

AN OUTBREAK OF MULTIDRUG-RESISTANT, QUINOLONE-RESISTANT *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM DT104

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ABSTRACT

Background Food-borne salmonella infections have become a major problem in industrialized countries. The strain of *Salmonella enterica* serotype typhimurium known as definitive phage type 104 (DT104) is usually resistant to five drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. An increasing proportion of DT104 isolates also have reduced susceptibility to fluoroquinolones.

Methods The Danish salmonella surveillance program determines the phage types of all typhimurium strains from the food chain, and in the case of suspected outbreaks, five-drug-resistant strains are characterized by molecular methods. All patients infected with five-drug-resistant typhimurium are interviewed to obtain clinical and epidemiologic data. In 1998, an outbreak of salmonella occurred, in which the strain of typhimurium DT104 was new to Denmark. We investigated this outbreak and report our findings here.

Results Until 1997, DT104 infections made up less than 1 percent of all human salmonella infections. The strain isolated from patients in the first community outbreak of DT104 in Denmark, in 1998, was resistant to nalidixic acid and had reduced susceptibility to fluoroquinolones. The outbreak included 25 culture-confirmed cases. Eleven patients were hospitalized, and two died. The molecular epidemiology and data from patients indicated that the primary source was a Danish swine herd. Furthermore, the investigation suggested reduced clinical effectiveness of treatment with fluoroquinolones.

Conclusions Our investigation of an outbreak of DT104 documented the spread of quinolone-resistant bacteria from food animals to humans; this spread was associated with infections that were difficult to treat. Because of the increase in quinolone resistance in salmonella, the use of fluoroquinolones in food animals should be restricted. (N Engl J Med 1999; 341:1420-5.)

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THE incidence of zoonotic food-borne salmonella infections has increased in most industrialized countries. Of particular concern is the spread of multidrug-resistant *Salmonella enterica* serotype typhimurium, known as definitive phage type 104 (DT104).^{1,2} In Denmark, efforts have been made to control salmonella in chickens (layers and broilers) and pigs.³ Intensive surveillance for salmonella in live animals and in food products is conducted during processing and distribution to wholesalers and retailers. These data, together with the data from surveillance of diseases in humans, are col-

lated and analyzed at the Danish Zoonosis Center, a network including the Danish Veterinary Laboratory, the Food and Veterinary Administration, and the Statens Serum Institut. In this report we describe how this surveillance system identified and controlled an outbreak of multidrug-resistant DT104. The investigation provided a unique opportunity to document the spread of an infectious agent through the food chain. Furthermore, the results indicate the reduced effectiveness of treatment of human disease with the spread of a quinolone-resistant strain of DT104.

METHODS

Surveillance

In Denmark the diagnosis of human salmonella infections is carried out at the Statens Serum Institut and at eight clinical microbiology laboratories. The Statens Serum Institut receives notification of positive findings as well as isolates from the microbiology laboratories. Surveillance for multidrug-resistant typhimurium DT104 is carried out by monitoring the antibiotic susceptibility of all strains of typhimurium and by phage typing of the isolates at the Danish Veterinary Laboratory.⁴⁻⁷ Telephone interviews are conducted prospectively with patients who are infected with typhimurium strains that are resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline — that is, strains indicative of five-drug-resistant typhimurium DT104. The aim is to obtain standardized clinical and epidemiologic information, and the interview includes data on food consumption and place of purchase of consumed food. In the present investigation, information on the place of purchase was compared with the distribution list from a slaughterhouse suspected as the source of the DT104 strain.

