Eppendorf tube and gently mixed with 20 mg/ml Proteinase K (Bioline GmbH, Germany). One percent SeaKem Gold (SKG) agarose (Cambrex Bio Science, Inc. Rockland, USA) in 0.5 X Tris-Borate EDTA (TBE) buffer was melted in a microwave oven on a low-medium power for 20-30 sec and 400 µl of the molten agarose mixed with the cell suspension in a microcentrifuge tube. The mixture was dispensed into the reusable plug mould where it was solidified at room temperature for 10-15 min.

3.3.2 Lysis and washing of cells in agarose plugs

Five millilitres of cell lysis buffer (CLB) (50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl), 0.5 mg/µl of Proteinase K, and solidified plugs were mixed into a 50 ml tube and incubated in a water bath for 120 min between 54 and 55ºC with constant agitation at 150-175 revolutions per minute (rpm). Following incubation, the supernatant was decanted, between 10 and 15 ml of pre-heated sterile water was added and the mixture incubated at the above conditions. This washing step was repeated one more time. Preheated TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the tube and the mixture incubated at the above conditions. This step was repeated three times and the plugs were transferred into a microcentrifuge tube and stored at 4ºC in 5-10 ml of TE buffer until further use.

3.3.3 Restriction digestion of DNA in agarose plugs with *Xba*I and *Spe*I

Plugs were removed from the TE buffer, placed on a glass slide and cut into 2.0 to 2.5 mm lengths. Each plug was transferred to a 1.5 ml Eppendorf tube and 175 µl of sterile water, 20 µl of restriction buffer, and 50 U of *Xba*I restriction enzyme (Hoffmann-La Roche Ltd., Germany) were added as recommended by the PulseNet PFGE manual (Centers for Disease Control and Prevention (CDC) 2009)). The above mixture was incubated in a water bath for 120 min at 37ºC. The same procedure was applied for *Spe*I 50 U (New England Biolabs Inc.) restriction enzyme.

3.3.4 PFGE electrophoresis and gel documentation

Electrophoresis was performed on a contour-clamped, homogenous electric field (CHEF) Mapper electrophoresis system (BioRad, USA), the cooling module of which was set at 14ºC with the pump turned on 30 min prior to the beginning of a gel run. The plugs, digested with either *Xba*I or *Spe*I, were loaded into the wells in an agarose gel (1% SKG agarose in 0.5 X TBE), ensuring an absence of air bubbles, and run for 18 hrs at electrophoresis conditions recommended for *Salmonella* restriction with *Xba*I by the PulseNet PFGE manual. Finally, the gel was stained with ethidium bromide for approximately 10 min followed by de-staining with distilled water for approximately 30 min. The DNA banding patterns were visualised with a Gel Doc and analysis performed with BioNumerics Version 2.0 software (Applied Maths NV, USA). PFGE analysis is based on a comparison between selected isolates and the percentage similarity between banding patterns. The percentage similarity was calculated using the Dice similarity coefficient method with a 1.5% optimisation and 2% band filtering

tolerance window. A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

4. Results

4.1. COLONY MORPHOLOGY AND SEROLOGY

The colonies of all *Salmonella* isolates were approximately 2-3 mm in size and appeared moist and raised on nutrient agar, and grey, round and non-haemolytic on blood agar regardless of their species of origin.

At the time of initial isolation, all isolates were serotyped by ESR using the Kauffmann-White Scheme. Serological confirmation was undertaken in this laboratory using poly-O and poly-H sera. Eighty six isolates showed agglutination with poly-O and poly-H sera from SIFIN. Three of the isolates, when tested with sera from SIFIN were negative for agglutination when tested with poly-H but positive for poly-O, and one isolate was negative when tested with poly-O but positive with poly-H. These four isolates agglutinated with poly-H and poly-O sera from BD and were also confirmed to be *Salmonella* spp. with the API20E biochemical, TSI and motility test (Table 2). The API20E profile index for the four isolates indicated 76% probability that the isolates are indeed *Salmonella* spp. On TSI agar the four isolates produced red (alkaline) slant, yellow (acid) butt with blackening of the medium as a result of hydrogen sulphide production which is indicative of members of *Enterobacteriaceae* family.

4.2. ANTIMICROBIAL SENSITIVITY TESTING

Due to the growing concerns related to antimicrobial susceptibility of *S.* Typhimurium DT160, all isolates were tested against 11 antimicrobials that are often used for treatment of human patients or animals (Tables 3 and 4). Except for tetracycline and oxytetracycline, all isolates were susceptible to these antimicrobials. Based on the reference standards for tetracycline described in CLSI M31-A3, 26 isolates fell within the intermediate range of susceptibility to tetracycline and 8 isolates within the same range for susceptibility to oxytetracycline (Table 5).

Kappa analysis for the antimicrobial susceptibility test indicated good agreement between the two technicians who each performed the tests for all antimicrobials, with the exception of ciprofloxacin for which the borders of the inhibition zones were not clear and therefore not easily measured (Table 6).

