Epidemiological investigation of *Salmonella tilene* by pulsed-field gel electrophoresis and polymerase chain reaction

Chandar M Anand MD, Kevin Fonseca PhD, Ken Longmore CPHIC, Robert Rennie PhD, Linda Chui MSC RM(CCM), Mike Lingley MD, David Woodward BSc

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Pulsed-field gel electrophoresis (PFGE) and DNA fingerprinting by the polymerase chain reaction (PCR) were performed on 11 isolates of *Salmonella tilene*. Five strains were from a cluster of human patients, six from sugar gliders and pygmy hedgehogs kept as family pets or from local pet retailers, and one isolate from the first North American case of *S tilene* described in Washington State in 1994. The PFGE restriction patterns showed all isolates to be similar. However, PCR using primers to the 16S and 23S rRNA genes of *Escherichia coli* demonstrated that the Washington State isolate differed from the rest of the other isolates, which were all similar based upon their DNA fingerprint. This study indicates that reliance on one technique alone may be insufficient to show nuances between strains that are, in many respects, closely related.

Key Words: *Polymerase chain reaction, Pulsed-field gel electrophoresis,* Salmonella tilene

Investigation épidémiologique de *Salmonella tilene* par électrophorèse sur gel à champ pulsé et amplification génique

RÉSUMÉ : L'électrophorèse sur gel à champ pulsé et des techniques d'empreintes génétiques (tests d'amplification génique) ont été appliquées sur onze isolats de *Salmonella tilene* : cinq souches provenaient de patients humains, six de phalangers du sucre et de hérissons nains gardés comme animaux de compagnie dans des familles ou dans des animaleries, et un isolat provenant du premier cas nord-américain de *S. tilene* décrit dans l'état Washington en 1994. L'électrophorèse sur gel à champ pulsé basée sur leur modèle de restriction a révélé que tous les isolats étaient semblables. Toutefois, les tests d'amplification génique à l'aide d'amorces des gènes de l'ARNr 16 et 23 d'*Escherichia coli* ont permis de démontrer que l'isolat de l'état de Washington différait des autres qui étaient tous semblables sur le plan de l'empreinte génétique de leur ADN. Cette étude indique qu'il est peut-être insuffisant de ne se fier qu'à une technique pour montrer les nuances entre des souches qui sont à plusieurs points de vue apparentées.

Provincial Laboratory of Public Health for Southern Alberta, Calgary, Alberta; Palliser Health Authority, Environmental Health Services, Medicine Hat, Alberta; Department of Microbiology and Public Health, University of Alberta Hospital, Edmonton, Alberta; National Reference Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, Ontario

Correspondence and reprints: Dr CM Anand, Provincial Laboratory of Public Health for Southern Alberta, 3030 Hospital Drive NW, PO Box 2490, Calgary, Alberta T2P 2M7. Telephone 403-670-1200, fax 403-270-2216

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Transmission of pathogens from infected pet animals to their caregivers has been documented, including transmission of salmonellae from pets to their caregivers (1-3). Although the rates of transmission are unknown, some pets are more likely to harbour and excrete salmonella than others, increasing the risk of transmission to pet owners. The importation of exotic pet species from abroad may pose a further risk because they may harbour unusual pathogens, potentially placing individuals who look after or keep these animals as pets at high risk of infection from such pathogens.

In 1994, a report implicated African pygmy hedgehogs (*Atelerix albiventris*) in a family case of *Salmonella tilene* because these animals, kept as pets, were also infected with this unusual salmonella serotype (4). Apart from serological typing, no additional studies were performed to show that the strains from both the animals and patients were similar.

We report the details of an investigation of *S tilene* isolates from different exotic pets and family members, which likely originated from a single source, and the use of pulsed-field gel electrophoresis (PFGE) and DNA fingerprinting by polymerase chain reaction (PCR) to assist with the discriminatory analysis of these isolates.

