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Outbreak of *Salmonella typhimurium* Infection Traced to Contaminated Chocolate and Caused by a Strain Lacking the 60-Megadalton Virulence Plasmid

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We describe an outbreak of *Salmonella typhimurium* infection, caused by contaminated chocolate produced by one Norwegian company, which occurred in Norway and Finland in 1987. A total of 349 bacteriologically verified cases were recorded in Norway, and 12 cases were recorded in Finland. There was a predominance of young children among the patients (median age, 6 years), many of whom developed acute hemorrhagic diarrhea. The outbreak strain exhibited a rare phage lysis pattern and a characteristic plasmid profile lacking the 60-MDa virulence-associated plasmid. DNA hybridization failed to demonstrate any DNA sequence homology between the outbreak strain and the virulence plasmid. The outbreak strain was nonlethal for orally infected mice. The finding of only ≤10 *S. typhimurium* cells per 100 g of chocolate in about 90% of the positive samples obtained from retail outlets suggested that an inoculum of fewer than 10 organisms may have been sufficient to cause symptomatic disease.

Salmonella typhimurium continues to be an important cause of human gastroenteritis throughout the world. In Norway, an annual average of 186 cases was recorded during the 5-year period from 1982 through 1986 (mean annual incidence, 4.50 cases per 100,000 people). However, 61.8% of these patients had acquired the infection in foreign countries.

Salmonellosis is a growing concern to the chocolate industry (3). Although the risk of acquiring salmonellosis from chocolate is comparatively low (3), several outbreaks have been reported in which chocolate products were incriminated as the source of infection (2, 4, 6, 7, 12, 22). In 1987, an outbreak of *S. typhimurium* infection that was caused by contaminated chocolate occurred in Norway and Finland (14). The purposes of this article are (i) to describe the outbreak and the investigations which led to identification of the source of infection and (ii) to describe the virulence properties of the outbreak strain.

(A preliminary report of this outbreak has been published previously [14].)

MATERIALS AND METHODS

Epidemiological investigations. Cases were identified by isolating *S. typhimurium* with the characteristic properties of the outbreak strain (see below) from stool samples submitted by general practitioners, health centers, and hospitals to medical microbiological laboratories in Norway and Finland. The laboratories forwarded the isolates to the Norwegian and Finnish salmonella reference centers for further characterization. Isolates from chocolate products were received from several municipal food control service laboratories. Epidemiological information (age, sex, geographical distribution) and clinical data were obtained from the patient registration forms which followed the samples to the laboratories.

A case series investigation which comprised eight patients who were 1.3 to 5.5 years old was carried out. Detailed personal interviews about the pattern of food consumption in the week prior to the onset of illness were conducted by using a structured questionnaire as described previously (10). Only symptomatic, primary cases from each household were included in the study.

Characterization of the outbreak strain. Phenotypic and genotypic characterization of *S. tyhimurium* isolates from human patients and chocolate products was done by using serotyping, biochemical fingerprinting, phage typing, antimicrobial susceptibility testing, plasmid profile analysis, restriction enzyme cleavage analysis, and multilocus enzyme electrophoresis. A detailed description of the methods employed and the results obtained has been presented elsewhere (15).

Determination of mouse virulence. The virulence of four isolates was determined by oral infection of BALB/c mice as described by Helmuth et al. (11). Control mice were challenged with strain 353/82, which harbored 60-MDa virulence plasmid pRQ28 (11).

Hybridization experiments. To ascertain whether the outbreak strain harbored any of the genetic determinants located on the 60-MDa virulence plasmid, colony hybridization and Southern hybridization experiments were conducted by using plasmid pRQ28 obtained from virulent *S. typhimurium* isolate 353/82 as a genetic probe. The plasmid DNA was purified by CsCl gradient centrifugation and labeled with ^{32}P by using a commercial nick translation kit (Amersham International PLC, Buckinghamshire, England). For colony hybridization, isolates from the outbreak were inoculated onto L-agar plates with a 48-point inoculator. After incubation at 37°C overnight, colonies were transferred to Schwarzband filters (no. 586; Schleicher & Schüll,

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Dassel, Federal Republic of Germany). The filters were processed further as described by Maas (16). For Southern hybridization, plasmid DNAs from test isolates were extracted by using the method of Kado and Liu (13) and were separated on 0.8% agarose gels. DNA was blotted onto nitrocellulose membranes as described by Southern (21). DNA hybridization and washing procedures were performed under stringent conditions at 42°C in a buffer containing 50% formamide as described previously (18). Control strains harboring the virulence plasmid were included in each experiment.

Bacteriological examinations. Qualitative determination of salmonellae in chocolate products and environmental samples was carried out by using a method recommended by The Nordic Committee on Food Analysis (19) and potassium tetrationate broth as the selective enrichment medium. Quantitative determination of salmonellae was performed by using a most-probable-number procedure according to the above-mentioned method, including preenrichment in buffered peptone water (pH 7.0) at 37°C overnight.

