Salmonella enterica subspecies II infections in England and Wales – the use of multilocus sequence typing to assist serovar identification

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Identifying rare Salmonella serotypes by conventional serotyping can be a problem for diagnostic or reference microbiology laboratories. We report two cases of the seldom encountered serovar Salmonella Dubrovnik, which is known as Salmonella subspecies II 41 : z : 1,5, in an elderly man and a young child. Multilocus sequence typing, a technique that is being used more frequently in our laboratory, was used to assist serovar identification as serotyping proved to be inconclusive. To our knowledge, these cases were not linked geographically and there were no known associations between them although they occurred within a very short time of each other.

INTRODUCTION

Classification and identification of Salmonella are complex, and are mainly based on serological detection of antigens. The complexity is often due to the presence of Salmonella strains that do not fully express their antigens and hence confuse the identification process (Grimont & Weill, 2007; Peters et al., 2010; Achtman et al., 2012). Therefore reference laboratories frequently receive isolates that are difficult to type and require a combination of microbiological and molecular methods for identification. Our laboratory screens Salmonella isolates using TaqMan real-time PCR for identification of the most common subspecies of Salmonella enterica (subsp. I and III) (Hopkins et al., 2009, 2011), as well as multilocus sequence typing (MLST) for problematic strains.

Between July and August 2010, three unusual isolates were referred to the Public Health England Salmonella Reference Service (formerly the Health Protection Agency Salmonella Reference Unit) for confirmation and typing. Two isolates came from the same elderly male patient (83 years of age) admitted to hospital with fever. The first isolate was from a blood culture and had a presumptive identification from the sending laboratory of Salmonella sp., while the second isolate from a faecal culture had a presumptive identification of Salmonella arizonae. The third isolate was from faeces of a 3-year-old female hospitalized with severe diarrhoea. There was no known link between the cases, and they were hospitalized in geographically separated regions. Despite treatment with appropriate antibiotics the elderly male deteriorated and died following admission.

In order to establish whether the isolates from these two cases belonged to the less common S. enterica subsp. salamae (subsp. II), S. enterica subsp. houtenae (subsp. IV) or Salmonella bongori (previously subsp. V), a range of biochemical tests were performed together with serum agglutination tests, MLST (Kidgell et al., 2002) and fjb and flic genotyping (Mortimer et al., 2004; McQuiston et al., 2004).

METHODS

Bacterial isolates and identification. The two isolates from the elderly male were submitted on 9 and 22 July 2010, and the isolate from the child was submitted on 11 August 2010, to the Salmonella Reference Service (SRS) of Public Health England (PHE). This is the national reference laboratory for salmonellas from humans in England and Wales. All isolates were typed phenotypically and were further tested genotypically.

A retrospective analysis of all the available clinical and epidemiological data was also carried out on every S. enterica subsp. salamae present in the SRS database between 2004 and 2012.

Serotyping. Isolates were cultured and serotyped by SRS staff using specific antisera. Biochemical analysis and serotyping were in accordance with the White-Kauffmann–Le Minor scheme (Grimont & Weill, 2007).

Real-time PCR. Salmonella isolates were screened using an in-house TaqMan real-time PCR assay for identification of the most common subspecies of S. enterica (Hopkins et al., 2009, 2011).

MLST. MLST was performed as previously described (Kidgell et al., 2002) by determining the sequences of seven housekeeping genes (aroC, dnaN, hemD, hisD, purE, recA and thrA). The data obtained were compared with the Salmonella MLST database at the University College, Cork (http://pubmlst.org) and isolates were assigned
sequence type (ST) numbers as defined by the database. Sequence types were assigned based on the set of allele types derived from each of the seven loci.

**Flagellar gene typing.** Genes encoding flagellin \([fliC] \text{ encodes the phase 1 flagellar (H1) antigen and } fliB \text{ encodes the phase 2 flagellar (H2) antigen}\) are typically conserved at the 5\(^\prime\) and 3\(^\prime\) ends but variable in the central region in *Salmonella* and can be used in molecular serology (Mortimer et al., 2004; McQuiston et al., 2004). Partial sequences of the *fliC* and *fliB* gene were obtained as previously described by Mortimer et al. (2004) and McQuiston et al. (2004).

