View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

ORIGINAL ARTICLE

Molecular characterisation of an outbreak strain of multiresistant *Salmonella enterica* serovar Typhimurium DT104 in the UK

A. J. Lawson[1], *M. Desai*[2], *S. J. O'Brien*[3], *R. H. Davies*[4], *L. R. Ward*[1] *and E. J. Threlfall*[1]

[1]Laboratory of Enteric Pathogens, Public Health Laboratory Service (PHLS) Central Public Health Laboratory, [2]Virus Reference Laboratory, PHLS Central Public Health Laboratory, [3]Gastrointestinal Diseases Division, PHLS Communicable Disease Surveillance Centre, London and [4]Veterinary Laboratories Agency, Weybridge, New Haw, Surrey, UK

ABSTRACT

A major national outbreak of multiresistant *Salmonella enterica* serovar Typhimurium definitive phage type 104 (MR DT104) occurred in England and Wales in the summer of 2000. Isolates of MR DT104 were characterised by antimicrobial resistance type (R-type), pulsed-field gel electrophoresis (PFGE), plasmid profiling and fluorescent amplified fragment length polymorphism (fAFLP) analysis. Results of R-type, PFGE and fAFLP showed that summer 2000 outbreak-associated isolates were indistinguishable from most MR DT104 isolates collected in England and Wales during the 1980s and 1990s. However, outbreak-associated isolates all had an additional 2-MDa plasmid (PP D), and this distinct profile allowed outbreak cases to be distinguished from background MR DT104 infections, thereby facilitating the epidemiological investigation by improving the specificity of the case definition. The study demonstrated the highly clonal nature of MR DT104 and the importance of a hierarchical approach to molecular subtyping for outbreak investigations.

Keywords *Salmonella* Typhimurium, DT104, molecular subtyping, fAFLP

Original Submission: 22 August 2002; **Revised Submission:** 16 January 2003; **Accepted:** 11 February 2003

Clin Microbiol Infect 2004; 10: 143–147

INTRODUCTION

Multiresistant (to four or more antimicrobial agents) *Salmonella enterica* serovar Typhimurium definitive phage type 104 (MR DT104) is a zoonotic pathogen isolated frequently from food animals. Initially, it was associated with cattle, but is now also isolated commonly from pigs, sheep and poultry [1]. Most MR DT104 isolates possess chromosomally-encoded resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (R-type ACSSuT). Additional antibiotic resistances, i.e., plasmid-mediated resistance to trimethoprim and/or point mutations of the *gyrA* gene causing low-level resistance to ciprofloxacin (MIC = 0.25–0.5 mg/L), are also common [2]. Between 1990 and 1996, MR DT104 became an increasingly prevalent cause of human gastroenteritis (Fig. 1), and although isolations have decreased since 1997 (in line with other *Salmonella* serotypes), it remains second only to *S.* Enteritidis PT4 as the most frequently reported agent of human salmonellosis in England and Wales [3].

Subtyping MR DT104 isolates for the purpose of outbreak investigation is problematic because of the highly clonal nature of these strains. For example, resistance type (R-type) ACSSuT predominates in between 60% and 70% of isolates [3]; pulsed-field gel electrophoresis (PFGE) with a standard *Xba*I protocol generates a single profile (xtm 1) in *c.* 90% of isolates [4]; and by plasmid profiling (PP), *c.* 70% of isolates are characterised by the presence of a single 60-MDa plasmid (PP A) [5]. In practice, >50% of MR DT104 isolates from humans in England and Wales

Corresponding author and reprint requests: A. J. Lawson Laboratory of Enteric Pathogens, Public Health Laboratory Service (PHLS) Central Public Health Laboratory, 61 Colindale Avenue, London, NW9 5HT, UK
Tel: +44 20 82004400
Fax: +44 20 89059929
E-mail: andy.lawson@hpa.org.uk