RESULTS

Detection of the outbreak and identification of the source of infection. During the late winter and early spring of 1987, the Norwegian Salmonella Reference Center received a markedly increased number of *S. typhimurium* isolates, a majority of which were from young children who had not recently travelled abroad. Laboratory investigations showed that these isolates could be distinguished from control isolates by means of phenotypic and genotypic characteristics (15) (see below). Since isolates with identical characteristics were obtained from most of the medical microbiological laboratories in the country, it was suggested that a common-source outbreak of nationwide distribution was taking place. During the same time period, the national surveillance system for infectious diseases detected an increased number of reported cases of *S. typhimurium* infection. By 20 March, the number of reported cases had reached 33, and the National Institute of Public Health issued a letter of information to all local Boards of Health and Municipal Food Control Services in Norway, in which their cooperation in identification of the source of infection was requested.

At that time, the Municipal Food Control Service in Trondheim had already initiated an individual case investigation. An analysis of food anamneses from eight children (ages, 1.3 to 5.5 years) identified 13 food items which had been consumed by at least four patients. All of these food items were promptly purchased from the stores identified during the interviews, regardless of whether they were considered to be potential sources of salmonellae. A total of 300 samples were cultured for salmonellae. On 30 March, isolation of *S. typhimurium* from three chocolate products manufactured by one factory in Trondheim was reported. The isolates were later shown to exhibit the same characteristics as the outbreak strain. By 31 March, 67 cases had been reported, and the National Health Department issued a public health warning through newspapers, radio, and television and prohibited all trade of the incriminated chocolate products. The following weekend, all chocolate produced by the factory in Trondheim was removed from about 13,500 retail outlets all over the country, with the active cooperation of the producer. About 600,000 kg of chocolate were destroyed. Following this action, the outbreak, which was still increasing when the chocolate products were recalled, quickly came to an end (Fig. 1). The last case, which was recorded in August, was a woman who had eaten a chocolate bar bought in March.

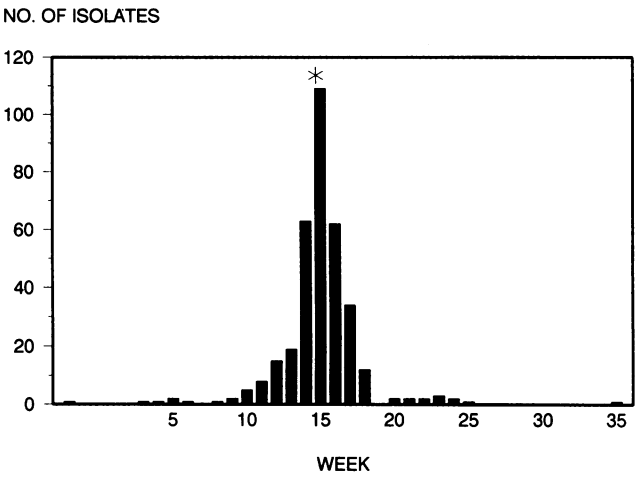

FIG. 1. Epidemic curve: seasonal distribution of 349 bacteriologically verified cases of *S. typhimurium* infection recorded during the chocolate-related outbreak in Norway in 1987, showing weeks when the bacterial isolates were received. Each bar represents the number of cases recorded during 1 week. The asterisk indicates the week when the public warning was issued and the incriminated chocolate products were recalled.

Characterization of the outbreak strain. The outbreak strain had the following characteristics: (i) O-antigen factors 4 and 12 were present, but O-antigen factors 1 and 5 were not; (ii) the phase 2 H antigens *1,2* predominated, whereas the phase 1 determinant *i* was suppressed and required phase reversal to become detectable; (iii) the strain was susceptible to 11 antimicrobial agents; (iv) the strain had a positive reaction for xylose (after 2 days of incubation), but negative reactions for inositol and rhamnose; (v) the strain exhibited an uncommon phage lysis pattern, tentatively designated U277 (courtesy of B. Rowe); and (vi) the strain had a characteristic plasmid profile lacking the 60-MDa virulence-associated plasmid (all of the plasmids detected were smaller than 35 MDa [Fig. 2]).

Outbreak in Norway. Between December 1987 and December 1988, the Norwegian Salmonella Reference Center received a total of 349 *S. typhimurium* isolates with the characteristic properties of the outbreak strain. All of the isolates were from patients with no reported history of travelling abroad. Since all microbiological laboratories in the country routinely forward all *Salmonella* isolates to the Reference Center, the number of isolates received corresponded closely to the total number of bacteriologically verified cases during the outbreak. The number of cases reached a peak in week 15, with 109 isolates, and declined rapidly after the incriminated chocolate products were withdrawn, suggesting that the discrete source of infection had been removed (Fig. 1).

Of the 349 bacteriologically verified cases, 182 (52.1%) were males, whereas females accounted for 163 cases (46.7%). Sex affiliation was not recorded for the remaining four patients. Although the age of patients ranged from 5 months to 83 years, 48.7% of the patients were younger than 5 years old. The median age was 6 years. Cases were reported from all 19 counties in Norway; the incidence ranged from 4.0 to 13.4 cases per 100,000 inhabitants during the outbreak, with a mean of 8.3 cases per 100,000 inhabitants.

