Characterization of Salmonella enterica Serovar Typhimurium DT104 Isolated from Denmark and Comparison with Isolates from Europe and the United States

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A total of 136 isolates of Salmonella enterica serovar Typhimurium DT104 from Denmark (n = 93), Germany (n = 10), Italy (n = 4), Spain (n = 5), and the United Kingdom (n = 9) were characterized by antimicrobial resistance analysis, plasmid profiling, pulsed-field gel electrophoresis (PFGE) with the restriction enzymes XbaI and BlnI, and analysis for the presence of integrons and antibiotic resistance genes. The isolates from Denmark were from nine pig herds, while the isolates from other countries were both of animal and of human origin. All but 10 isolates were resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfonamides, and tetracycline. Five isolates from the United Kingdom and Spain were sensitive to all antibiotics examined, whereas four isolates from the United Kingdom and the United States were also resistant to one or more of the antibiotics, namely, gentamicin, neomycin, and trimethoprim. All but two strains had the same PFGE profiles when the XbaI restriction enzyme was used, while seven different profiles were observed when the BlnI restriction enzyme was used. Different dominating BlnI types were observed among European isolates compared with the types observed among those from the United States. All the isolates harbored common 95-kb plasmids either alone or in combination with smaller plasmids, and a total of 11 different plasmid profiles were observed. Furthermore, all but one of the multidrug-resistant isolates contained two integrons, ant (3′)-Ia and pse-1. Sensitive isolates contained no integrons, and isolates that were resistant to spectinomycin,streptomycin, and sulfonamides had only one integron containing ant (3′)-Ia. When restriction enzyme BlnI was used, the 14 isolates from one of the nine herds in Denmark showed unique profiles, whereas isolates from the remaining herds were homogeneous. Among isolates from seven of nine herds, the same plasmid profile (95 kb) was observed, but isolates from two herds had different profiles. Thus, either PFGE (with BlnI) or plasmid profiling could distinguish isolates from three of nine pig herds in Denmark. The epidemiological markers (antimicrobial susceptibility testing, plasmid profiling, and PFGE) applied demonstrated high in vivo stability in the Danish herds. This may indicate that some different strains of multidrug-resistant S. enterica serovar Typhimurium DT104 have been introduced into Danish food animal herds. The presence of isolates from six different countries with similar profiles by PFGE with XbaI and highly homogeneous profiles by PFGE with BlnI indicate that multidrug-resistant S. enterica serovar Typhimurium DT104 has probably been spread clonally in these countries. However, some minor variation could be observed by using plasmid profiling and profiling by PFGE with BlnI. Thus, a more sensitive technique for subtyping of strains of DT104 and a broader investigation may help in elucidating the epidemiological spread of DT104 in different parts of the world.

During the last few decades, infections with Salmonella enterica have been recognized as a major hazard to humans in most developed countries, and the main source of infections has been contaminated food of animal origin (11). The genus Salmonella covers more than 2,400 different serotypes (21), and although all serotypes must be considered potential human pathogens, only a limited number of serotypes are attributed to as the cause of infection in humans and animals. In addition, some specific clones of S. enterica have been very dominant in one or more host species and have been able to spread worldwide. The establishment and spread of S. enterica serovar Enteridis PT4 and PT8 in poultry and humans and of multidrug-resistant S. enterica serovar Typhimurium DT204 and DT193 in cattle and humans are typical examples of this (25, 26, 29).

In the 1990s, a new multidrug-resistant strain of S. enterica serovar Typhimurium, strain DT104, emerged and was first recognized in the United Kingdom (31), but in the following years the strain has been isolated in other countries as well, such as Germany (1), the United States (6), Canada (22), Italy (27), Belgium (15), Israel (19), and Denmark (5). However, this type may occur more widely, as all countries do not have an organized salmonella surveillance system that reports the resistance patterns and phage types of S. enterica strains isolated from different sources. The multidrug-resistant strain S. enterica serovar Typhimurium DT104 was initially characterized as having chromosomally located genes for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (resistance type, ACSSuT), but in recent years strains with additional resistance or decreased susceptibility to gentamicin, trimethoprim, and/or fluoroquinolones have been observed (17, 31, 32). Multidrug-resistant strain DT104 was originally isolated from cattle but has now been isolated from a wide range of animal host species (6, 17, 31), and the organism has become the second most common course of human salmonellosis after S. enterica serovar Enteridis PT4 in the United Kingdom and Germany (1, 32).