4.3. VIRULOTYPING AND PFGE

S. Typhimurium SL1344 was chosen as one of the two control strains as it is well characterised, and the genome is completely sequenced and available for analysis. As expected, SL1344 was positive for the *sopE gene* whilst F98 was PCR negative. Sequence analysis of our 12 PCR products confirmed that the primers amplified the desired target genes and demonstrated that they shared a high degree of similarity to the published gene sequences for *S.* Typhimurium SL1344 and, that the products were of the expected length. The reference strain SL1344 was the only strain to carry *sopE.* Most of the isolates carried the 11 virulence genes. The genes *misL, orfL, pipD, prgH, sitC, spiC* and *sopB* were identified in every isolate. In addition to the *sopE* gene, six isolates did not have one of the following genes; *invA, iroN, pefA* and *sifA* (Figure 2) and of these isolates, four originated from sparrows and two from humans.

The pulsed field profiles for all 90 isolates using both *Xba*I and *Spe*I restriction enzymes were analysed using Bionumerics. Visual inspection of the PFGE gels revealed at least five different *Spe*I and six different *Xba*I patterns, but there was evidence of a single common two-enzyme profile that accounted for the majority of isolates.

Cluster analysis, using a cut-off value of 95% similarity based on the Dice index, identified five *Spe*I and five *Xba*I clusters. When combined with the virulence gene PCR assay there were a total of 11 different genotype profiles. However, genotype profile AA1 accounted for 78/90 (86.7%) of isolates. The second most common was genotype profile AA2 which accounted for 3/90 (3.3%). These isolates were PCR negative for *sifA* and comprised two sparrow isolates and one human isolate. The remaining nine profiles were represented by single isolates (Figure 2).

5. Discussion

Ninety isolates were compared in an attempt to identify possible clonal relatedness between isolates obtained from avian species, from environments associated with poultry, and from humans. The relatedness was determined through a combination of techniques including virulotyping, PFGE patterns and antibiotic susceptibility.

Proper characterisation of *Salmonella* isolates is essential for investigations of *Salmonella* outbreaks as non-typhoidal *Salmonella* strains have reached epidemic proportions in many countries despite improvements in sanitation and hygiene. Precise strain identification is a prerequisite for implementing efficient surveillance and preventative measures aimed to reduce the spread and eradicate the source of infection (Threlfall & Frost 1990).

While four *Salmonella* isolates tested negative for either for poly-H or poly-O sera from SIFIN, all of them were positive to poly-H and poly-O sera from BD. The conventional serotyping method is based on bacterial surface antigens and has a few limitations, such as subjective interpretation of the results based on the level of experience of the individual performing the test and quality of the antisera. The ambiguity of the results could also be due to the different preparations of the antisera. The polyclonal anti-*Salmonella* rabbit sera from BD may have broader antigen coverage than the mouse serum from SIFIN which is a mixture of monoclonal antibodies. Also, some *Salmonella* isolates may lack the antigen specificity needed for this product resulting in a negative reaction. In addition, the nature of the antibodies might have been affected by the different animal species in which the antibodies were generated.

Results from the antimicrobial tests provided no indication of multidrug resistance among the DT160 isolates tested. However, a total of 34 DT160 isolates characterised in this study fell within the intermediate range of susceptibility to either tetracycline or oxytetracycline. This result is slightly different to the one reported in 2003 for the same definitive type in NZ (Thornley *et al.* 2003) where the isolates were found to be susceptible to all antimicrobials tested, including tetracycline. This may be a concern since resistance towards the tetracycline group has already been reported in Taiwan for both human and animal isolates (Tsen *et al.* 2002). Due to the rapid development of multi-drug resistance (MDR) among *Salmonella* strains, it is possible that DT160 may acquire resistance to a number of antibiotics used frequently to treat gastrointestinal infections both in humans and in farm animals. The reported rates of drug-resistant *Salmonella,* including MDR strains, have increased considerably. There is a considerable concern that once MDR develops in some of the variants the resistance genes are retained in the population even when antimicrobial drugs are withdrawn (WHO 2005). The use of chloramphenicol as an antibiotic growth promoter was banned in the US and European Union partly because of its overuse in animal production and the likely contribution to increased resistance in *Salmonella* Typhi (Hughes & Heritage 2004). To establish infection, *Salmonella* spp. may require up to 200 pathogenic genes (Bowe *et al.* 1998). A panel of 12 potential virulence genes were targeted for PCR-based virulotyping. One human and two sparrow isolates were PCR negative for the *sifA* gene that is responsible for invasion and the survival of the *Salmonella* in macrophages (Brumell *et al.* 2001; Galan *et al.* 1992). However, the apparent absence (or sequence divergence) of either *invA* or *sifA* genes does not necessarily mean that *Salmonella* would be unable to either invade or survive respectively, as other genes may also encode for these functions. The *iroN* gene is associated with iron acquisition and, while present in all phylogenetic lineages of *S. enterica,* it is absent in other closely related species like *S. bongori* and *E. coli* (Baumler *et al.* 1998). Interestingly in this study, only one isolate was PCR negative for the *pefA* gene. This plasmid-encoded gene is essential for *Salmonella* attachment to intestinal epithelial surfaces (Baumler *et al.*

1997). The absence of targeted genes has to be interpreted with caution as a nucleotide mismatch between the primers and the target genomic sequence may lead to a false negative result. The *sopE* gene coding for translocator effector protein can be present in *Salmonella* isolates related to clinical disease, particularly in humans (Hopkins & Threlfall 2004). This gene, however, appeared to be absent from all tested isolates except the control SL1344 strain.