**RESULTS**

All three isolates were negative in a simplex 5\(^\prime\) nuclease TaqMan assay that targets the *hilA* gene, indicating that the isolates were not *S. enterica* subsput. *enterica* (subsp. I). A duplex 5\(^\prime\) nuclease PCR assay for identification of the Arizona group was negative for the *LacZ* locus but positive results were obtained for the *trtRSBCA* locus involved in tetrathionate respiration. This confirmed the presence of *Salmonella* DNA but indicated that the isolates were not *S. enterica* subsp. *arizonae* (subsp. IIIa) or *S. enterica* *diarizonae* (subsp. IIIb).

A range of biochemical tests were performed together with serum agglutination tests to confirm the presence of less common subspecies of *S. enterica*. The three isolates were unreactive with a polyvalent *Salmonella* somatic [O] serum but tested positive with a polyvalent flagellar [H] serum indicative of the presence of *Salmonella* flagella antigens. Additional agglutination testing identified the phase 1 and phase 2 flagellar antigens (H antigens) to be H1=Z and H2=1,5. This was confirmed by amplification and partial sequencing of the *fliC* and *fliB* genes (data not shown). Due to the complexity/difficulty in identifying the [O] antigen, further titration against a wider range of group-specific antisera covering the Group C and Group S serogroups revealed the somatic [O] antigen to be either 6,7 or 41 according to the White–Kauffmann–Le Minor classification.

MLST was then used to obtain the possible identity of the *Salmonella*ae involved. MLST clustered the isolates with other subsp. II serotypes within the *Salmonella* MLST database, with an identical match to ST1082. The ST1082 isolate in the MLST database is 1709K, the Kauffmann reference strain of *S. enterica* subsp. *salamae* 41:Z:1,5 (formerly serotype Dubrovnik, isolated from a reptile in Yugoslavia in 1964) (F.-X. Weill, Institut Pasteur, personal communication).

Although biochemical results were not atypical and confirmed *S. enterica* subsp. *salamae* (subsp. II), the techniques were laborious and time consuming, and required long incubation times. Eventually the isolates were serologically classified with the formula 41:Z:1,5 (somatic antigen [O]; flagellar antigen [H] phase 1:phase II) and named as *Salmonella* subsp. II 41:Z:1,5 according to current nomenclature (Grimont & Weill, 2007).

The possibility of cross-contamination between the isolates from the elderly man and the child was ruled out because the isolates were received and processed by our laboratory at different times.

Between January 2004 and December 2012, our laboratory identified 280 isolates of *S. enterica* subsp. *salamae* of which 223 were from human infections including three cases of bacteraemia. The majority of these isolations were single occurrences of extremely rare salmonellas, although a few notable exceptions appeared with greater frequency during that period (Table 1).

Of the human-derived isolates, 90 % (200/223) were fully sensitive to a wide range of antimicrobials using standard methods of determination (Frost, 1994). Where resistance was present it was usually to a single antimicrobial, with only two records of multidrug resistance (SRS in-house data).

The highest incidence of infection with *S. enterica* subsp. *salamae* was seen in young children. For isolates from patients of known age, 52 % were from children 4 years of age or less, of which 33 % were from infants younger than 1 year (Fig. 1).

Of the 57 non-human isolates, 23 were from animal sources, of which only three were listed as reptiles. Interestingly, of the 16 food isolates, eight were from either herbs or spices. Although six isolates were listed as ‘source unknown’, they all originated from private food laboratories, thus

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**Table 1.** The most prevalent *Salmonella enterica* subsp. *salamae* isolated in England and Wales, 2004–2012

<table>
<thead>
<tr>
<th>Former serovar name</th>
<th>New name</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em></td>
<td>Subsp. II 58:1Z13,28:Z6</td>
<td>26</td>
</tr>
<tr>
<td><em>Salmonella Sofia</em></td>
<td>Subsp. II 4,1,2:bc-</td>
<td>20</td>
</tr>
<tr>
<td><em>Salmonella Tranoroa</em></td>
<td>Subsp. II 55;k:Z39</td>
<td>19</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Subsp. II 30;L28:Z6</td>
<td>15</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Subsp. II 50;b:Z6</td>
<td>12</td>
</tr>
<tr>
<td><em>Salmonella Hagenbeck</em></td>
<td>Subsp. II 48:d:Z6</td>
<td>11</td>
</tr>
<tr>
<td><em>Salmonella Nairobi</em></td>
<td>Subsp. II 42:r-</td>
<td>10</td>
</tr>
</tbody>
</table>
implicating their origin as from an unknown food. Environmental samples accounted for ten of the remaining isolates.

One hundred and seventy-nine of the 223 human cases had no history of foreign travel.