The surveillance of food animals and food of animal origin is described in the annual report of the Danish Zoonosis Center.³ Every commercial flock of layers and broilers and every herd producing more than 100 pigs for slaughter per year is regularly tested for salmonella by a combination of serologic and bacteriologic methods. Cattle herds are tested only when there is suspicion of infection. All slaughterhouses take part in salmonella monitoring. Every flock of broilers is tested after slaughter, and approximately 30,000 end-product samples of pork and 3000 end-product samples of beef are tested yearly. The number of samples tested from each slaughterhouse is proportional to the number of animals slaughtered. Approximately 12,000 to 15,000 samples from retail outlets are tested by municipal food-control units. A total of more than 2 million samples from living animals and food of animal origin are tested for salmonella annually in Denmark. All isolates of salmonella are submitted to the Danish Veterinary Laboratory for serotyping and additional characterization.

The 1998 Outbreak

In June 1998, an unusual strain of typhimurium was identified in specimens or cultures from five patients and from samples of

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TABLE 1. PATIENTS INFECTED WITH QUINOLONE-RESISTANT, MULTIDRUG-RESISTANT *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM DT104 IN DENMARK, FEBRUARY THROUGH AUGUST 1998.

PATIENT No.	DATE OF ONSET	AGE (YR)/ SEX	HOSPITAL ADMISSION	ANTIMICROBIAL TREATMENT AFTER ONSET OF DISEASE	ASSOCIATION WITH SLAUGHTERHOUSE ESTABLISHED
1	2/16/98	<1/M	Yes	Cefotaxime	Not relevant
2	5/10/98	1/F	No	No	Not relevant
3	5/29/98	82/F	Yes	Ampicillin, then ciprofloxacin	No data
4	5/30/98	11/M	No	No	No
5	5/31/98	47/F	No	No	Yes
6	6/01/98	43/M	No	No	Yes
7	6/01/98	39/F	No	No	Yes
8	6/01/98	22/F	No	No	No
9	6/02/98	54/F	No	No	Yes
10	6/05/98	74/M	Yes	Ciprofloxacin	Yes
11	6/07/98	44/M	No	No	No data
12	6/07/98	34/F	Yes	Fleroxacin	Yes
13	6/08/98	45/F	No	No	Yes
14	6/15/98	62/F	Yes	Ciprofloxacin, then gentamicin and ceftriaxone, and then ciprofloxacin	No data
15	6/19/98	13/F	No	No	No
16	6/30/98	23/M	No	Clarithromycin	No
17	7/03/98	36/F	No	Ciprofloxacin	No
18	7/04/98	58/M	No	No	Exposure at work
19	7/11/98	58/F	Yes	Ciprofloxacin, then mecillinam	No
20	7/16/98	25/M	No	No	Yes
21	7/17/98	88/F	Yes	Yes (type unknown)	Yes
22	7/22/98	71/F	Yes	Gentamicin, ampicillin, and metronidazole	No
23	7/26/98	47/F	No	No	Exposure at work
24	8/27/98	82/F	Yes	Ampicillin, then metronidazole	No data
25	8/27/98	82/F	Yes	Yes (type unknown)	Nosocomial transmission
26	8/27/98	40/F	Yes	No	No
27	8/31/98	46/M	Yes	Ciprofloxacin and erythromycin	No

pork obtained from a slaughterhouse. We used epidemiologic and microbiologic methods to investigate the source of this outbreak, to search for additional cases, and to determine the mode of transmission of infection.

Microbiologic Examination

Antibiotic-susceptibility testing of isolates from humans was performed by tablet diffusion on Danish Blood Agar (SSI Diagnostica, Hillerød, Denmark) with the use of Rosco Neosensitabs (Rosco, Roskilde, Denmark). Nalidixic acid-resistant strains were tested for susceptibility to ciprofloxacin with the E test (Biodisk, Solna, Sweden). The antibiotic susceptibility of isolates from animals and food was examined as previously described.⁸

Strains of DT104 from animals, food, and humans were analyzed by pulsed-field gel electrophoresis (PFGE), carried out in a contour-clamped homogeneous electric-field system (Pulsaphor Plus, Pharmacia LKB, Uppsala, Sweden). The preparation of DNA blocks and digestion with restriction enzymes were carried out as previously described.⁹ All strains were analyzed with the use of the restriction enzymes *Xba*I and *Bln*I. The electrophoretic running conditions for analysis with both enzymes were 12 V per centimeter at 14°C for 23 hours. Pulse times were increased in steps, as follows: 10 seconds for 22 hours, 15 seconds for 52 hours, 20 seconds for 6 hours, 40 seconds for 5 hours, and 60 seconds for

4 hours. Polymerized phage lambda DNA (Pharmacia LKB) was used as a molecular-size marker.