PFGE sub-typing can be used to trace the clonal relationship between isolates. Interpretation of PFGE data can be highly subjective so, to minimise potential inter-laboratory variation, certification from the PulseNet Aotearoa NZ, which is recognised by the CDC, USA, was obtained. The enzyme initially selected for PFGE was *Xba*I as this infrequently-cutting enzyme produces well defined and reliable restriction DNA banding patterns of the *Salmonella* genomes. Due to the expected high genetic similarity among the isolates, a second restriction digestion was performed with *Spe*I enzyme to increase the overall discriminatory power of PFGE (Ribot *et al.* 2006). Analyses of the isolates digested with either *Xba*I or *Spe*I enzymes revealed five *Spe*I and five *Xba*I clusters that, when combined with the virulence gene PCR assays, resulted in 11 different genotype profiles. However, as 86.7% of isolates grouped to the AA1 genotype profile, it suggests that the majority of isolates were genetically very similar and that this has remained relatively stable over the time period sampled. Our findings are consistent with a recent report (Dyet *et al*.2010) using multilocus variable-number tandem-repeat analysis (MLVA). There was no obvious correlation between genotype profile, virulotype, source or date of isolation (Figure2), although most of the uncommon genotypes were found in human and wild bird sources.

6. Conclusion

Our data suggest that there are no indications for multi-drug resistance among the 90 DT160 isolates examined in this study. PFGE profiles, virulence gene PCR assay results and sensitivity to the various antimicrobials tested indicate the majority of the *Salmonella* DT160 isolates were very closely related and that isolates grouping in the genotype profile AA1 were predominant (87% of isolates), representing a dominant epidemiologically-important clone that has persisted in multiple hosts, including avian wildlife and poultry. The small proportion of isolates that were distinguishable from genotype AA1 each occurred at low prevalence and in multiple hosts, indicating that genomic variants arose in the population, but this did not result in the evolution and emergence of a new dominant, host-associated clone. No obvious correlation was found between the source and the date of isolation and any of the other characteristics examined. Finally, the overall genetic similarity between the majority of isolates from avian, human and poultry environments in NZ in the 1999 and 2009 time period suggests that either the same or, very closely related isolates, circulated freely between a range of animal species.

7. Acknowledgements

We would like to thank Dr Phil Carter and Dr Muriel Dufour from ESR Ltd for supplying the isolates used in this study. We thank Sharina Omar for her contribution to report writing as well as laboratory work and Errol Kwan, Lynn Rogers, Rebecca Pattison and Rukhshana Akhter for their excellent technical assistance, and for Dr Eve Pleydell for technical advice and proof reading. This work was supported by grant 11406 from the Ministry of Agriculture and Forestry, Biosecurity, NZ.

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9. Appendices

Table 1. Virulence genes with the expected PCR product sizes used to virulotype *Salmonella* isolates.

Table 2. Results of the four isolates tested negative for either poly-H or poly-O sera from SIFIN and further evaluated with sera from BD, TSI and motility tests.

Table 3. Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates.

Table 4. Zone diameter interpretive standard and our quality control ranges of antimicrobial disc susceptibility test for the reference strain *Escherichia coli* ATCC25922 on Mueller Hinton Agar.

Table 5. Summary of the 26 isolates that were within the intermediate range for sensitivity to tetracycline and 8 isolates within the same range for sensitivity to oxytetracycline.

Table 6. Twenty five percent randomly selected *Salmonella* isolates were retested for antibiotic sensitivity against all antibiotics used in the first experiment. Measurements were taken from the same two technicians independently from each other.

Figure 1. Virulotyping and representative gels for the twelve virulent genes and the two *Salmonella* control strains SL1344 and F989 used in this study.

Figure 2. Genotypes of Salmonella DT160 based on two-enzyme PFGE and virulence gene PCR.

Table 1. Virulence genes with the expected PCR product sizes used to virulotype *Salmonella* isolates.

Gene	Salmonella Pathogenicity Island (SPI)	Gene function	Primers (5' to 3')	Predicted product size (bp)	Reference
iroN	na	Iron acquisition	*F:ACTGGCACGGCTCGCTGTCGCTCTAT **R:CGCTTTACCGCCGTTCTGCCACTGC	1205	Skyberg et al. 2006
invA	SPI-1	Type III secretion system	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC	1070	Skyberg et al. 2006
sitC	SPI-1	Iron transport	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	768	Skyberg et al. 2006
prgH	SPI-1	Type III secretion system	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC	756	Skyberg et al. 2006
sopE	SPI-1	Type III secreted effector protein	F:TCCAAAAACAGGAAACCACAC R:TCAGTTGGAATTGCTGTGGA	642	Hopkins &Threfall 2004
misL	SPI-3	Survival in macrophages	F:GTCGGCGAATGCCGCGAATA R:GCGCTGTTAACGCTAATAGT	561	Gassama-Sow et al. 2006

N.B.: PCR product size is approximate and based on SL1344 genome; may vary between strains and forward and reverse primer designation is based on orientation within the SL1344 published genome.

Table 1. (cont.) Virulence genes with the expected PCR product sizes used to virulotype *Salmonella* isolates.