**DISCUSSION**

MLST as a replacement for serotyping in *S. enterica* has been demonstrated recently (Achtman et al., 2012). In this study, MLST was used to confirm the identity of three strains that were difficult to serotype in a reference laboratory setting. This clearly shows the potential of using MLST in support of routine reference work.

In the 1960s, the International Subcommittee on the Taxonomy of the *Enterobacteriaceae* renamed serovars belonging to *S. enterica* subsp. *salamae* (Carpenter, 1968). These serovars are now known as *Salmonella* subsp. II followed by their antigenic formula (e.g. *Salmonella* Dubrovnik is now known as *Salmonella* subsp. II 41 : z : 1,5) (Table 1). Since it was first identified as *Salmonella* Dubrovnik in 1964 (Kelterborn, 1967) there had been no reports of this serovar in England and Wales until 2004. To date there have only been six isolated cases (one with foreign travel to Italy) of this extremely rare serovar reported in our laboratory although there were four cases reported in Italy between 1994 and 1996 based on a SALM-NET study (Scuderi et al., 2000).

Serovars of *S. enterica* subsp. *salamae* are typically reported as being associated with cold-blooded hosts, and are cited as rarely causing human infections, but a review of the existing literature does not provide much information on this subspecies, and only one publication specifically names *Salmonella* Dubrovnik (Wuthe, 1969). Whilst it is noted that turtles, snakes, iguanas and lizards may all carry *S. enterica* subsp. *salamae*, the carrier rates and relative importance as sources of human salmonellosis are not well described and much of the available literature about reptiles and salmonellas focuses on *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae* and *S. enterica* subsp. *houtenae* (Warwick et al., 2001; Mermin et al., 2004; Pedersen et al., 2009).

While the large majority of human salmonellosis in England and Wales is due to infection with *S. enterica* subsp. *enterica* (subsp. I), *S. enterica* subsp. *salamae* can still cause gastrointestinalis and occasionally systemic disease in warm-blooded hosts. It may also be responsible for severe infection in the young, very old or immunocompromised patients who then require hospitalization (Angulo & Swerdlow, 1995; CDC, 1999). Most of the infections with this subspecies that we saw in our laboratory were accompanied by gastroenteritis, with systemic infection apparent in only a very few cases. Although the outcome of bacteraemia cases may occasionally be fatal, there did not appear to be a problem with antibiotic resistance that would have implications for treatment.

The fact that the highest incidence of *S. enterica* subsp. *salamae* infection was seen in young children was not unexpected. As noted by Bertrand et al. (2008) the prevalence of infants younger than one year of age may be partly due to the bias in investigating childhood salmonellosis cases more thoroughly. However, our data show that when *S. enterica* subsp. *salamae* was compared with other non-typhoidal serovars the incidence was still higher than would be expected for simple age-bias (C. Lane, PHE, personal communication).

Most literature states that isolates belonging to subspecies other than *S. enterica* subsp. *enterica* (subsp. I) are almost exclusively obtained from exotic sources such as reptiles. It has been suggested that whenever *Salmonella* belonging to subspecies other than *enterica* are found in humans, an exotic pet source should be suspected. There are no specific data on reptile ownership in our retrospective dataset, as this information has not been routinely gathered in England and Wales.

**CONCLUSION**

The source of infection in single cases of salmonellosis is rarely identified, especially when the strain is a seldom-encountered serovar without any apparent endemic bias preference. Although 44 of the human cases here were known to be associated with foreign countries, especially the African continent (22/44), the majority of cases had no history of foreign travel. Thus it is difficult to identify management strategies without being able to identify potential environmental or food sources. We are not sure whether the case of *Salmonella* subsp. II 41 : z : 1,5 bacteraemia described here was due to the virulence of the serovar alone or to the underlying health of the elderly patient. As the only link between the two cases is loosely temporal we have to assume that the appearance of both...
cases in a short period of time was coincidental. Although it is generally believed that cold-blooded animals are the major reservoir of S. enterica subsp. salamae, it is also important to consider food-borne infections. If it is assumed that such cases are always sporadic infections from reptiles, either directly or indirectly, they may never be reported to the relevant public health authorities.

The identification of rare Salmonella serotypes can be hindered due to the difficulty of identifying the somatic [O] antigen or flagella [H] antigens, which might be caused by varying expression levels of the genes involved. MLST was used in this study for rapid and accurate identification of the strains involved. With the current trend for using whole genome sequencing for diagnostic and public health microbiology, MLST will be used in real-time for identifying all Salmonellas within our laboratory in the near future.

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REFERENCES