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases

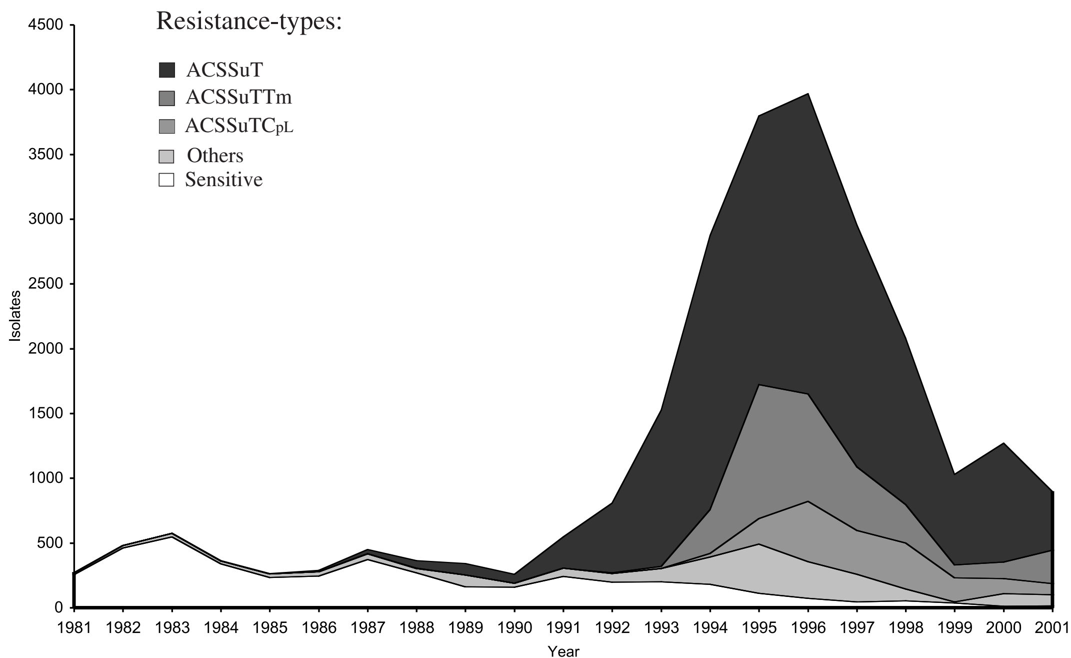

Fig. 1. Multiple antibiotic resistance in *S*. Typhimurium DT104 isolates from humans in England and Wales 1981–2001.

are indistinguishable by the above subtyping methodologies, emphasising the need for the development of new high-resolution subtyping techniques such as fluorescent amplified fragment length polymorphism (fAFLP) analysis.

In summer 2000, a national outbreak of MR DT104 occurred in England and Wales with over 360 laboratory-confirmed cases, including one fatality [6,7]. To facilitate the outbreak investigation, all MR DT104 isolates were characterised by R-type and plasmid profiling, and selected isolates were analysed by PFGE and fAFLP. Furthermore, outbreak-associated strains from 2000 were compared with MR DT104 isolates from humans and animals collected between 1984 and 2000 to explore changes which may have occurred since these strains were first identified.

MATERIALS AND METHODS

Bacterial strains

Human isolates of MR DT104 ($n = 500$) were collected in summer and autumn 2000, including: 63 from June to July (pre-outbreak); 403 from August to September (the months during which outbreak samples were received); and 34 from October to November (post-outbreak). In addition, 126 MR DT104 isolates collected from food production animals in 1999–2000, and 40 isolates from either humans or animals collected in 1984–98 (all from England and Wales), were examined. This latter group was included so that the relationship between the current (2000) outbreak strain and earlier isolates could be examined.

All isolates were characterised phenotypically by standard methodologies (serotype, phage type). Before analysis, isolates of MR DT104 were stored on nutrient agar slopes (Oxoid, Basingstoke, UK) and grown overnight on nutrient agar or nutrient broth (Oxoid) at 37 °C when required.

Antibiotic susceptibility testing

Isolates were screened for resistance to 12 antimicrobial agents by the breakpoint method as described previously [8] and levels defined by British Society for Antimicrobial Chemotherapy guidelines [9], with the exception of low-level ciprofloxacin resistance testing [10]. The following concentrations (mg/L) were used: ampicillin (A) 8; cefotaxime (Ct) 1; chloramphenicol (C) 8; gentamicin (G) 4; kanamycin (K) 16; streptomycin (S) 16; sulphonamides (Su) 64; tetracycline (T) 8; trimethoprim (Tm) 2; furazolidone (Fu) 8; nalidixic acid (Nx) 16; low-level ciprofloxacin (Cp_L) 0.125; high-level ciprofloxacin (Cp_H) 1.0.

PFGE profiles

DNA preparation was as described previously [11]. Chromosomal DNA contained in agarose plugs was digested with 10–20 U of *Xba*I (Roche Diagnostics, Mannheim, Germany), and PFGE was performed with a CHEF DR II system (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5× Tris-borate–EDTA buffer (Severn Biotech, Kidderminster, UK). DNA restriction fragments were resolved in agarose (Bio-Rad) 1% w/v gels, and a low-range PFGE marker (New England BioLabs, Beverly, MA, USA) was used as a size standard. Electrophoresis conditions were: 5.4 V/cm for 44 h, with pulse times ramped at 6–72 s.