After electrophoresis, gels were stained in aqueous bromide (Sigma, St. Louis) at 2 µg per milliliter for 15 minutes and photographed under ultraviolet light (300 nm). Each fragment pattern that differed from the others in one or more fragments of more than 100 kilobases was assigned a type number.

A 342-base-pair fragment of the *gyrA* gene was amplified with the primers P1 (5'TACCGTCATAGTTATCCACGA) and P2 (5'GTACTTTACGCCATGAACGT). The amplification products were sequenced on an ABI 373A automatic sequencer with the AmpliTaq FS dye terminator kit (Applied Biosystems, Foster City, Calif.). The sequences were compared and analyzed by DNAsis software (Hitachi Software Engineering, Yokohama, Japan).

RESULTS

Identification of the Outbreak

The outbreak came to light on June 18, 1998, when an unusual resistance pattern appeared in typhimurium found in specimens or cultures from five patients received between June 9 and June 16, 1998 (Patients 5, 6, 9, 10, and 13) (Table 1). On the same

day, a sample of pork collected on May 26 at a slaughterhouse on the island of Zealand was recognized to be positive for typhimurium with the same resistance profile as that seen in the isolates from the five patients. This resistance profile was also found in typhimurium strains from two pork samples routinely collected by municipal food inspectors. In both cases, the wholesalers had received pork from the slaughterhouse on Zealand. Besides being resistant to five drugs, the strains were resistant to nalidixic acid. This resistance profile had not previously been detected in salmonella from Danish animals or animal-derived foods, and it had been detected only twice in salmonella from humans (Table 1, Patients 1 and 2). Because of the unusual resistance pattern, we hypothesized that the five cases of disease in humans were caused by multidrug-resistant typhimurium in pork from the slaughterhouse.

Tracing the Source

Investigation at the slaughterhouse revealed that the pork sample positive for multidrug-resistant typhimurium probably came from pigs that had been delivered to the slaughterhouse on May 20, 22, or 25. During these three days, animals from a total of 37 herds had been delivered, and samples from each pen of animals were screened bacteriologically. One herd, a fattening swine herd, was found to be infected with the strain identified in the outbreak. On subsequent investigation of contacts of the infected herd, the outbreak strain was found in another swine herd that had delivered piglets and shared machinery with the fattening herd. Nearly 90 herds were examined in connection with this investigation, and only these 2 herds tested positive. The veterinarian responsible for the implicated herds gave a written statement that fluoroquinolones had not been used in the herds in 1998.

During the period of approximately four months when the outbreak was under investigation, the outbreak strain was not detected in any of 3296 follow-up fecal samples collected from 313 herds in which pigs were seropositive for salmonella or in any of approximately 5000 samples of pork products collected at all slaughterhouses in the country. Furthermore, it was not detected in any other animal herds or samples from slaughterhouses.

Microbiologic Investigations

The unusual resistance pattern was found in isolates from all the patients, the slaughterhouse, the two pork samples from the food-inspection agencies, and the two swine herds. It was also found in one isolate obtained later during the outbreak from a sample of smoked tenderloin and in an isolate from pork from the kitchen of Patient 19. All these isolates were of DT104, with the same resistance phenotype. The strains were susceptible to ciprofloxacin, as judged by

tablet diffusion. The minimal inhibitory concentration of ciprofloxacin in the isolates from the outbreak ranged from 0.064 to 0.124 mg per liter. These isolates were $1/10$ as susceptible to ciprofloxacin as the isolates that were susceptible to nalidixic acid.