The identification of multidrug-resistant S. enterica serovar Typhimurium DT104 in Danish food production animal herds in 1996 resulted in an agreement between animal producer
organizations and veterinary authorities that contamination of foodstuffs of Danish origin with multidrug-resistant strain DT104 should be considered unacceptable (5). Therefore, the Danish salmonella surveillance system has been updated and adapted to identify infected animal herds aimed at eradicating multidrug-resistant strain DT104 from food production animal herds.

It would thus be important to investigate whether multidrug-resistant strain *S. enterica* serovar Typhimurium DT104 was introduced into Danish food production animal herds by imported animals or whether the resistance has emerged within the country as a result of selection of resistance genes. The aim of this study was to characterize multidrug-resistant *S. enterica* serovar Typhimurium DT104 isolated from Danish food production animal herds by the use of epidemiological markers and to compare these isolates with those from other countries in order to describe the clonality of these pathogens. In addition, selected antibiotic resistance genes have been characterized, and the localization of these genes has been investigated.

**RESULTS**

*S. enterica* serovar Typhimurium DT104 isolates were isolated at different times from nine Danish food animal herds infected in 1996, 1997, and early 1998 and were found to be homogeneous since all but one shared the resistance profile AChSpStSuT. A single DT104 isolate from herd 1 and two isolates from herd 8 had the resistance profile SpStSu. All but one of the isolates had a common PFGE profile when the restriction enzyme *Xba*I was used (*Xba*I type 1). The different PFGE profile obtained with *Xba*I, which was found for a single isolate from an animal in herd 9, differed from the common type described above by two bands. When the restriction enzyme *Bln*I was used, 14 isolates from herd 6 showed a unique profile (*Bln*I type V), whereas isolates from the remaining herds were homogeneous since all but two were of a common type. One isolate of *Bln*I type III differed from the common type by two bands. This isolate was identified among the 42 isolates in herd 1 isolated over a period of 10 months. The second isolate (herd 5) was of *Bln*I type X, and the profile of this isolate, which originated from a dog, also differed from the common plasmid profile for isolates from the herd. The electrophoretic conditions applied gave a good separation of the minor fragments but gave an insufficient separation of the larger fragments. An additional analysis under other electrophoretic conditions applied for separation of fragments larger than 800 kb, however, showed variation in only one of all strains included in the study (data not shown). This single strain (*Bln*I type X) was from herd 5 and also differed from the other isolates in several other characteristics.

The common plasmid profile for isolates from pigs in herd 5 was fragments of 95 and 6.8 kb, but the plasmid profile of the isolate from the dog was fragments of 115, 90, 4.4, and 3.0 kb. Isolates from herd 2 harbored a 95-kb plasmid and an additional plasmid of approximately 2.2 kb, whereas isolates from all other herds contained a single plasmid of 95 kb.

The Danish isolates collected from 1991 to 1995 originated from four different pig herds. One of the four isolates had a resistance profile identical to the profile of those found during 1996 to 1998 (AChSpStSuT), another isolate had the SpStSu resistance profile, and the remaining two isolates were fully sensitive to all the antibiotics investigated. These four isolates had the common *Xba*I type (*Xba*I type 1). Only the two resistant isolates were of the common *Bln*I type (type I), while the two sensitive isolates had another *Bln*I profile (type V) that was separated from the *Bln*I type I profile by a two-band difference. Three isolates (isolates with the SpStSu resistance profile and sensitive isolates) harbored the 95-kb plasmid alone, whereas the fourth multidrug-resistant isolate harbored plasmids of 95 and 2.2 kb.