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 143–147

Plasmid analysis

Plasmid DNA was isolated by the method of Kado and Liu [12] and analysed by electrophoresis at 110 V for 3.5 h in an agarose (Sigma-Aldrich Company, Poole, UK) 0.8% w/v gel. Gels were stained with ethidium bromide, and plasmid DNA was visualised under UV light. Plasmids were sized (in MDa) in relation to plasmids of known molecular mass carried in *Escherichia coli* K12 strain 39R861 [5].

fAFLP methodology

Genomic DNA was extracted using the MagNa Pure LC Total Nucleic Acid Isolation Kit on the MagNa Pure LC automated instrument (Roche Diagnostics, Lewes, UK). fAFLP analysis was performed on genomic DNA (500 ng) with the enzyme combination *Hin*dIII and *Hha*I [13]. The forward primer (*Hin*dIII adapter-specific), labelled with the blue fluorescent dye FAM, contained an extra selective base, A, at the 3′ end (*Hin*dIII + A), while the reverse primer, unlabelled, also contained an extra selective base, A, at the 3′ end (*Hha*I + A); these were used for PCR with touchdown conditions [14]. fAFLP products were separated on an ABI 377 automated DNA sequencer (Applied Biosystems, Warrington, UK) with polyacrylamide 5% w/v gels. Each fAFLP reaction was loaded with an internal size marker, GeneScan-2500 labelled with ROX (Applied Biosystems) [13]. Fluorescent amplified fragments (AF) were sized with GeneScan 3.1.0 software and analysed by GenoTyper 2.5 software (Applied Biosystems). Dice coefficients of similarity were calculated with in-house software, and cluster analysis was performed by UPGMA (NEIGHBOR program in PHYLIP) [15]. A dendrogram was displayed with the TreeView program [16].

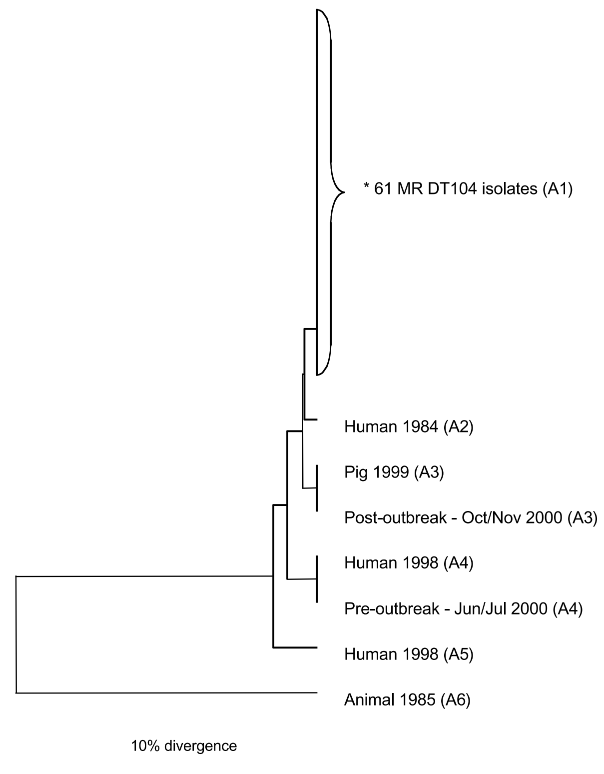

Fig. 2. Relationship between MR DT104 isolates by fAFLP profile. The dendrogram was derived from fAFLP data by UPGMA and the PHYLIP program. fAFLP profiles were designated A1–A6 (shown in parentheses). *61 MR DT104 isolates: 3/4 pre-outbreak—June to July 2000; 10/10 outbreak—August to September 2000; 2/3 post-outbreak—October to November 2000; 10/11 animal—1999–2000; and 36/40, 1984–98.

RESULTS

Summer 2000 outbreak

Most of the 500 isolates examined, and all outbreak-associated isolates, were found to be R-type ACSSuT, PFGE profile xtm 1. However, outbreak-associated isolates were found to possess a 2-MDa plasmid in addition to the typical 60-MDa *S.* Typhimurium 'serovar-specific' plasmid (PP D). The 2-MDa plasmid from selected outbreak-associated isolates was indistinguishable from the 2-MDa plasmids of earlier PP D isolates [5] by restriction digestion with *Eco*RI and *Hin*dIII (data not shown). During the outbreak months, the incidence of PP D was 64% (259/403), compared with 6% (4/63) pre-outbreak, and 9% (3/34) post-outbreak.