PFGE patterns for the majority of strains investigated, including the isolates from animals and food of animal origin, were indistinguishable. Two strains from humans varied in either the *Xba*I profile (Patient 8) or the *Bln*I profile (Patient 19).

Sequence analysis of the polymerase-chain-reaction-amplified region of the *gyrA* gene identified a base substitution at codon 87 (according to *Escherichia coli* numbering¹⁰) in all isolates from humans. The substitution (GAC→AAC) causes an amino acid change from aspartate to asparagine. All isolates from food related to the outbreak contained the same substitution, as did isolates from two swine herds related to the outbreak. Nalidixic acid-resistant DT104 isolates from food originating in Germany and the United Kingdom and from a Danish swine herd, found at a later stage and not epidemiologically related to the Danish outbreak, contained a base-pair substitution at codon 83. The isolates from food from the United Kingdom and from the Danish swine herd contained a TCC→TTC substitution (serine to phenylalanine), and the isolate from food from Germany contained a TCC→TAC substitution (serine to tyrosine).

Epidemiologic Investigations

Table 1 summarizes the characteristics of 27 registered cases of quinolone-resistant, multidrug-resistant typhimurium DT104 infection in Denmark through August 1998, and Figure 1 shows the course of the epidemic. Two patients probably acquired infection by occupational transmission. Patient 18 was a worker at the incriminated slaughterhouse, and Patient 23 was a nurse who cared for Patient 21. The nurse was exposed in the hospital ward where the patient was admitted. She had malaise and low-grade fever during the week after exposure and diarrhea seven days after exposure. Patient 25 acquired salmonella infection at the hospital, where she shared a room with Patient 21. Among the remaining 23 patients, data on food exposure were available for 19; 18 of these had consumed pork products, including meatballs, tenderloin, and roast pork. Patient 19 had tasted a raw meatball before cooking it. She became ill with diarrhea and vomiting 4.5 hours after exposure. The outbreak strain was later found in the patient's kitchen in leftover frozen pork.

An association with the consumption of pork originating from the slaughterhouse was established for cases with disease onset after May 26, the day multidrug-resistant typhimurium was found in the slaughterhouse (Fig. 1). All patients had acquired gastroenteritis in Denmark, and 9 of 18 patients had eaten

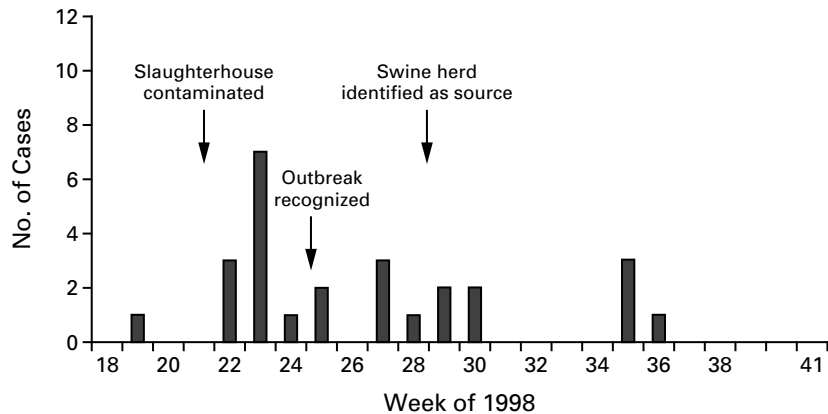


Figure 1. Number of Cases of Quinolone-Resistant, Multidrug-Resistant *Salmonella enterica* Serotype Typhimurium DT104 in Denmark, According to Week of Onset, May through August 1998.

