JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2002, p. 4450–4456 Vol. 40, No. 12
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.12.4450–4456.2002

Multiple Genetic Typing of *Salmonella enterica* Serotype Typhimurium Isolates of Different Phage Types (DT104, U302, DT204b, and DT49) from Animals and Humans in England, Wales, and Northern Ireland

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Received 3 May 2002/Returned for modification 30 June 2002/Accepted 30 August 2002

Salmonella enterica **serotype Typhimurium is a common cause of salmonellosis among humans and animals in England, Wales, and Northern Ireland. Phage types DT104 and U302 were the most prevalent types in both livestock and humans in 2001. In addition, *Salmonella* serotype Typhimurium DT204b was responsible for a recent international outbreak involving England. A total of 119 isolates from humans ($n = 28$) and animals or their environment ($n = 91$), belonging to DT104 ($n = 66$), U302 ($n = 33$), DT204b ($n = 12$), and DT49 ($n = 8$), were fingerprinted by a combination of well-established genetic methods (pulsed-field gel electrophoresis [PFGE], *Pst*I/*Sph*I [PS] ribotyping, and plasmid profiling). The different techniques identified different degrees of polymorphism (from greatest to least, plasmid profiling [40 types], PS ribotyping [34 types], and PFGE [23 types]). It seems clear that a prevalent genomic clone, as well as a variety of less frequent clones, is present for each of the phage types. In most cases, the prevalent clones appeared within isolates from several animal species and from several geographical locations. We did not find clear evidence of a higher degree of diversity for any of the animal species included, or of any link between isolates from particular animal species and humans. The data presented show the inaccuracy of drawing epidemiological conclusions based on a single fingerprinting method. Strains that share one of the markers do not necessarily belong to the same clone, and a multiple typing approach is required to enable enough discrimination to track strains for epidemiological investigations.**

Salmonella enterica serotype Typhimurium is a common cause of salmonellosis among humans and animals in many countries (6). *Salmonella* serotype Typhimurium is primarily a pathogen of cattle, but other species such as sheep, goats, pigs, birds, and humans can be affected. Phage typing has enabled differentiation into more than 200 definitive phage types (DTs) (1). Some of these types appear with high prevalence in certain geographical areas, and therefore this method may not be sufficiently discriminatory for epidemiological studies and does not necessarily reflect clonality (11).

The results of passive surveillance by the Veterinary Laboratories Agency in Great Britain indicated that 602 *Salmonella* serotype Typhimurium incidents were reported for animals in 2000 (7). Phage types DT104 and U302 were the most prevalent phage types in livestock and represented 53 and 10%, respectively, of the *Salmonella* serotype Typhimurium isolates in that year. The multidrug-resistant DT104 emerged in cattle populations in the United Kingdom in the 1990s (27), and soon it was isolated in other countries (2) and from a wide range of animal species including humans (17). In England, Wales, and Scotland, *Salmonella* serotype Typhimurium DT104 has been the second most common *Salmonella* phage type isolated from humans and animals in the past decade. However, the substantial fall in the numbers of cases reported for both humans and animals in the past 2 years suggests that the epidemics may be getting close to an end (6, 27). Data provided by the Laboratory of Enteric Pathogens (Public Health Laboratory Service) indicate that 2,085 *Salmonella* serotype Typhimurium infections were reported in England and Wales during 2001. *Salmonella* serotype Typhimurium DT204b has been of particular relevance during 2002. This phage type was responsible for a recent international outbreak of salmonellosis (15). For this reason, it was considered interesting to include some isolates of this phage type and of the related phage type DT49 in the present study.