A high degree of homogeneity was observed among the isolates from Denmark, Germany, Italy, Spain, the United Kingdom, and the United States. All but two strains had the same PFGE profiles when the restriction enzyme *Xba*I was used, whereas seven different profiles were observed when the restriction enzyme *Bln*I was used (Table 2; Fig. 1). The dominating *Bln*I type among European isolates was type I, whereas...
it was type V among isolates from the United States. AmChSpStSuTe was the most common resistance profile among isolates from each country, but one isolate from the United Kingdom and three isolates from the United States had additional resistance markers. Four of the 11 isolates from the United Kingdom and I of the 5 isolates from Spain were sensitive to all antibiotics examined, and 4 of these sensitive isolates were of BlnI type IV whereas the fifth isolate, from the United Kingdom, was BlnI type X. All isolates possessed a single 95-kb plasmid either alone or in combination with smaller plasmids, and a total of 11 different plasmid profiles were seen (Table 2).

Analysis of integrons and antibiotic resistance genes showed that isolates with the AmChSpStSuTe resistance profile had two integrons, indicated by two fragments of 1,008 and 1,133 bp generated by PCR. All isolates with these two integrons were also positive for products by PCR with the primers for integrons used in combination with primers for ant (3')-Ia and pse-I. Isolates with the SpStSu resistance profile had only one integron.

<table>
<thead>
<tr>
<th>Herd no.</th>
<th>No. of isolates</th>
<th>Period of isolation (mo.day/yr)</th>
<th>Source(s)</th>
<th>Characterization of the herd type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stability of the herd type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>1991 Pig</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95, 2.2</td>
<td>One of 41 isolates had SpStSu resistance profile</td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>1</td>
<td>1994 Pig</td>
<td>Resistance profile, SpStSu; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>One of 41 isolates was BlnI type III</td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>1</td>
<td>1994 Pig</td>
<td>Resistance profile, sensitive; PFGE profile, XbaI type I and BlnI type IV; plasmid profile (approx. kb): 95</td>
<td>No variations in two isolates</td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>1</td>
<td>1995 Pig</td>
<td>Resistance profile, sensitive; PFGE profile, XbaI type I and BlnI type IV; plasmid profile (approx. kb), 95</td>
<td>No variations in two isolates</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>11.20.96–9.10.97 Pigs</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>No variations in four isolates</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>7.5.96–9.17.97 Pigs</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile XbaI type I and BlnI type I; plasmid profile (approx. kb), 95 and 2.2</td>
<td>No variations in three isolates</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1.19.96–5.9.97 Calf, pig</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>No variations in two isolates</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>8.6.96–5.12.97 Pigs</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>No variations in two isolates</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>3.10.97–11.7.97 Pigs, dog, environment</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95 and 6.8</td>
<td>No variations in isolates from pigs or the environment</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>10.31.97–12.19.97 Pigs, cattle</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type V; plasmid profile (approx. kb), 95</td>
<td>No variations in 14 isolates</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1.7.98 Pig</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>Two isolates with SpStSu resistance profile</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>1.9.98–1.23.98 Pigs, dog</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>One isolate was XbaI type V</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>12.1.97–12.22.97 Pigs</td>
<td>Resistance profile, AmChSpStSuTe; PFGE profile, XbaI type I and BlnI type I; plasmid profile (approx. kb), 95</td>
<td>One isolate was XbaI type V</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Herd type is defined as the type most frequently isolated from the herd.

<sup>b</sup> HM, historical material for comparison.
integron, indicated by a PCR product of 1,008 bp, and were positive for a product by PCR with the primers for integrons in combination with primers for ant (3’)-Ia. Susceptible isolates did not yield any products by PCR. No integrons could be detected in a single multidrug-resistant isolate from a dog from herd 5.

**DISCUSSION**

In the 1990s, a new strain of *S. enterica* emerged in many countries. This multidrug-resistant strain, *S. enterica* serovar Typhimurium DT104, has been reported from the United Kingdom (31), Germany (1), the United States (6), Canada (22), Italy (27), Belgium (15), Israel (19), and Denmark (5). The spread may, however, be more extensive since only a limited number of countries have surveillance systems that report on the resistance patterns and phage types of *Salmonella* strains. Reports on resistance patterns alone have indicated that 69.8% of *S. enterica* serovar Typhimurium strains of bovine origin in France had a resistance profile which was compatible with that of multidrug-resistant strain DT104 (7).