The numbers on the x axis indicate the weeks of the year.

pork originating from the slaughterhouse concerned. In the same period, 13 patients with quinolone-sensitive DT104 were interviewed. Four had travel-associated infections ($P=0.02$ for the comparison with patients with quinolone-resistant DT104 infection, by Fisher's exact test), and none of the nine patients with domestically acquired infections had consumed pork from these retailers ($P=0.01$ for the comparison with patients with quinolone-resistant DT104 infection, by Fisher's exact test).

The median age of the patients listed in Table 1 was 45 years, and they were ill for a median of 14 days (interquartile range, 10 to 21). At least seven had a history of antibiotic use before the onset of disease. Eleven patients were admitted to the hospital with gastroenteritis or septicemia. Fluoroquinolone treatment was reported to lack clinical effect in at least four cases. Patients 10, 12, and 19 had persistent diarrhea despite treatment with ciprofloxacin or fleroxacin; they recovered after treatment with mecillinam (Patient 19) or discontinuation of treatment (Patients 10 and 12). From Patient 19, three strains isolated after treatment were tested for susceptibility to ciprofloxacin, and the minimal inhibitory concentration for all isolates remained 0.09 mg per liter.

Patient 14 was a 62-year-old woman without chronic or malignant disease. She was admitted to the hospital after nine days with gastrointestinal symptoms. During five days of treatment with ciprofloxacin (250 mg twice daily), intestinal perforation developed, and laparotomy was performed. The patient was treated before and after the operation with ceftriaxone and gentamicin, and again with ciprofloxacin for four days, but she died of multiorgan failure. *Salmonella* was not cultured from samples taken at the time of operation. Autopsy revealed peritonitis and a perforation of the colon.

Patient 25 was an 82-year-old woman with diabe-

tes mellitus and enteropathy after radiotherapy for uterine cancer, who was admitted to the hospital after eight days with malabsorptive symptoms. In the hospital she became infected with DT104 and intestinal perforation developed. She died more than two months after the onset of gastroenteritis. Diagnoses at autopsy included perforations of both the ileum and the urinary bladder and peritonitis. *Salmonella* was cultured from the peritoneal fluid at autopsy. Patient 3, who had fecal, urinary, and blood cultures positive for salmonella, was treated with ciprofloxacin eight days after the onset of gastroenteritis. The patient recovered after 10 days of treatment with intravenous ciprofloxacin (400 mg twice daily). *Salmonella* was isolated from blood cultures taken two days after the initiation of treatment. Patient 27 had a dual infection with campylobacter species and underwent an appendectomy. Antimicrobial treatment was initiated after the operation.

DISCUSSION

Since the beginning of the 1990s, infections with the zoonotic salmonella typhimurium DT104 have been recognized in several countries.^{1,2} It has a broad host spectrum and the potential of spreading to large numbers of domestic as well as to wild animals. Because of its extensive reservoir, DT104 is difficult to control in animal husbandry. It is often resistant to five antibacterial agents and can also be resistant to others, including the fluoroquinolones. Since fluoroquinolones are the drugs of first choice for extraintestinal and serious intestinal complications of human salmonellosis, fluoroquinolone resistance has the potential of causing problems with therapy. There is, however, little documented evidence of the effect of quinolone-resistant salmonella on human health.¹¹

Denmark has active surveillance of salmonella at farms, including nearly all commercial food-animal

producers, and all slaughterhouses. Isolates generated through the surveillance system are collected, characterized, and stored centrally. This system yields detailed information on the prevalence of and trends in the distribution of different salmonella types in national food production, and the emergence of new types of salmonella can quickly be detected. Until 1997, DT104 infections accounted for less than 1 percent of the total number of human salmonella infections, and apart from a small hospital outbreak in 1996, only sporadic cases had been recorded.³ The present emergence of a quinolone-resistant strain is the first community outbreak in Denmark.