Numerous genotypic methods have been used to subtype *Salmonella* serotype Typhimurium isolates, among them plasmid profiling (28, 31), IS*200* restriction fragment length polymorphism (3, 19), ribotyping (8, 21), pulsed-field gel electrophoresis (PFGE) analysis of genomic DNA (5, 24), random amplification of polymorphic DNA (18), and amplified fragment length polymorphism (16, 26). Molecular typing analysis has indicated that DT104 isolates from different hosts and geographical origins are highly clonal (18, 23). The aim of the present study was to assess the use of a multiple typing approach for sensitive differentiation of clones within animal and human *Salmonella* serotype Typhimurium isolates from some of the most relevant phage types in the United Kingdom. This approach uses three well established methods (PFGE, *Pst*I/

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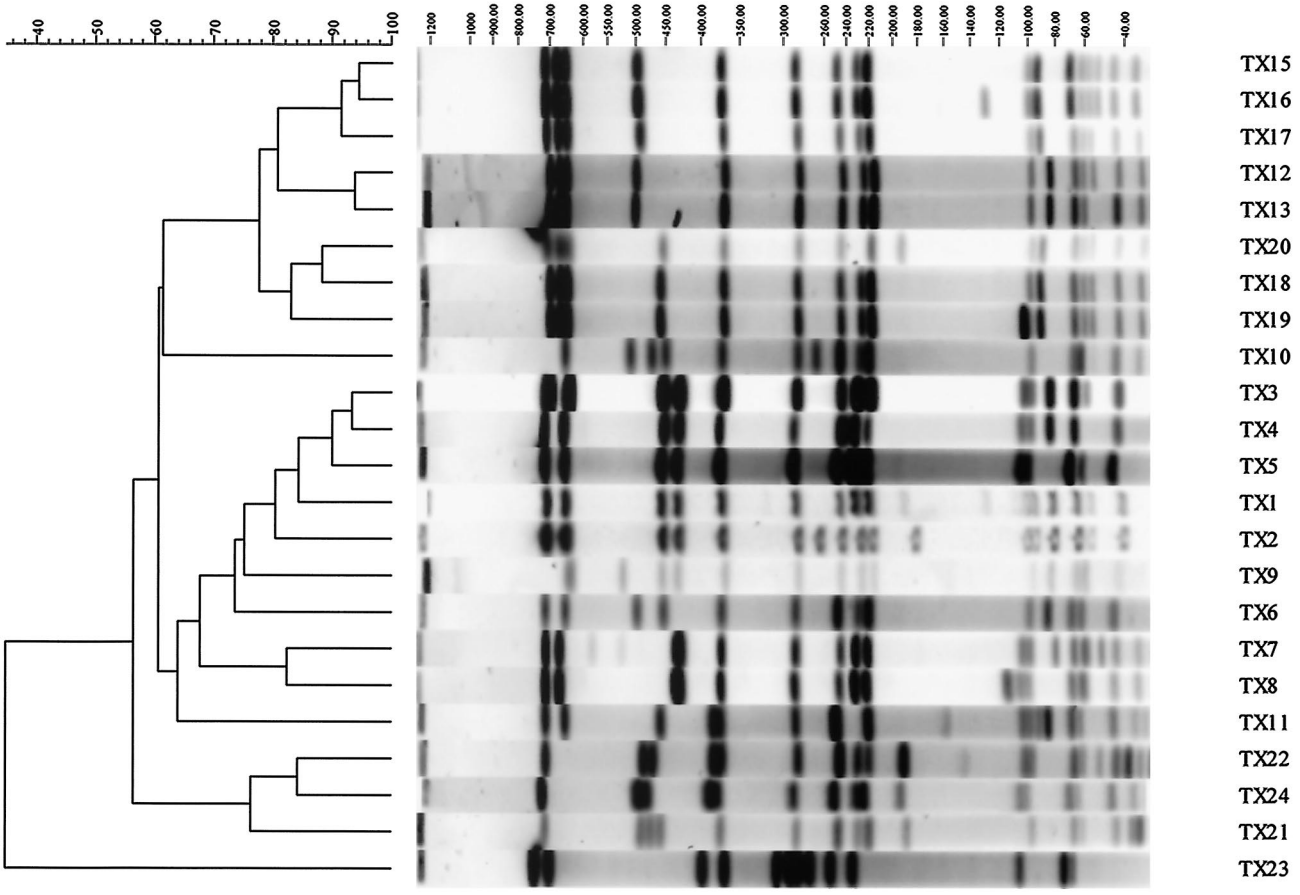

FIG. 1. Dendrogram generated by GelCompar II software showing the relationships of 23 representative fingerprints (*Xba*I-PFGE or TX types) for 119 *Salmonella* serotype Typhimurium isolates. The bands generated were analyzed by using the Dice coefficient and the unweighted pair group method with arithmetic averages. Numbers above the gel are molecular sizes in kilobase pairs.