In the present study, isolates from Danish food production animal herds were compared with the isolates from Germany, Italy, Spain, the United Kingdom, and the United States by using different epidemiological markers. Danish *S. enterica* serovar Typhimurium DT104 isolates were isolated from nine different herds within periods of up to 16 months. These isolates were homogeneous and showed (with few exceptions) identical genomic profiles by PFGE when restriction enzymes *Xba*I and *Bln*I were used, as well as possessed identical resistance patterns (the *AmChSpStSuTe* resistance profile). By PFGE with the restriction enzyme *Bln*I, isolates from one herd (herd 6) could be separated from isolates from the other herds, and by plasmid profiling, isolates from two herds (herds 2 and 5) could be distinguished. The unique PFGE and plasmid profiles were stable among isolates within the isolation period. In all, the results showed a high degree of in vivo stability of the different epidemiological markers used in these investigations.

In the present study, either PFGE (with *Bln*I) or plasmid profiling could separate isolates from three of the nine Danish pig herds. The dominant plasmid profile consisted of a single plasmid of 95 kb. A plasmid of this size is known as the serotype-associated plasmid, and a plasmid profile consisting of this plasmid alone is dominant in *S. enterica* serovar Typhimurium strains (4, 33). The discriminatory power of this profile is therefore low compared to that of other plasmid profiles. The three different plasmid profiles observed among the isolates in this investigation and the unique *Bln*I profiles demonstrated a high degree of in vivo stability in the Danish herds for up to 16 months. This may indicate that different strains of multidrug-resistant strain *S. enterica* serovar Typhimurium DT104 have been introduced into Danish food production animal herds over time. Eleven different plasmid profiles were demonstrated in the total material investigated in this study. Such diversity has also been reported among isolates from the United Kingdom (17, 33, 34). Plasmid profiling has previously been used to differentiate a group of closely related, multidrug-
resistant S. enterica serovar Typhimurium DT204c strains (36), with which the method has proved its usefulness, despite the potential instability of plasmids. In general, the discriminatory power of the epidemiological markers applied in the present study was low, which reflected the high degree of clonality among the Danish isolates of multidrug-resistant strain DT104.

The Danish isolates of multidrug-resistant strain S. enterica serovar Typhimurium DT104 recovered from 1991 to 1995 were very similar to those found from 1996 to 1998. These results may indicate that the multidrug-resistant strain DT104 has been present in Denmark for several years without causing a tremendous spread of infection, which has also been seen in Germany and the United Kingdom (1, 37). In contrast, sensitive isolates of strain DT104 (two Danish isolates, four isolates from the United Kingdom, and one isolate from Spain) differed from the multidrug-resistant isolates in their Bin1 profiles and by the absence of the two integrons coding for resistance to ampicillin, streptomycin, and sulfonamide. Even though only minor differences in Bin1 types were observed between sensitive and resistant isolates, they may indicate that the resistant isolates represent a unique clonal line which has spread among herds and countries. Recently, Ridley and Threlfall (24) have reported that sensitive and multidrug-resistant strains of S. enterica serovar Typhimurium DT104 can be separated by PFGE with XbaI, generating a fragment of approximately 10 kb which hybridizes to the integrons. This differentiation was not shown in the present study, as only fragments of >100 kb were used to determine the PFGE type, but the presence and absence of integrons in sensitive and resistant strains correlate with observations from the United Kingdom.

Multidrug-resistant strains of S. enterica serovar Typhimurium DT104 from different countries in Europe and from the United States had a common XbaI profile and a basic resistance pattern. This homogeneity is likely to be caused by a common origin, and thus, a new worldwide epidemic of S. enterica infection seems to have appeared. In the 1980s and 1990s, S. enterica serovar Enteritidis PT4 was the most dominant type among humans and was especially dominant in layer birds. In the 1980s, S. enterica serovar Enteritidis PT4 is still persistent worldwide and is the most common cause of human salmonellosis, but a number of scientists have pointed out that in the future, strain PT4 may be replaced by the multidrug-resistant S. enterica serovar Typhimurium DT104 (1, 35).

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