Our investigation suggested that the source of the outbreak was pork from a slaughterhouse. The peak of the epidemic curve (during weeks 22 and 23 of 1998) coincided with the demonstration of the outbreak strain in the slaughterhouse, and the data on food exposure were corroborated by the results of PFGE typing and sequence analysis of a fragment of the *gyrA* gene. All isolates from the outbreak contained the same mutation, whereas isolates from another pig herd and from pork from Germany and the United Kingdom did not. A number of different base-pair substitutions in the *gyrA* gene of salmonella have been found to result in resistance to nalidixic acid. The GAC→AAC substitution (aspartate to asparagine) at codon 87 that was found in this outbreak has been infrequently detected in salmonella isolates.¹²⁻¹⁵ However, Ridley and Threlfall¹⁶ found that 11 of 15 nalidixic acid-resistant DT104 isolates contained this substitution.

It is likely that the reservoir of the outbreak clone was the two identified swine herds. Surveillance for salmonella identified no other isolates of the outbreak strain in Danish food animals, end products from slaughterhouses, or other meat products.

The outbreak strain was resistant to nalidixic acid but would be considered susceptible to fluoroquinolones according to standard cutoff points for the drugs' minimal inhibitory concentrations.¹⁷ The outbreak strain had one mutation in the gyrase gene, and no strains with two or more mutations were recovered during treatment. Nevertheless, an impaired response to fluoroquinolone treatment was reported. Fluoroquinolones frequently fail to change the natural course of diarrhea due to salmonella, especially if the diarrhea has lasted for more than a week before treatment is instituted.¹⁸ However, in the present study, diarrhea resolved promptly when the treatment was changed from ciprofloxacin to mecillinam in one patient, and an intestinal perforation developed in another patient during treatment with ciprofloxacin. It is important to emphasize that it would have been impossible to predict the clinical course of these patients even if they had been infected by a quinolone-sensitive strain.

Careful epidemiologic studies are warranted to de-

termine the effect of reduced fluoroquinolone susceptibility on human health and its implications for treatment options. Despite this note of caution, our data suggest that salmonella strains with one mutation in the gyrase gene may respond poorly to fluoroquinolones, as suggested by others.^{19,20} Because reduced susceptibility cannot be detected by disk or tablet diffusion with newer fluoroquinolones, susceptibility to nalidixic acid may be used as a surrogate marker.¹⁹ Minimal inhibitory concentrations of ciprofloxacin should be determined for nalidixic acid-resistant strains.

Fluoroquinolones were licensed for veterinary use in Denmark in 1993. In 1998 fluoroquinolones accounted for 400 kg of a total of 57,300 kg of antimicrobial agents consumed by all food-producing animals, and there was no indication of fluoroquinolone use in 1998 in the implicated herds. These observations suggest that selection pressures were not extensive in this outbreak. It is impossible to determine whether the *gyrA* mutant was introduced by pigs from outside Denmark, was introduced by environmental spread (e.g., from wild animals or equipment), or was related to the use of fluoroquinolones at the suspected farms before 1998.

The incubation period ranged from 4.5 hours to 7 days, although the incubation period of salmonella is said to be usually 16 to 72 hours.²¹ Although most patients were infected by food products, we also demonstrated occupational and nosocomial transmission. Several of the patients had taken antimicrobial drugs before the onset of disease; this observation is in line with studies suggesting that antibiotic treatment is an important risk factor for antimicrobial-resistant infections.²²

In conclusion, our investigation documented how quinolone-resistant, multidrug-resistant *S. enterica* serotype typhimurium DT104 spread from food animals to humans. Through an integrated surveillance system, it was possible to detect the outbreak and mitigate the spread of this strain of salmonella. Furthermore, in vitro resistance to nalidixic acid in isolates was associated with reduced efficacy of fluoroquinolones in vivo. Because fluoroquinolones remain a standard empirical treatment for suspected extraintestinal salmonella infections and serious gastroenteritis, particularly in patients with underlying health problems, the occurrence of quinolone-resistant salmonella in animals is of great concern. To avoid the selection of quinolone-resistant strains, with a potential for clonal spread, fluoroquinolones should not be used in food animals unless other therapeutic options have been ruled out.

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