*Sph*I [PS] ribotyping, and plasmid profiling) and has proven successful in our laboratory for other *Salmonella* serotypes (13, 14).

MATERIALS AND METHODS

***Salmonella* isolates.** A total of 119 isolates from humans ($n = 28$) and animals or their environments ($n = 91$), belonging to DT104 ($n = 66$), U302 ($n = 33$), DT204b ($n = 12$), or DT49 ($n = 8$), were randomly selected to cover a variety of geographical locations within England and Wales (Midlands [$n = 14$], Northeast [$n = 28$], Northwest [$n = 11$], Southeast [$n = 15$], and Southwest [$n = 34$]) and Northern Ireland ($n = 17$). Among the animal isolates, a variety of species were represented (chickens [$n = 39$], cattle [$n = 20$], pigs [$n = 13$], horses [$n = 5$], sheep [$n = 4$], dogs [$n = 3$], a cat [$n = 1$], a turkey [$n = 1$], a duck [$n = 1$], exotics [$n = 1$], a wild bird [$n = 1$], and feed [$n = 2$]). The *Salmonella* cultures were serotyped by following a microagglutination method (25) and were phage typed (1) at the Veterinary Laboratories Agency (Weybridge, United Kingdom).

Plasmid analysis. Plasmid DNA was isolated by the alkaline lysis method as described previously (12). Samples were analyzed by electrophoresis in 1× Tris-borate-EDTA buffer (with recirculation at 20°C) at 150 V for 4.5 h on 0.8% agarose gels. Plasmid-containing *Escherichia coli* strain 39R861 and a supercoiled DNA ladder (Gibco BRL, Paisley, United Kingdom) were used as size markers. Plasmids were compared by use of GelCompar II software. The molecular weights of the plasmids were calculated by comparison with the external markers, and images were normalized accordingly.

Restriction fragment length polymorphism. Genomic DNA was extracted from approximately 200 mg (wet weight) of bacteria; then restriction enzyme digests (*Pst*I plus *Sph*I) of *Salmonella* DNAs were prepared and were fractionated by electrophoresis as described previously (13). Fractionated DNA was transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Lewes, United Kingdom) with 0.4 mM NaOH in a vacuum blotting apparatus (Pharmacia Biotech, St. Albans, Herts, United Kingdom) connected to a variable pump set at 4×10^3 Pa for 1 h. Membranes were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and air dried before DNA was fixed to the membranes by cross-linking under UV light. Membranes were prehybridized for 4 h at 42°C in 20 ml of DIG Easy Hyb (Roche Molecular Biochemicals). Plasmid pKK3535 carrying the *rrnB* rRNA operon from *E. coli* was extracted by using a QIAfilter plasmid Midi purification kit (Qiagen, Crawley, United Kingdom) and was labeled with digoxigenin-11-dUTP by a random-primed DNA labeling technique by use of the DIG-High Prime kit (Roche Molecular Biochemicals). Probes were denatured by boiling and added to fresh hybridization fluid at 20 ng/ml, and hybridizations were performed overnight at 42°C in a Hybaid oven. The presence of the labeled probe was detected by using the alkaline phosphatase-conjugated antibody DNA detection kit (Roche Molecular Biochemicals) and the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) as recommended by the supplier. Images produced on X-ray film were computer analyzed using GelCompar II 1.01 software (Applied Maths, Kortrijk, Belgium). Molecular weights of the probed fragments were calculated by comparison with the external markers, and images from different gels were normalized accordingly. For the purposes of this study, different PS types were assigned to strains when a genetic difference could be detected.