Investigation of food animal isolates

All 126 isolates from food animals were R-type ACSSuT and PFGE profile xtm 1. Plasmid profile PP D was present at an overall frequency of 9% (11/126). The distribution of profile PP D by animal type was: 13% (4/31) pigs; 12% (5/41) poultry; 6% (1/17) sheep; and 3% (1/37) cattle.

fAFLP analysis

In total, 68 MR DT104 isolates of R-type ACSSuT and PFGE profile xtm 1 were examined by fAFLP. These comprised: 17 human isolates from 2000 with plasmid profile PP D (ten from the outbreak, four pre-outbreak, and three post-outbreak); 11 food animal isolates from 1999 to 2000 (all PP D); and the group of 40 isolates (all PP A) collected in 1984–98. When examined by fAFLP, 90% (61/68) constituted a single fAFLP profile, termed A1. This group included 15 of 17 summer 2000 human isolates (all ten from the outbreak, three of

four pre-outbreak, and two of three post-outbreak), ten of 11 food animal isolates, and 36 of 40 of the 1984–98 isolates. The remaining seven of 68 (10%) comprised five fAFLP profiles termed A2–A6. A dendrogram showing the relationship between the fAFLP profiles is shown in Fig. 2. Among all the isolates in this study, fAFLP data consisted of 61 AFs in the size range 60–750 bp; 27 of these AFs were polymorphic, while the remaining 34 were present in all isolates tested. Each fAFLP profile consisted of between 44 and 49 AFs. The number of AF differences between the six distinct profiles (A1–A6) ranged from one to 25.

DISCUSSION

Since its first emergence in the early 1980s, MR DT104 has become an increasingly common cause of human salmonellosis in England and Wales. Infections reached a peak of over 4000 human cases in 1996, but subsequently declined to 1030 cases in 1999 [3]. Nevertheless, MR DT104 remains the second most common *Salmonella* phage type isolated from humans after *S*. Enteritidis PT4 [1]. In 2000, reported cases of MR DT104 once again began to increase. This increase reflected a national outbreak of MR DT104 involving over 360 laboratory-confirmed cases. The outbreak began in early August, and statistical analysis of a case-control study indicated that the most likely cause was the consumption of contaminated lettuce distributed to fast-food outlets, although no isolates from foodstuffs were obtained [17].

The outbreak-associated strain was typical of MR DT104, being both R-type ACSSuT and PFGE profile xtm 1. However, it was characterised by the presence of a 2-MDa plasmid in addition to the serovar-specific 60-MDa plasmid. This plasmid profile, PP D, has previously been reported to be present in *c.* 5% of MR DT104 isolates from humans [5]; during the outbreak period, the incidence of this profile type rose to 64%. The incidences of PP D immediately before and after the outbreak were 6% and 9%, respectively, i.e., in line with previously reported levels. In food animals, the overall incidence of PP D was 9%. There was no concomitant rise in food animal cases of MR DT104 during 2000. The epidemiological investigation of outbreaks associated with common strains of *Salmonella* is often hampered by the fact that it is difficult to distinguish outbreak cases from background sporadic disease. In this investigation, inclusion of the plasmid profile increased the specificity of the case definition, thereby facilitating a successful outcome for the case-control study.

The genotype of the outbreak-associated strain, as determined by PFGE and fAFLP, was identical to that found in most MR DT104 isolates. The predominant MR DT104 genotype (PFGE, xtm 1; fAFLP, A1) was present in 90% (36/40) of the isolates collected between 1984 and 1998, including the first MR DT104 human isolate with R-type ACSSuT in the UK in 1984. Eighteen years later, the same genotype still appears to be prevalent, having caused outbreaks of human illness throughout the 1990s and a major outbreak in summer 2000 in the UK. A small proportion (*c.* 10%) of MR DT104 isolates appear to be non-clonal, in that they differ from the predominant MR DT104 genotype as defined above. While these isolates have not been associated with outbreaks in this study (perhaps because of the small number investigated), they are associated with human illness and seem capable of persisting from year to year (i.e., fAFLP profiles A3 and A4) and in different hosts (fAFLP A3). These non-clonal patterns include two of the earliest UK isolates: the second human isolate from 1984 (fAFLP A2), and an animal isolate from 1985 (fAFLP A6), the latter being the most divergent from the predominant type (fAFLP A1). This suggests that the earliest MR DT104 strains were more heterogeneous than those of today, but further work on archived isolates would be required to pursue this point.