TABLE 1
Origin of isolates, patient demographics and clinical history

Patient isolates	Sex, age (years)	Clinical history
44950	Female, 32	Vomiting, soft stools
44951	Female, 5	Stomach ache, soft stools
44209	Female, 3	Stomach ache, soft stools
44952	Male, 13	No symptoms
E2212	Female, 4 months	Diarrhea, sepsis, meningitis
Animal isolates	**Animal**	**Comments**
93116	Hedgehog	Family pet
93120	Hedgehog	Family pet
97315	Hedgehog	Local retailer
R763	Sugar glider	Household pet of female E2212
R849	Sugar glider	Household pet of female E2212
22/77	Hedgehog	Washington state isolate (4)

PATIENTS AND METHODS

Outbreak investigation: The investigation of this incident was prompted by the isolation and serotyping of human strains as *S tilene*, a strain rarely isolated in Canada. During the initial investigation while the serotype of the *Salmonella* species was being determined, it was assumed that a family of three females – one aged 32 years, one five years and one three years – (44950,44951,44209) and a 13-year-old male had acquired the infection from contact with chicks that had been raised in their home (Table 1). The family also maintained a breeding farm for exotic birds and other animals. Within two months of the first cases, another individual (E2212), a four-month-old female, from a geographically separate town and unrelated to this family, was also investigated because of the isolation of *S tilene* from her stool. This individual lived in a household where sugar gliders (*Petaurus breviceps*) were kept as pets (Table 1). Based upon a 1994 *Morbidity and Mortality Weekly Report* (4), it was determined that the more likely sources of this salmonella serotype in both incidents were pygmy hedgehogs and sugar gliders. Consequently, fecal samples from animals bred at the exotic pet farm and the sugar gliders were tested for the presence of this organism. In addition a number of local retailers of these exotic animal pets were contacted, and stool samples from pygmy hedgehogs were obtained and processed for the isolation of salmonella.

Isolation procedures: Stool samples submitted to the laboratory were processed according to standard protocols for the isolation, identification and serotyping of *Salmonella* species (5).

Provisional serotyping for somatic (0) antigens by slide agglutination and flagellar (H) antigens by tube agglutination was performed using antisera from Wellcome (Murex Diagnostics Ltd, Dartford, England) and Difco (Difco Laboratories, Michigan). For complete serotyping all isolates were referred to the National Reference Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, Ontario.

Antibiotic susceptibility testing: Minimal inhibitory concentration values (MICs) were determined using Vitek (Biomerieux Vitek, Missouri).

PFGE: A single colony was inoculated into Luria broth, and incubated overnight to give a density of between 2 and 2.5 OD units at 600 nm, which corresponded to the late log phase of growth. The culture was washed twice in SB buffer (10 mM Tris-HCl, containing 20 mM sodium chloride and 100 mM EDTA, pH 7.5), and resuspended in the same buffer. Next, 60 μL of this suspension was mixed with an equal volume of 1.6% low-melting point agarose, poured into a plug and set aside to solidify. The plugs were incubated for 1 h at 37°C in lysing solution (10 mM Tris-HCl, 50 mM sodium chloride, 100 mM EDTA, 0.5% sarkosyl and 1 mg/mL lysozyme), followed by deproteinization (1 mg/mL of proteinase K in 500 mM EDTA, 0.5% sarkosyl) at 44°C for 15 to 24 h, then washed once in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA), and then four additional times in TE buffer with the addition of 1 mM phenylmethylsulphonyl fluoride to remove cell debris and excess reagents. These plugs were stored at 4°C pending treatment with the appropriate restriction enzyme.

Genomic DNA was digested with *Xba1* or *Not1* (GIBCO BRL), using the respective buffers provided by the manufacturer for each enzyme. Each plug was immersed in 200 μL of buffer containing 48 U of *Xba1* or 25 U of *Not1*, and incubated at 37°C for 17 h.

The plugs were embedded in a 1.2% agarose gel (Sigma-Aldrich), and run in a Chef DR2 pulsed gel electrophoresis apparatus (Bio-Rad Laboratories) for 22 h at 14°C, with Tris-borate-EDTA (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH

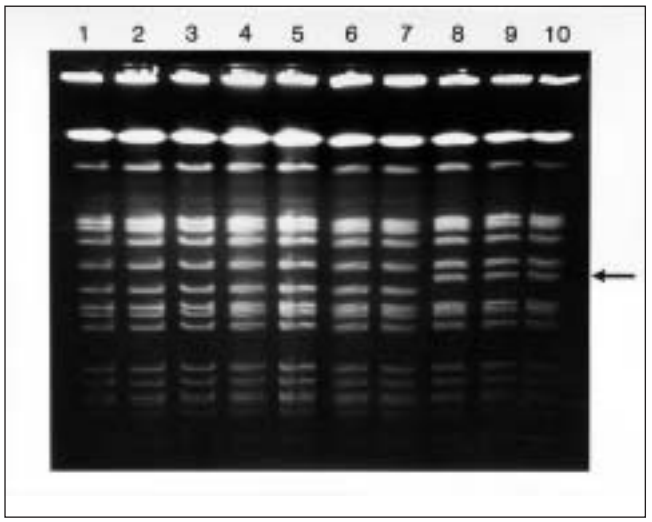

Figure 1) *Pulsed-field gel electrophoresis pattern of* Xba1 *digests of genomic DNA from* Salmonella tilene *isolates. Lanes 1 to 10: 22/77 (Washington State isolate), 44950, 44951, 44952, 93116, 93120, 97315, E2212, R763, R849 (see Table 1 for description of strains). Arrow indicates band with different migration rate*

8.0) as the running buffer. The voltage setting was 6 V/cm, with the initial pulse set at 5 s and stepped to 40 s. The gel was stained in ethidium bromide (1 μg/mL) for 30 mins, destained in water for 1 to 2 h, and then photographed.

Band patterns were distinguished based upon the mobility and numbers of bands seen unaided for each strain and were assessed for similarity according to the criteria of Tenover et al (6).

PCR: Eleven isolates of *S tilene* (Table 1) and two additional salmonella strains, *Salmonella johannesburg* and *Salmonella newport*, which served as internal controls, were tested.

Growth from 24 h bacterial cultures, inoculated from a single colony onto blood agar plates, was washed twice with 12mM Tris-HCl buffer (pH 7.4), and the cells were resuspended in 500 μL of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) with RNAse and lysozyme (Boehringer Mannheim Biochemicals, Indiana) added to give final concentrations of 0.08 mg/mL and 0.2 mg/mL, respectively. The mixtures were incubated at 37°C for 1 h, then frozen at –70°C for 30 mins. After thawing, freshly prepared sodium dodecyl sulphate and proteinase K (Boehringer Mannheim Biochemicals) were added to a final concentration of 6% and 0.12 mg/mL, respectively. The samples were incubated at 65°C for 1 h, after which sodium chloride and cetylammonium bromide were added to final concentrations of 1.6%, followed by further incubation at 65°C for 10 mins. Proteins were removed with phenol-chloroform and chloroform/isoamyl alcohol treatment, and the DNA was precipitated by the addition of isopropanol. The purity and quantitation of bacterial DNA were assessed by ultraviolet absorbance and agarose gel electrophoresis.

PCR was performed on the bacterial DNA using primers based to the conserved regions of *Escherichia coli* 16S (primer P1) and 23S rRNA (primers R1 and R3), respectively.

The sequences of these primers are as follows: P1 (1046-63) 5 -AGGTGCTGCATGGCTGTC-3 , R1 (12-30) 5 -TAACCGTAC-ACGGTGGATC-3 , R3 (1516-35) 5 -GGCGTGATGACGAGGCACTA--3 , and were synthesized by a PCR MATE 391 DNA synthesizer (Applied Biosystems). Twenty nanograms of bacterial DNA were added to a final volume of 50 μL of a PCR mixture containing 40 pmol of each primer, 50 μmols each of dNTPs (Pharmacia Biotechnology, Quebec), 1 U *Taq* polymerase, 1X reaction buffer, and 1 mM (final concentration) magnesium chloride (Gibco BRL). A negative control with no DNA was included with all runs. After denaturing the template at 94°C for 1 min, DNA was amplified for 45 cycles in a Perkin-Elmer 9600 thermal cycler with the following parameters: 1 min at 94°C, 1 min at 55°C, 5 mins at 72°C. The amplified products together with a 1 kb molecular weight marker (Gibco BRL) were subjected to electrophoreses for 60 mins at 150 V in 1.5% agarose gels (Ultrapure, Gibco BRL), containing 0.05 μg/mL ethidium bromide and visualized by ultraviolet transillumination.

RESULTS

Table 1 presents patient demographics and clinical histories, and details of animal isolates. Four of five patients were symptomatic. Five isolates were from pet animals, of which three were from the pygmy hedgehogs and two from sugar gliders; the reference *S tilene* hedgehog isolate was obtained from the Washington State outbreak.