PFGE. A single colony of each *Salmonella* isolate was incubated overnight at 37°C in 3-ml amounts of Luria-Bertani (LB) broth with moderate shaking. One-milliliter aliquots of the cultures were transferred into microcentrifuge tubes and washed twice with 1 ml of saline solution (0.85% [wt/vol] NaCl); finally, cells were resuspended in 0.8 ml of saline solution and equilibrated at 40°C. This suspension was mixed in equal parts with molten 2% agarose (CleanCut; Bio-

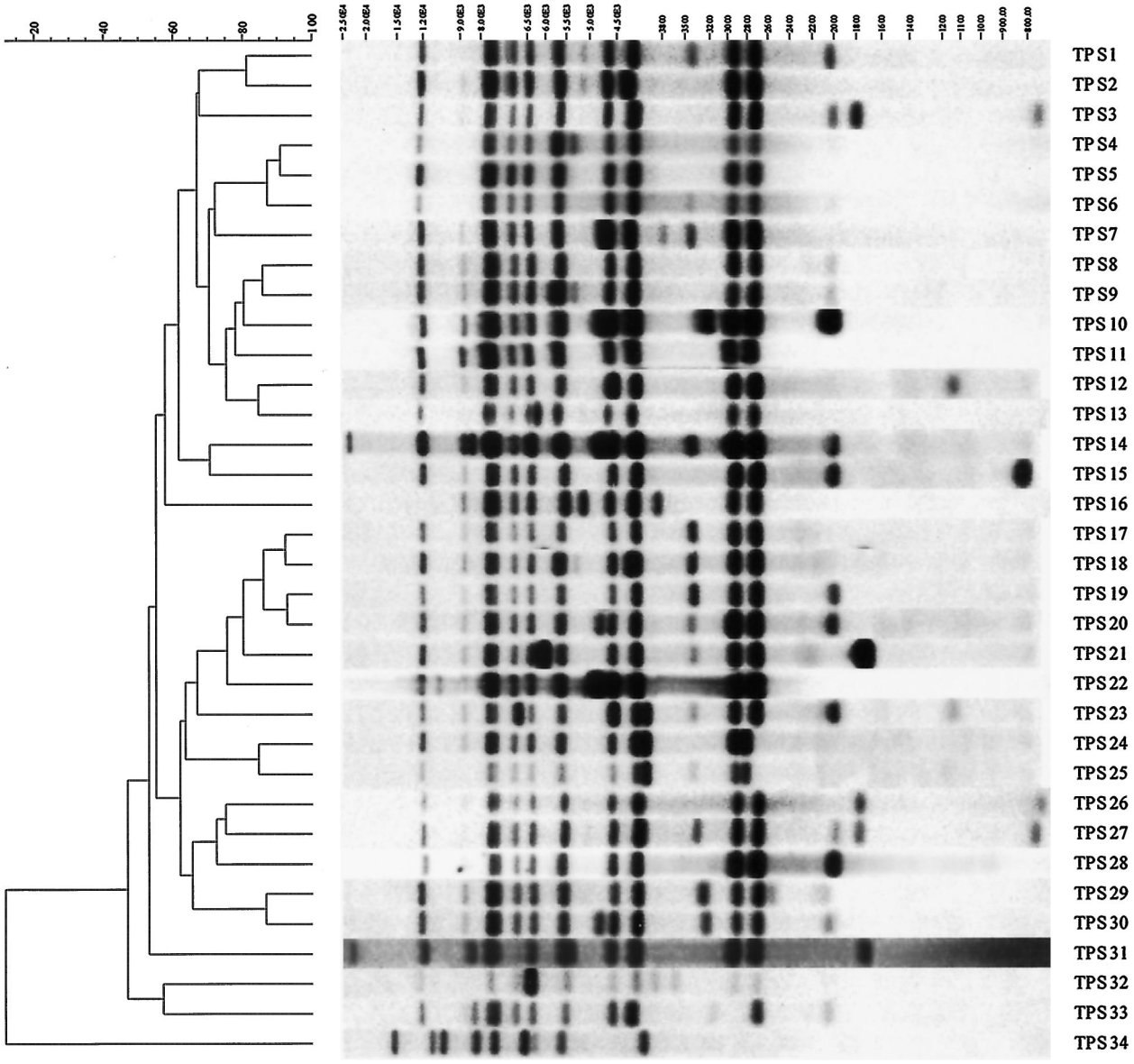

FIG. 2. Dendrogram generated by GelCompar II software showing the relationships of 34 representative fingerprints (PS ribotypes) for 119 *Salmonella* serotype Typhimurium isolates. The bands generated were analyzed by using the Dice coefficient and the unweighted pair group method with arithmetic averages. Numbers above the gel are molecular sizes in base pairs.