Gene	Salmonella Pathogenicity Island (SPI)	Gene function	Primers (5' to 3')	Predicted product size (bp)	Reference
sifA	SPI-2	Type III secretion system	F:TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	Skyberg *et al.* 2006
pipD	SPI-5	Type III secreted effector-associated with SPI-1 system	F:CGGCGATTCATGACTTTGAT R:CGTTATCATTCGGATCGTAA	399	Gassama-Sow *et al.* 2006
orfL	SPI-4	Adhesin /Autotransporter	F:GGAGTATCGATAAAGATGTT R:GCGCGTAACGTCAGAATCAA	332	Gassama-Sow *et al.* 2006
spiC	SPI-2	Type III secretion system	F:CCTGGATAATGACTATTGAT R:AGTTTATGGTGATTGCGTAT	301	Gassama-Sow *et al.* 2006
sopB	SPI-1	Type III secreted effector protein	F: CGGACCGGCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTGAGTGTATT	220	Skyberg *et al.* 2006
pefA	plasmid	fimbriae	F:GCGCCGCTCAGCCGAACCAG R:CAGCAGAAGCCCAGGAAACAGTG	156	Skyberg *et al.* 2006

N.B.: PCR product size is approximate and based on SL1344 genome; may vary between strains and forward and reverse primer designation is based on orientation within the SL1344 published genome.

Table 2. Results of the four isolates tested negative for either poly-H or poly-O sera from SIFIN and further evaluated with sera from BD, TSI and motility tests.

Internal laboratory number	Sources	Serotyping				API20E test	TSI agar	Motility
		SIFIN		BD				
		poly-H	poly-O	poly-H	poly-O			
DT22	*Human*	N	P	P	P	*4305542=SSP	**R/Y/H_2S+	NM
DT50	*White eye*	P	N	P	P	4305542=SSP	R/Y/H_2S+	NM
DT86	*Sparrow*	N	P	P	P	4305542=SSP	R/Y/H_2S+	NM
DT89	*Human*	N	P	P	P	4305542=SSP	R/Y/H_2S+	NM

*4305542=SSP is an API20E analytical profile index
** R, Alkaline, Y, Acid, H_2S hydrogen sulphide production
NM, Non-motile
P, positive; N, negative
TSI, Triple Sugar Iron agar

Table 3. Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates.

Internal lab. no.	Antibiotics										
	AK	AMC	AMP	C	FOX	CIP	CPD	NA	OT	SXT	TET
DT1	*22	27	24	29	27	28	26	21	21	23	21
DT2	25	24	23	27	24	36	26	22	18	27	19
DT3	24	27	26	25	27	34	26	22	16	27	19
DT4	25	28	27	26	28	37	28	24	19	27	21
DT5	24	27	25	25	26	36	27	25	18	25	19
DT6	24	27	25	26	26	39	25	24	18	26	20
DT7	21	22	22	23	23	34	23	19	16	20	20
DT8	21	25	24	26	26	33	26	24	17	23	20
DT9	22	23	20	27	26	36	26	22	19	23	20
DT10	21	21	22	25	23	33	24	20	18	21	21
DT11	24	27	24	28	25	37	26	24	23	26	21
DT12	23	25	24	26	25	35	26	24	23	24	22
DT13	19	23	22	24	23	33	22	22	19	22	21
DT14	21	24	21	27	23	34	25	23	18	21	20
DT15	22	25	26	22	25	34	25	23	21	26	20
DT16	21	24	24	29	24	37	26	25	21	24	20
DT17	22	28	24	27	25	37	29	24	22	25	19
DT18	23	27	24	29	26	38	27	23	22	25	20
DT19	22	27	24	26	27	37	28	26	21	25	21
DT20	23	27	24	26	26	38	28	26	21	25	20
DT21	24	25	24	26	28	35	29	25	23	24	20
DT22	25	26	23	29	26	37	28	25	24	21	21
DT23	24	26	23	26	26	37	27	25	23	25	20
DT24	26	28	26	29	28	39	28	26	25	28	19
DT25	22	28	23	26	26	36	28	23	23	27	19
DT26	21	24	21	25	22	34	23	22	21	21	19
DT27	22	24	21	25	23	34	25	23	22	22	18
DT28	22	23	23	25	25	34	25	24	22	21	20
DT29	23	23	23	26	24	34	24	22	21	23	20
DT30	22	24	22	25	24	34	25	24	21	21	21
DT31	20	24	21	23	23	32	23	22	20	20	19
DT32	22	23	22	25	22	33	24	22	20	22	21
DT33	23	24	23	26	24	35	25	24	21	23	19
DT34	21	23	22	24	24	33	24	22	21	22	19
DT35	20	24	21	23	23	32	23	22	20	20	20
DT36	24	25	24	27	25	38	28	25	23	27	18
DT37	25	27	24	29	27	39	28	27	24	29	20
DT38	24	26	24	27	26	37	27	25	22	27	22
DT39	23	26	23	27	27	36	27	24	22	25	19
DT40	23	25	23	26	26	36	27	25	23	24	19

Table 3. (cont.) Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates.