Biochemical and serotyping reactions for all these strains were similar and met the identification criteria for salmonella and serotype as *S tilene* (40: e,h: 1,2). Susceptibility tests showed uniform MIC values to the following antibiotics: amoxicillin/clavulanic acid (less than 8 mg/L), ampicillin (1.0 mg/L), carbenicillin (less than 16 mg/L), cefonicid (less than 4 mg/L), ceftriaxone (less than 8 mg/L), cephalothin (less than 2 mg/L), ciprofloxacin (less than 0.5 mg/L), nalidixic acid (less than 16 mg/L), nitrofurantoin (less than 32 mg/L), norfloxacin (less than 4 mg/L), tetracycline (less than 1 mg/L) and trimethoprim/sulphamethoxazole (less than 10 mg/L).

PFGE was used to discriminate the *S tilene* strains following digestion of the genomic DNA with restriction enzymes, namely *Xba1* and *Not1*. A maximum of 10 isolates could be accommodated on each gel run, and consequently the band patterns for isolate 44209 are not shown here. In Figure 1 the band pattern after treatment with *Xba1* is shown. The Washington State isolate (22/77) and those from family members and the three hedgehogs were identical (44950, 44951, 44952, 93116, 93120, 97315), including isolate 42209 (results from other runs). Strains from the female patient (E2212) and her two pet sugar gliders (R763 and R849) were also identical based upon patterns from *Xba1* treatment. There was a small but discernible difference between the patterns of the last three isolates and the preceding group of seven isolates, indicated by a single band with a slower migration rate. This difference was reproducible in a number of separate runs. In all other respects the pattern was identical. The band patterns for restriction enzyme *Not1* are also similar for all strains tested including 44209 (data not shown). No minor band migration differences were observed after treatment with this enzyme as was noted with *Xba1*.