Rad, Hempstead, United Kingdom) and pipetted into disposable molds. Three of these agarose plugs were incubated overnight at 56°C in 2 ml of ES lysis buffer (0.5 M EDTA–1% *N*-laurylsarcosine [Sigma, Poole, United Kingdom]) with proteinase K (Sigma) at a final concentration of 250 μg/ml. The next morning, the lysis buffer was replaced with fresh ES buffer-proteinase K solution, followed by a second overnight incubation at 56°C. Thereafter, DNA-containing plugs were thoroughly washed in TE buffer (10 mM Tris-HCl–1 mM EDTA [pH 8]) and stored at 4°C. Chromosomal DNA was digested with 30 U of *Xba*I (Promega, Southampton, United Kingdom), and PFGE was performed with a CHEF DRIII system (Bio-Rad) in 0.5× Tris-borate-EDTA extended-range buffer (130 mM Tris, 45 mM boric acid, 2.5 mM EDTA; Bio-Rad) with recirculation at 14°C. DNA macrorestriction fragments were resolved on 1% agarose gels (PFGE-certified agarose [Bio-Rad]), and a lambda ladder pulsed-field gel marker (New England Biolabs, Hitchin, United Kingdom) was used as a size standard. Pulse times were ramped from 5 to 60 s during a 48-h run at 5.1 V/cm. The preparation and digestion of DNA from a proportion of the strains were repeated, and samples were electrophoresed under the same conditions to assess the reproducibility of the method. Macrorestriction patterns were compared by use of GelCompar II software. The molecular weights of the restriction fragments were calculated by comparison with the external markers, and images were normalized accordingly. Different profiles were assigned to *Xba*I-PFGE types (X types) according to differences in the restriction patterns. A difference of at least one restriction fragment in the patterns was considered the criterion for distinguishing between different clones or strains.

RESULTS

PFGE of *Xba*I restriction digests of DNAs from 120 isolates generated 23 PFGE profiles containing 13 to 18 restriction fragments. The most prevalent type was TX3, found in 67.5% of the isolates. Figure 1 represents a dendrogram with all PFGE types for the isolates included in the study. Five differ-