Internal lab. no.	Antibiotics										
	AK	AMC	AMP	C	FOX	CIP	CPD	NA	OT	SXT	TET
DT41	25	28	24	27	26	36	30	27	23	27	19
DT42	23	26	24	27	26	37	27	25	24	26	20
DT43	24	26	25	27	26	36	28	25	24	27	18
DT44	26	29	26	29	27	39	30	27	24	28	18
DT45	24	27	23	27	27	39	28	27	24	26	20
DT46	26	29	26	29	27	37	29	26	25	28	19
DT47	24	27	25	26	26	36	27	25	25	26	18
DT48	24	26	24	27	26	35	27	25	24	25	19
DT49	23	28	24	28	27	36	28	26	25	26	19
DT50	23	27	23	26	27	36	25	24	24	23	19
DT51	24	26	25	29	26	36	28	24	23	29	18
DT52	24	27	24	27	26	36	29	26	25	26	18
DT53	22	28	25	27	27	37	28	26	25	25	19
DT54	24	26	24	26	26	35	26	26	23	26	18
DT55	24	26	25	27	26	35	28	26	24	25	19
DT56	23	26	25	27	26	37	29	26	25	26	19
DT57	24	26	26	27	27	37	28	26	25	26	18
DT58	23	26	25	28	27	38	27	25	25	27	18
DT59	24	26	25	28	27	38	29	25	25	26	19
DT60	24	26	25	26	27	35	27	24	25	26	18
DT61	24	28	25	24	28	38	29	27	24	28	18
DT62	24	26	25	26	27	38	28	26	25	27	18
DT63	24	28	25	29	27	37	28	27	25	28	17
DT64	24	27	24	25	27	36	24	25	23	26	18
DT65	25	26	25	26	27	38	27	26	26	26	20
DT66	25	28	26	27	28	38	28	26	26	27	19
DT67	24	27	24	26	28	36	29	25	25	27	20
DT68	24	26	25	25	28	37	27	26	25	26	19
DT69	25	27	25	25	27	34	27	25	25	26	18
DT70	24	27	24	25	27	36	28	24	23	27	18
DT71	25	27	25	26	28	39	26	28	24	29	20
DT72	25	27	24	27	28	37	29	26	24	27	17
DT73	24	26	23	27	27	36	28	26	25	28	18
DT74	23	25	25	28	26	35	27	25	24	19	19
DT75	24	27	25	26	26	38	27	25	25	29	16
DT76	23	26	25	27	25	37	26	24	21	24	17
DT77	23	26	23	26	25	35	28	23	23	20	16
DT78	24	27	22	25	27	35	27	25	24	23	18
DT79	25	27	24	24	26	36	28	24	24	24	19
DT80	23	26	26	29	27	36	27	25	23	26	18

Table 3. (cont.) Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates.

Internal lab. no.	Antibiotics										
	AK	AMC	AMP	C	FOX	CIP	CPD	NA	OT	SXT	TET
DT81	24	25	23	26	25	35	26	24	23	23	19
DT82	23	27	23	24	27	35	27	25	25	22	17
DT83	24	27	25	24	27	36	27	26	25	24	19
DT84	23	27	23	28	27	37	28	25	25	26	19
DT85	23	26	23	28	27	35	28	25	24	24	19
DT86	24	27	24	26	27	37	26	25	25	26	19
DT87	23	26	23	25	25	36	28	26	25	25	19
DT88	23	25	22	26	25	36	26	23	23	25	18
DT89	23	25	22	25	25	35	26	25	23	22	19
DT90	23	25	22	25	25	35	26	24	24	21	19
*E. coli***	23-25	20-23	16-21	23-25	26-28	33-39	25-28	23-27	22-25	24-29	20-23

AK, Amikacin; AMC, Amoxicillin-clavulanic acid; AMP, Ampicillin; C, Chloramphenicol; FOX, Cefoxitin; CIP, Ciprofloxacin; CPD, Cefpodoxime; OT, Oxytetracycline; NA, Nalidixin acid; SXT, Trimethoprim-sulfamethazole; TET, Tetracycline
*zone diameter in millimetres
***E. coli* = ATCC 25922

Table 4. Zone diameter interpretive standard for *Enterobacteriaceae* and our quality control ranges of antimicrobial disc susceptibility test for the reference strain *E. coli* ATCC25922 on Mueller Hinton Agar.

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			*E. coli*
		S	I	R	
Amikacin, AK*	30	≥17	15-16	≤14	19-26
Amoxicillin-clavulanic acid, AMC	20/10	≥18	14-17	≤13	18-24
Ampicillin, AMP	10	≥17	14-16	≤13	16-22
Cefoxitin, FOX**	30	≥18	15-17	≤14	23-29
Cefpodoxime, CPD	10	≥21	18-20	≤17	23-28
Chloramphenicol, C	30	≥18	13-17	≤12	21-27
Ciprofloxacin, CIP**	5	≥21	16-20	≤15	32-40
Nalidixic acid, NA**	30	≥19	14-18	≤13	na
Tetracycline	30	≥19	15-18	≤14	18-25
Trimethoprim-sulfamethoxazole, SXT	1.25/23.75	≥16	11 15	≤10	23-29

S, susceptible; I, Intermediate; R, resistant
*CLSI M31-A3; **CLSI M100-S20
µg, microgram; mm, millimetres
na, not available

Table 5. Summary of the 26 isolates that were within the intermediate range for sensitivity to tetracycline and 8 isolates within the same range for sensitivity to oxytetracycline.