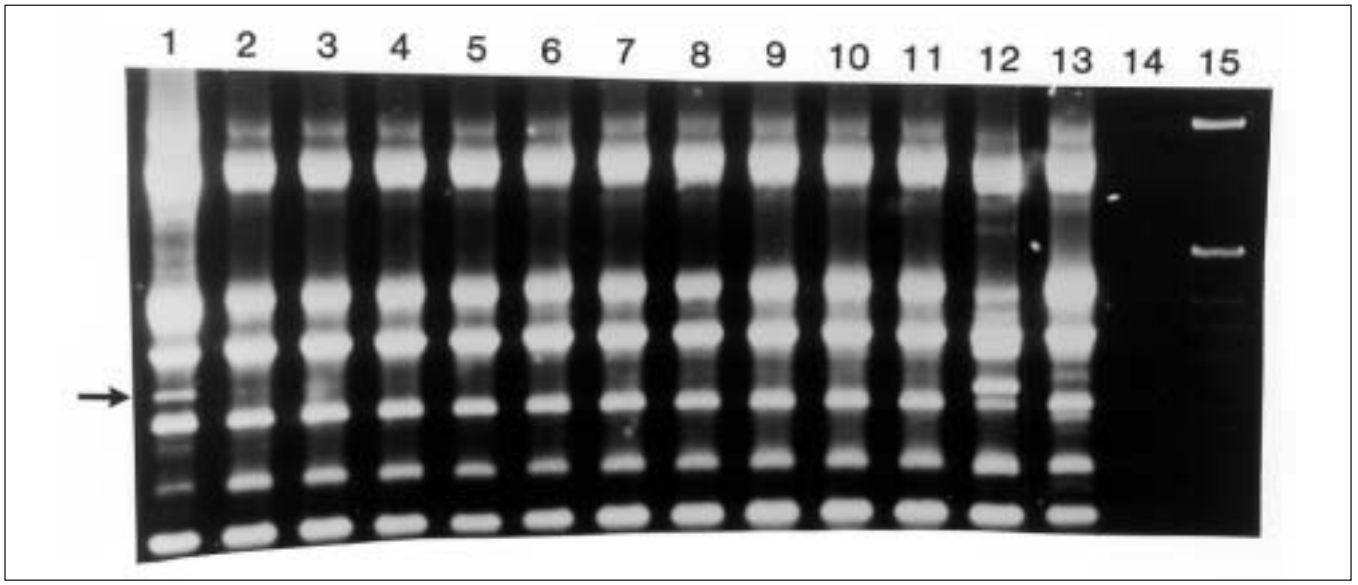

Figure 2) *DNA fingerprint profile of* Salmonella tilene *isolates generated to conserved regions of* Escherichia coli *16/23S rRNA by polymerase chain reaction. Lanes 1 to 11: 22/77 (Washington State isolate), 44950, 44951, 44952, 44209, 93116, 93120, 97315, E2212, R763, R849 (refer to Table 1 for description of strains). Lanes 12, 13, 14 and 15 are* Salmonella johannesburg, Salmonella newport, *negative control and 1 kb molecular weight marker*

The DNA fingerprinting profile of the isolates generated by PCR to the conserved region of *E coli* 16/23S rRNA is shown in Figure 2, where an additional band is present for the Washington State strain (22/77). The DNA fingerprints for the other *S tilene* human and animal isolates were similar. The minor difference in the band pattern observed in the PFGE with *Xba1* for some of the isolates was not evident in this assay. *S newport* was included because it has a flagella antigen (6,8: eh: 1,2) similar to that of *S tilene* (1,40:eh:1,2) which may explain the similarity in the banding pattern observed in Figure 2.

DISCUSSION

Serotyping of human strains salmonella as *S tilene*, a serotype rarely isolated in Canada, prompted investigation of this incident. The isolation of *S tilene* from both the pygmy hedgehogs and humans at an exotic pet farm provided further support for cross-infection between human and animals. Follow-up samples from other animals kept on this farm, such as rheas and ostriches, were negative for this serotype, although other serotypes, *Salmonella icturi* and *Salmonella typhimurium*, were also isolated. Furthermore, pygmy hedgehogs from a local pet retailer were found to be a reservoir of *S tilene*. All the source animals appeared asymptomatic because their stools were formed, despite the fact that *S tilene* could be easily recovered from these samples. The likely source of the young female's (E2212) salmonella infection was from sugar gliders, kept as pets by the parents because *S tilene* was isolated from both the child and the pets. The child was from a geographically separate location and unrelated to the family at the exotic animal farm. However, subsequent epidemiological investigations showed that the sugar gliders and pygmy hedgehogs were likely cross-infected at a common point, a breeding stock farm. The clinical history of these patients showed that all but one of the *S tilene*-infected individuals were symptomatic, a feature that was also noted in the 1994 *Morbidity and Mortality Weekly Report* (4).

PFGE and DNA fingerprinting by PCR were used to assess how closely these strains were related to each other and to one of the *S tilene* isolates from the 1994 Washington State outbreak. Based upon the PFGE results alone, all the strains were found to be very similar, if not identical, and thus could be regarded as having originated from a single source. PFGE also showed that the isolates from the child and the pet sugar gliders were identical, based upon the minor band migration after digestion with *Xba1*. In contrast, the DNA fingerprint pattern clearly showed a difference between the Canadian isolates and the Washington State strain. The epidemiological evidence supported a common focus of cross-infection between the sugar gliders and pygmy hedgehogs, which most likely occurred in Alberta because the pygmy hedgehogs and sugar gliders originated from the same stock farm. An American source originally supplied the stock farm, and the evidence suggested that the pygmy hedgehogs were the primary source of *S tilene*. Clearly, reliance alone on the PFGE results would have led to the supposition that these strains were closely related to the Washington State strain.

DNA fingerprinting using arbitrarily selected primers as an aid can discriminate between strains of the same species with a similar phage type or serotype. However, the band pattern may not always provide unequivocal proof of dissimilarity because the results of this test may vary depending upon the conditions chosen (7,8). In our study, DNA fingerprinting differentiated between the Washington and Canadian isolates, but PFGE identified minor difference between the two subgroups of the Canadian isolates, which was consistent with the epidemiological and geographical data. Thus in some circumstances, it may be prudent to apply more than one technique for additional discrimination, especially when the isolates are geographically distinct.

This study emphasizes the possibility of transmission of pathogens from animals to humans. The importation of exotic pets that have not been extensively tested for unusual pathogens may pose additional risk of unknown infections to the caregivers and retailers. Educating both pet retailers and pet owners about the risks of acquiring infections when petting or handling animals is necessary; perhaps screening these animals for likely pathogens before importation or sale should also be required.

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