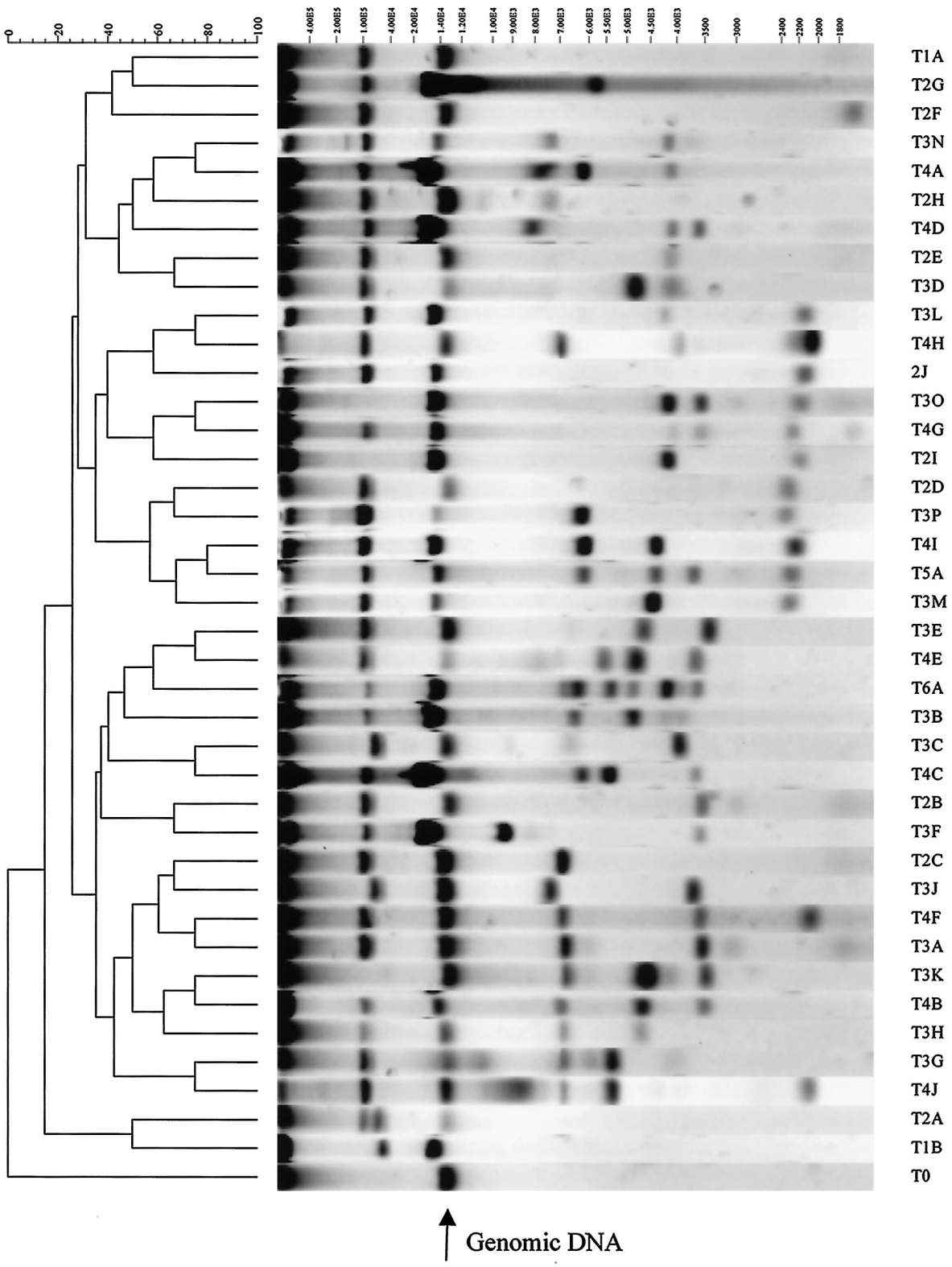

FIG. 3. Dendrogram generated by GelCompar II software showing the relationships of 40 representative plasmid types for 119 *Salmonella* serotype Typhimurium isolates. Clustering analysis was performed by using the Dice coefficient and the unweighted pair group method with arithmetic averages. Numbers above the gel are molecular sizes in base pairs.

TABLE 1. Genomic types (PFGE type and ribotype) present in two or more *Salmonella* serotype Typhimurium isolates

Genomic type	Fingerprint[a]	No. of isolates	Phage type(s)	Animal species	Area(s)[b]
5	TX12/TPS11	6	DT49	Cow, chicken, horse	NE, SW
14	TX18/TPS3	4	DT204b	Chicken	M, SW
23	TX3/TPS11	49	DT104, U302	Cow, chicken, horse, pig, sheep, turkey, dog, human	NE, NW, M, SE, SW, NI
24	TX3/TPS12	2	U302	Human	SE
26	TX3/TPS17	5	DT104	Chicken, pig, human	NE, SW, M
27	TX3/TPS19	3	DT104	Chicken, human	SE
35	TX3/TPS5	3	DT104	Chicken	NW, SE, SW
38	TX3/TPS8	7	DT104, U302	Cow, chicken, pig, wild bird	NE, SE, SW, NI

[a] PFGE type/ribotype.
[b] NE, Northeast; NW, Northwest; SE, Southeast; SW, Southwest; M, Midlands; NI, Northern Ireland.

ent clusters (similarity percentage, <60%) were found after cluster analysis of these fingerprints. PFGE types within clusters B, C, D, and E were found only among isolates of phage types DT104 and U302; similarly, PFGE types within cluster A were found only among isolates of phage types DT204b and DT49. The prevalent type TX3 was found only in DT104 and U302 isolates from a variety of animal species as well as from humans; similarly, it was widespread in all geographical locations. Types TX12 and TX18 were the most common types among DT204b and DT49 isolates, respectively.

PS ribotyping allowed identification of 34 ribotypes with 9 to 16 hybridizing bands, and the most prevalent type, TPS11, was found in 52.5% of the isolates. Figure 2 represents a dendrogram with all PS types for the isolates included in the study. Five separated clusters (similarity percentage, <55%) were identified from the clustering analysis. The cluster analysis did not show a clear association of phage types with clusters. The prevalent type TPS11 was found in isolates from all phage types, from several species (including humans), and from all geographical locations.