While high-resolution genotyping methodologies such as fAFLP provide important insights into the microepidemiology of MR DT104, demonstrating the highly clonal nature of these strains, the present study also showed the importance of a hierarchical approach to the molecular subtyping of MR DT104 and other salmonellas. Older fingerprinting methodologies such as plasmid profiling may still be particularly valuable in outbreak investigations. The present study also confirmed the value of close collaboration between molecular microbiologists, epidemiologists and veterinarians to establish the source(s) of outbreak strains.

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 143–147

ACKNOWLEDGEMENTS

This work was presented, in part, at the International Conference on Emerging Infectious Diseases 2002, Atlanta, GA, USA. The study was partially funded by a grant (FS3114) from the Food Standards Agency, London, UK. We thank our colleagues in the Laboratory of Enteric Pathogens.

REFERENCES

1. Threlfall EJ. Epidemic *Salmonella typhimurium* DT104—a truly international multiresistant clone. *J Antimicrob Chemother* 2000; **46**: 7–10.
2. Ridley A, Threlfall EJ. Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT 104. *Microb Drug Resist* 1998; **4**: 113–118.
3. Threlfall EJ, Lawson AJ, Walker RA, Ward L. *Salmonella typhimurium* DT104: the rise and fall of a multiresistant epizootic clone. *SCIEH Week Commun Dis Rep* 2001; **35**: 142–144.
4. Prager R, Liesegang A, Rabsch W *et al*. Clonal relationship of *Salmonella enterica* serovar Typhimurium phage type DT104 in Germany and Austria. *Zentralbl Bakteriol* 1999; **289**: 399–414.
5. Threlfall EJ, Hampton MD, Schofield SL, Ward LR, Frost JA, Rowe B. Epidemiological application of differentiating multiresistant *Salmonella typhimurium* DT104 by plasmid profile. *Commun Dis Rep CDR Rev* 1996; **6**: 155–159.
6. Anonymous. Increase in laboratory reports of *Salmonella typhimurium* DT104. *Commun Dis Rep CDR Weekly* 2000; **10**: 301.
7. Horby PW, O'Brien SJ, Adak GK *et al*. A national outbreak of multiresistant *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. *Epidemiol Infect* 2003; **130**: 169–178.
8. Frost JA. Testing for resistance to antimicrobial drugs. In: Chart H, ed. *Methods in practical laboratory bacteriology*. Boca Raton, FL: CRC Press, 1994; 73–82.
9. Working Party of the British Society for Antimicrobial Chemotherapy. A guide to sensitivity testing. *J Antimicrob Chemother* 1991; **27**(suppl D): 1–50.
10. Threlfall EJ, Ward LR, Skinner JA, Graham A. Antimicrobial drug resistance in non-typhoidal salmonellas from humans in England and Wales in 1999: decrease in multiple resistance in *Salmonella enterica* serotypes Typhimurium, Virchow, and Hadar. *Microb Drug Res* 2000; **6**: 319–325.
11. Powell NG, Threlfall EJ, Chart H, Rowe B. Subdivision of *Salmonella enteritidis* PT 4 by pulsed-field gel electrophoresis: potential for epidemiological surveillance. *FEMS Microbiol Lett* 1994; **119**: 193–198.
12. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365–1373.
13. Desai M, Logan JM, Frost JA, Stanley J. Genome sequence-based fluorescent amplified fragment length polymorphism of *Campylobacter jejuni*, its relationship to serotyping, and its implications for epidemiological analysis. *J Clin Microbiol* 2001; **39**: 3823–3829.
14. Desai M, Threlfall EJ, Stanley J. Fluorescent amplified-fragment length polymorphism subtyping of the *Salmonella enterica* serovar Enteritidis phage type 4 clone complex. *J Clin Microbiol* 2001; **39**: 201–206.
15. Felsenstein J. Phylip-Phylogeny Inference Package. *Cladistics* 1989; **5**: 164–166.
16. Page RDM. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 1996; **12**: 357–358.
17. Anonymous. Case control study links salad vegetables to national increase in multiresistant *Salmonella typhimurium* DT104. *Commun Dis Rep CDR Weekly* 2000; **10**: 333.

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 143–147