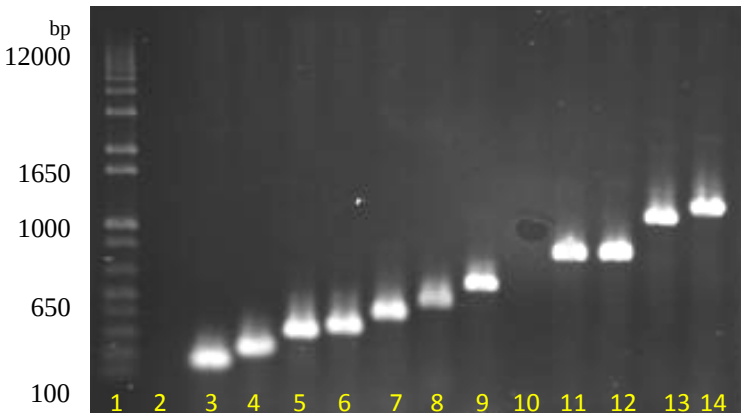
Internal laboratory number	TET	Internal laboratory number	TET	Internal laboratory number	OT
DT27	*18	DT63	17	DT2	18
DT36	18	DT64	18	DT3	16
DT43	18	DT69	18	DT5	18
DT44	18	DT70	18	DT6	18
DT47	18	DT72	17	DT7	16
DT51	18	DT73	18	DT8	17
DT52	18	DT75	16	DT10	18
DT54	18	DT76	17	DT14	18
DT57	18	DT77	16		
DT58	18	DT78	18		
DT60	18	DT80	18		
DT61	18	DT82	17		
DT62	18	DT88	18		

*zone in millimetres
**CLSI M31- A3
OT, Oxytetracycline disc content 30µg; TET, Tetracycline disc content 30µg.

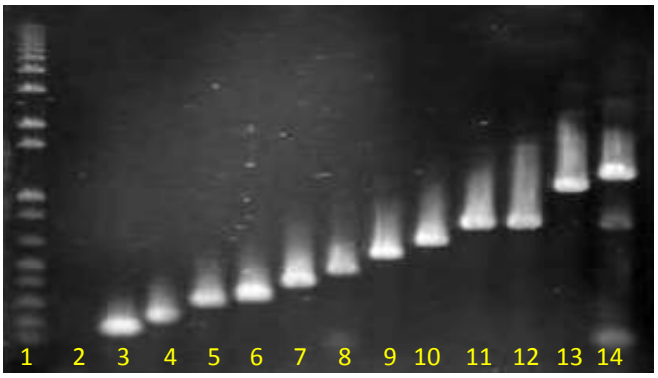
Table 6. Twenty five percent randomly selected *Salmonella* isolates were retested for antibiotic sensitivity against all antibiotics used in the original panel of antimicrobials. Measurements were taken from the same two technicians independently from each other.

Internal laboratory number	Antibiotics																			
	AK		AMC		AMP		C		FOX		CIP		CPD		NA		OT		SXT	
	*1	**2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
DT1	***21	21	26	25	19	18	29	29	24	23	33	35	26	26	24	23	22	21	26	26
DT3	24	24	26	25	19	18	27	26	25	26	24	37	26	26	25	24	21	18	24	24
DT10	24	23	25	26	20	20	25	26	25	26	35	37	27	27	25	24	23	24	26	25
DT12	23	23	27	27	21	19	26	25	26	26	36	35	26	24	25	25	25	24	24	24
DT14	22	22	24	23	20	19	25	27	22	23	33	37	25	25	22	23	21	21	23	23
DT15	22	22	25	25	20	19	25	26	25	24	33	36	26	26	23	22	21	20	23	24
DT27	22	23	25	25	20	18	22	24	24	25	32	35	24	24	24	23	25	24	23	22
DT32	22	22	24	23	19	19	26	26	24	23	34	35	25	25	23	22	22	22	23	22
DT38	21	21	25	25	20	20	24	24	22	23	29	34	26	24	23	23	22	21	23	22
DT43	21	22	23	24	19	19	24	26	22	22	34	36	25	24	22	22	21	21	24	23
DT46	21	23	23	24	19	17	24	25	24	23	33	36	26	26	23	23	22	20	24	25
DT47	22	22	24	23	19	17	23	26	21	23	32	36	23	25	22	21	20	20	22	23
DT50	24	24	27	27	19	18	24	25	25	25	31	34	23	25	24	25	23	23	24	25
DT54	22	23	22	25	17	18	23	25	22	23	33	35	24	25	24	23	22	21	22	23
DT56	22	22	25	25	19	17	26	26	25	24	31	36	23	24	24	23	22	22	23	23
DT62	24	24	25	25	19	18	26	26	25	24	34	36	24	25	25	24	24	22	24	24
DT68	21	22	28	28	21	20	26	26	24	25	35	37	26	25	26	25	25	23	24	23
DT72	22	23	25	24	19	18	26	25	24	24	33	36	26	26	24	23	19	21	23	23
DT79	24	24	27	26	20	18	26	26	25	26	36	37	27	27	24	24	25	22	26	26
DT85	23	23	25	25	18	18	23	27	24	25	35	36	27	26	24	24	22	21	24	24
DT87	23	22	24	24	18	17	23	23	24	23	32	36	25	26	24	24	21	21	23	23
DT90	24	24	25	25	18	16	24	24	24	24	35	34	24	24	24	23	22	22	21	22
E.coli	20	21	19	19	16	16	23	24	24	25	30	32	23	24	22	22	19	20	21	22

*1, Technician; **2, Technician; ***millimetres
AK, Amikacin; AMC, Amoxicillin-clavulanic acid; AMP, Ampicillin; C, Chloramphenicol; FOX, Cefoxitin; CIP, Ciprofloxacin; CPD, Cefpodoxime; OT, Oxytetracycline; NA, Nalidixin acid; SXT, Trimethoprim-sulfamethazole.