Forty different plasmid profiles with 0 to 6 plasmids were identified. The plasmid profile type comprised a numeral indicating the number of plasmids observed followed by a letter corresponding to the order in which the type was encountered. The most prevalent plasmid profile (T1A), containing only the serotype-specific plasmid (approximately 90.6 kb), was found in 33.3% of the isolates. Figure 3 represents a dendrogram with the types for the isolates included in the study. The prevalent type 1A was highly associated with DT104 (50% of the DT104 isolates belonged to plasmid type 1A), while only 11% of the isolates from the other phage types contained only the serotype-specific plasmid.

A combination of the two methods identifying polymorphism in genomic DNA allowed clear differentiation of 48 clones, among which type 23 (TX3/TPS11) was the most frequent, found in 41.2% of the isolates. This prevalent clone was found in isolates from all geographical locations and from both animals and humans. Inclusion of the plasmid-profiling results in the analysis allowed differentiation of 64 combined types or strains, among which type 26 (TX3/TPS11/1A) was the most frequent, found in 28.6% of the isolates. This prevalent clone was found only among DT104 isolates and in a single U302 isolate, and it was found in isolates from all geographical locations and from both animals and humans. Tables 1 and 2 summarize the fingerprinting results for the genomic and combined types (respectively) present in more than one isolate.

DISCUSSION

The purpose of this study was to investigate clonal diversity among human and animal *Salmonella* serotype Typhimurium isolates from a variety of geographical areas in England, Wales, and Northern Ireland. The isolates were fingerprinted by a combination of well-established genetic methods, an approach that has been shown to provide adequate discriminatory power for other *Salmonella* serotypes (13, 14).

The different techniques identified different degrees of polymorphism, with plasmid profiling identifying the most (40 types), followed by PS ribotyping (34 types) and PFGE (23 types). There are considerable differences in the literature

TABLE 2. Combined types (PFGE type, ribotype, and plasmid profile) present in two or more *Salmonella* serotype Typhimurium isolates

Combined type	Fingerprint[a]	No. of isolates	Phage type(s)	Animal species	Area(s)[b]
7	TX12/TPS11/T3L	4	DT49	Cow, horse	NE, SW
16	TX18/TPS3/T4I	4	DT204b	Chicken	M, SW
26	TX3/TPS11/T1A	34	DT104, U302	Cow, chicken, horse, pig, sheep, turkey, human	NE, NW, M, SE, SW, NI
27	TX3/TPS11/T2A	4	U302	Cow	SE, SW
30	TX3/TPS11/T2D	5	DT104, U302	Cow, chicken, pig	NE, SW
35	TX3/TPS12/T3B	2	U302	Human	SE
37	TX3/TPS17/T2B	3	DT104	Pig, human	NE, SW
41	TX3/TPS19/T3A	2	DT104	Human	NW, SE
49	TX3/TPS5/T2D	3	DT104	Chicken	M, SW
52	TX3/TPS8/T2C	4	DT104	Chicken, pig, wild bird	NE, SE, NI

[a] PFGE type/ribotype/plasmid profile.
[b] NE, Northeast; NW, Northwest; SE, Southeast; SW, Southwest; M, Midlands; NI, Northern Ireland.

regarding the sensitivity of each of these methods, which may be due to differences in the protocols used and also to differences in the composition of the panels of isolates under study. From our results it seems clear that a prevalent genomic clone is present for each of the phage types included in the study (genomic type 23 was found for 61% of the DT104 isolates, genomic type 14 was found for 42% of the DT204b isolates, genomic type 5 was found for 75% of the DT49 isolates, and finally genomic type 23 was found for 27% of the U302 isolates). In addition to these prevalent clones, a variety of less frequent clones are present within each of the phage types included in the study. The PFGE data suggest a relationship between phage types DT104 and U302 and between phage types DT204b and DT49, which is in agreement with previous reports (4, 22, 30).

It has been reported that the majority of *Salmonella* serotype Typhimurium strains carry the serotype-specific plasmid of 60 MDa, alone or in combination with other plasmids. Plasmid profiling seems to present a reasonable degree of sensitivity when applied to *Salmonella* serotype Typhimurium (2, 18, 29). However, the inherent mobility of the plasmid DNA suggests instability of the characteristic under scrutiny. This is a limitation which must be recognized in epidemiological research. In a 4-year longitudinal study on a pig farm, we have recovered *Salmonella* serotype Typhimurium DT104 strains with identical genomic fingerprints (assessed by PFGE with several restriction enzymes and by ribotyping) but different plasmid profiles; some of these plasmids were transferred in vitro to *Salmonella* recipient strains. This may indicate that at least some of the plasmid markers are not stable and could be shared between strains (unpublished data). Therefore, plasmid profiling may not be an adequate way of identifying clones, especially over long periods.

In view of the above, we have presented the results in two ways: (i) including only genomic information (PFGE and ribotyping) and (ii) combining genomic fingerprinting and plasmid profiling. If we consider only the genomic fingerprints, 40 of the 48 different types were found in individual isolates and 8 types were found in more than one isolate (Table 1). In most cases, these clones appeared in isolates from several animal species and from several geographical locations. In addition, types 23, 26, and 27 were found in both animal and human isolates. Two U302 isolates from humans from two different hospitals in the Southwest presented the same fingerprint (type 24), indicating a possible link between these isolates. If we include the plasmid profile information in the analysis, then 54 of 64 combined types were found only in individual isolates, while the remaining 10 were present in two or more isolates (Table 2). Of these, combined types 7, 26, 30, 37, and 52 were found in more than one animal species and geographical area. Furthermore, combined types 26 and 37 were found among both animal and human isolates. Combined type 52 was found in isolates from chickens, pigs, and wild birds. This evidence may indicate that wildlife could be an important factor involved in dissemination of *Salmonella* serotype Typhimurium infections among livestock, as has been suggested previously (6, 10). Unless a greater number of isolates are tested (ideally within surveillance schemes), it is difficult to comment on the significance of the great variety of individual sporadic clones found in the study. It seems likely that with a wider sample, these clones may be shown to be widely distributed among hosts and locations. In the year 2000, the number of reports of *Salmonella* serotype Typhimurium in livestock in Great Britain fell by 13.7%, to the lowest number of incidents reported for many years. However, there was a 12.3% increase in the numbers of reports of this serotype in humans (7). It has been suggested that a considerable percentage of the human cases in the United Kingdom are travel related, and this may explain the differences in the fingerprints found among human and animal isolates. However, this is only speculation at present, and testing of a significant number of animal and human isolates would be necessary to clarify this question. It seems clear that a very prevalent clone of phage type DT104 is present within the animal and human pools of *Salmonella* serotype Typhimurium isolates in the United Kingdom. This seems to be the same clone that has been reported elsewhere (18).

An Irish study (20) found diversity to be greatest among isolates from cattle and to a lesser extent among isolates from pigs and sheep. Also, a French study (9) revealed clonal similarity between cattle and human isolates, and diversity among human, poultry, and pig strains. In our study, we did not find clear evidence of a higher degree of diversity for any of the animal species included, or of any link between isolates from particular animal species and humans.

The data presented here show the inaccuracy of epidemiological conclusions drawn on the basis of a single fingerprinting method. Strains that share one of the markers do not necessarily belong to the same clone, and for populations with a high degree of clonality a multiple typing approach is required to enable enough discrimination to track clones or strains for epidemiological investigations. However, it is not always true that highly discriminatory methods have more value for deriving relationships among the population under study. It is necessary to validate molecular typing data by use of conventional epidemiological information. In an area such as food-borne zoonoses, where the traceability of a strain from farm to fork to patient is crucial, it is important to recognize the need for a harmonized methodology for testing and analysis. Such an approach would allow the establishment of "compatible" databases for human and veterinary isolates, which would facilitate enormously the investigation of outbreaks and the prospective use of surveillance data.

ACKNOWLEDGMENTS

This work was supported by grants from the Test Development Program (TDP) (FZ2003) and Seedcorn Program (SC0096) of the Veterinary Laboratories Agency, Weybridge, United Kingdom.

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