***Salmonella enterica* Typhimurium F98**



***Salmonella enterica* Typhimurium SL1344**

Lane	Gene	Size (bp)	Results (-/+)		Lane	Gene	Size (bp)	Results (-/+)	
			F98	SL1344				F98	SL1344
1	DNA marker		+	+	8	*sifA*	449	+	+
2	PCR mix (-) DNA		-	-	9	*misL*	561	+	+
3	*pefA*	156	+	+	10	*sopE*	642	-	+
4	*sopB*	220	+	+	11	*prgH*	756	+	+
5	*spiC*	301	+	+	12	*sitC*	768	+	+
6	*orfL*	332	+	+	13	*invA*	1070	+	+
7	*pipD*	399	+	+	14	*iroN*	1205	+	+

Figure 1. Virulotyping and representative gels for the 12 virulence genes and the 2 *Salmonella* control strains SL1344 and F989 used in this study.

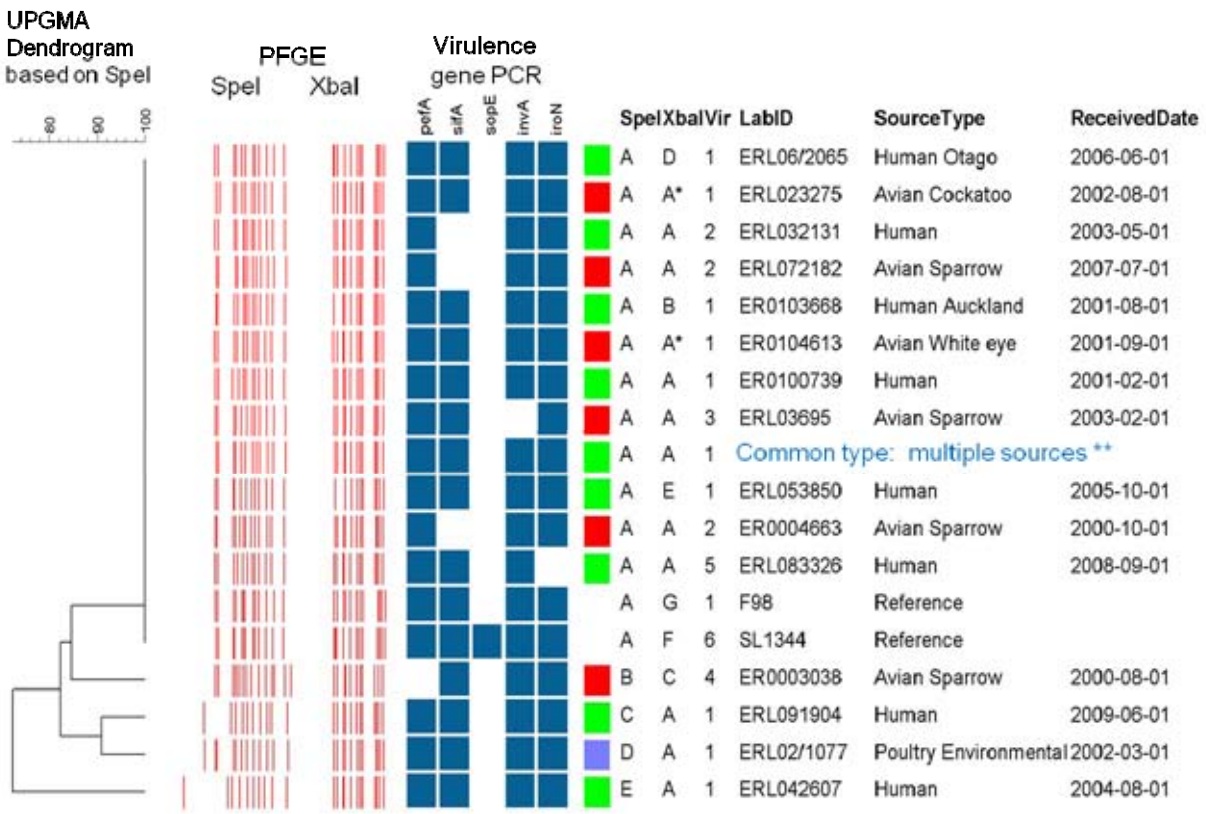


Figure 2. Genotypes of Salmonella DT160 based on two-enzyme PFGE and virulence gene PCR. The UPGMA dendrogram was based on the *Spe*I band pattern, using a Dice similarity coefficient calculated using 1.5% optimisation and 2% band filtering tolerance. Combined profile AA1 was the most common, accounting for 78/90 (87%) of isolates (only four representatives of this profile are provided in the diagram).

10. Supplementary Information

Table 7. Key table showing corresponding Environmental Science and Research (ESR) isolate number from the ERL, Enteric Reference Laboratory (ERL) to our internal laboratory identifier number (DT).

Internal laboratory number	ESR isolate number	Received date	Source type
DT1	ERL062065	08-Jun-06	Human
DT2	ERL052816	22-Aug-05	Poultry Environmental
DT3	ER003370	18-Aug-00	Human
DT4	ERL021077	08-Mar-02	Poultry Environmental
DT5	ERL091067	12-Mar-09	Poultry Environmental
DT6	ERL091081	17-Mar-09	Human
DT7	ERL042607	27-Aug-04	Human
DT8	ERL063767	28-Sep-06	Sparrow
DT9	ERL053052	31-Aug-05	Sparrow
DT10	ER016216	19-Dec-01	Poultry Environmental
DT11	ERL063449	12-Sep-06	Human
DT12	ERL06257	19-Jan-06	Human
DT13	ERL033614	04-Sep-03	Poultry Environmental
DT14	ERL091904	04-Jun-09	Human
DT15	ERL06292	24-Jan-06	Poultry Environmental
DT16	ERL073108	05-Oct-07	Human
DT17	ERL073368	26-Oct-07	Poultry Environmental
DT18	ERL05998	23-Mar-05	Poultry Environmental
DT19	ERL052645	10-Aug-05	Sparrow
DT20	ERL050172	25-Jan-05	Poultry Environmental
DT21	ERL064025	25-Oct-06	Poultry Environmental
DT22	ER004560	09-Oct-00	Human
DT23	ER012226	24-May-01	Poultry Environmental
DT24	ER015215	18-Oct-01	Cockatoo
DT25	ER015709	15-Nov-01	Sparrow
DT26	ERL023783	10-Sep-02	Human
DT27	ERL084557	18-Dec-08	Human
DT28	ERL021519	27-Mar-02	Poultry Environmental
DT29	ERL091580	28-Apr-09	Poultry Environmental
DT30	ERL022680	17-Jun-02	Sparrow

Table 7. (cont.) Key table showing corresponding Environmental Science and Research (ESR) isolate number from the ERL, Enteric Reference Laboratory (ERL) to our internal laboratory identifier number (DT).

Internal laboratory number	ESR isolate number	Received date	Source type
DT31	ERL023275	08-Aug-02	Cockatoo
DT32	ERL043701	29-Nov-04	Duck
DT33	ERL09880	03-Mar-09	Sparrow
DT34	ERL022681	17-Jun-02	Sparrow
DT35	ERL005619	12-Dec-00	Poultry Environmental
DT36	ERL082664	08-Aug-08	Sparrow
DT37	ERL064109	01-Nov-06	Poultry Environmental
DT38	ERL053554	29-Sep-05	Human
DT39	ERL072176	24-Jul-07	Human
DT40	ERL082092	11-Jun-08	Poultry Environmental
DT41	ERL032131	07-May-03	Human
DT42	ERL0919	05-Jan-09	Avian
DT43	ERL072182	24-Jul-07	Sparrow
DT44	ERL050427	10-Feb-05	Human
DT45	ER013668	16-Aug-01	Human
DT46	ERL032893	11-Jul-03	Poultry Environmental
DT47	ERL084152	18-Nov-08	Cockatoo
DT48	ERL09395	28-Jan-09	Human
DT49	ERL063325	04-Sep-06	Sparrow
DT50	ER014613	19-Sep-01	White eye
DT51	ERL081730	08-May-08	Human
DT52	ERL083326	12-Sep-08	Human
DT53	ERL052925	26-Aug-05	Duck
DT54	ERL072372	13-Aug-07	Poulty Environmental
DT55	ERL081168	26-Mar-08	Cockatoo
DT56	ERL082847	19-Aug-08	Poultry Environmental
DT57	ERL052818	22-Aug-05	Poultry Environmental
DT58	ERL061789	17-May-06	Poultry Environmental
DT59	ERL042806	10-Sep-04	Poultry Environmental
DT60	ERL033073	30-Jul-03	Greenfinch

Table 7. (cont.) Key table showing corresponding Environmental Science and Research (ESR) isolate number from the ERL, Enteric Reference Laboratory (ERL) to our internal laboratory identifier number (DT).

Internal laboratory number	ESR isolate number	Received date	Source type
DT61	ER005391	22-Nov-00	Poultry Environmental
DT62	ERL082846	19-Aug-08	Poultry Environmental
DT63	ERL072196	25-Jul-07	Sparrow
DT64	ERL030011	06-Jan-03	Human
DT65	ER010739	13-Feb-01	Human
DT66	ERL043592	16-Nov-04	Poultry Environmental
DT67	ER012224	24-May-01	Poultry Environmental
DT68	ERL043718	30-Nov-04	Poultry Environmental
DT69	ERL04205	21-Jan-04	Human
DT70	ER003038	3-Aug-00	Sparrow
DT71	ERL033362	21-Aug-03	Sparrow
DT72	ER004952	26-Oct-00	Poultry Environmental
DT73	ER011172	08-Mar-01	Human
DT74	ERL043779	06-Dec-04	Sparrow
DT75	ERL03695	13-Feb-03	Sparrow
DT76	ERL08372	24-Jan-08	Blue duck
DT77	ERL062557	12-Jul-06	Cockatoo
DT78	ER005001	31-Oct-00	Sparrow
DT79	ERL025021	20-Dec-02	Human
DT80	ERL041935	01-Jul-04	Human
DT81	ERL02312	22-Jan-02	Poultry Environmental
DT82	ERL033068	30-Jul-03	Poultry Environmental
DT83	ERL072197	25-Jul-07	Kaka
DT84	ER004783	18-Oct-00	Human
DT85	ERL053850	20-Oct-05	Human
DT86	ERL042332	12-Aug-04	Sparrow
DT87	ERL025008	19-Dec-02	Human
DT88	ERL034404	11-Nov-03	Human
DT89	ERL07266	31-Jan-07	Human
DT90	ER004663	12-Oct-00	Sparrow

Note: There is some inconsistency in the numbering format in the ESR database. Some numbers have a forward slash separating the year from the isolate number for that year, others have a varying number of zeros before the allocated isolate number. The reference numbers should therefore be interpreted as the ER or ERL prefix followed by two digits representing the year of isolation, followed by the isolate number for that year.