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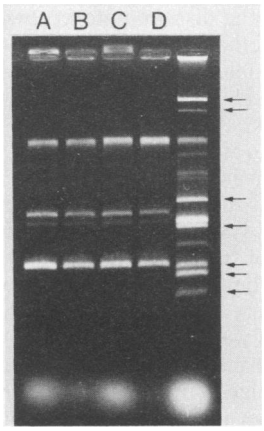

FIG. 2. Plasmid profiles of four outbreak isolates: agarose gel (0.7%) electrophoresis of partially purified plasmid DNAs from *S. typhimurium* isolates. Lanes A through D show the results obtained for isolates 224/87 (human, Norway), 976/87 (human, Finland), 364/87 (chocolate, Norway), and 968/87 (chocolate, Finland), respectively. The size markers on the right are standard plasmids (from top to bottom, 62.0, 35.8, 4.8, 3.4, 2.0, 1.8, and 1.4 MDa). All of the plasmids detected in lanes A through D were smaller than 35 MDa.

Outbreak in Finland. In February, the chocolate company exported one brand of chocolate bars to Finland, where it was distributed to retail stores in the beginning of April. The product was recalled 1 week later after notification of the Norwegian outbreak had been received. The public was warned through television. Between 3 April and 10 May, the outbreak strain was recovered from 12 persons (9 females and 3 males) from six counties. All except one remembered having eaten the incriminated product. The ages ranged from 3 to 65 years (median age, 15 years).

Clinical observations. No systematic investigation of the clinical manifestations was attempted. However, it is notable that a significant number of patients who were less than 2 years old developed acute, hemorrhagic diarrhea, which in many cases required hospitalization for intravenous rehydration therapy. No fatal cases were reported.

Virulence of the outbreak strain. The finding that the outbreak isolates lacked the 60-MDa virulence-associated plasmid (Fig. 2) prompted us to investigate the virulence properties of the outbreak strain. To this end, five mice were infected orally with more than 1.0×10^8 CFU of each of the following isolates of the outbreak strain: 224/87 (human, Norway), 976/87 (human, Finland), 364/87 (chocolate, Norway), and 968/87 (chocolate, Finland). None of these mice died within 24 days after infection. In contrast, all of the mice infected with the plasmid-bearing control strain (strain 353/82) died within 10 days. Furthermore, colony hybridization and Southern hybridization experiments failed to demonstrate any DNA sequence homology between the virulence plasmid and the four outbreak isolates listed above. These results suggest that the outbreak strain did not possess any of the plasmid-encoded virulence determinants, including the genes responsible for mouse lethality, either on plasmids or on the chromosome.

Examination of chocolate products. The combined results from interviewing patients and examining food items from retail stores led to the isolation of the epidemic strain from three chocolate products manufactured by a factory in Trondheim. On the basis of these results, a total of ca. 1,400 10-g samples of chocolate produced by the factory in question were cultured for salmonellae. A majority of the samples were from recalled chocolate products. In all, the outbreak strain was recovered from 73 (ca. 5%) of the samples and from 27 of 65 products examined.

Fifty-seven positive samples were selected for quantitative examination by the most-probable-number method. Quantitative estimates of the level of contamination ranged from 0 to 60 CFU/100 g of chocolate. Fifty-two (91.2%) of these samples were found to contain 10 CFU/100 g or less. This remarkably low contamination level was confirmed by quantitative examinations done at the National Veterinary Institute in Finland. The outbreak strain was isolated from 4 of 27 samples examined at this institute, and all positive samples contained less than 10 CFU/100 g.

S. typhimurium could not be isolated from 77 samples of 11 different raw products and dry ingredients, including cocoa beans, or from 64 samples of environmental dust and debris from the factory. All of these samples were collected during weeks 14 and 15, at the same time that the outbreak reached a peak (Fig. 1). On the other hand, three other *Salmonella* serovars were isolated from dust samples collected in rooms in which cocoa beans were stored or rinsed (*Salmonella brancaster*, *Salmonella onireke*, and *Salmonella stockholm*). We are not aware of any human isolates of these serovars in Norway.

DISCUSSION

The 349 bacteriologically verified cases reported from Norway probably represent only a small proportion of all persons infected during the outbreak. In other *Salmonella* outbreaks, a ratio of reported cases to total cases of 1:100 has been estimated (5). Since the outbreak was still increasing when the public health warning was issued, it is likely that many thousands of cases were prevented by recalling the chocolate products. We are not aware of any previously reported chocolate-related outbreak of salmonellosis in which such a high number of patients were involved.

The finding of only $\leq$10 *S. typhimurium* cells per 100 g of chocolate in a majority of the positive samples obtained at the stage of retail sale suggests that an inoculum of less than 10 organisms may have been sufficient to produce symptomatic infection. This inoculum is several logs less than the infective dose estimated for *Salmonella* spp. in previous investigations (1, 17). In agreement with our results, however, very small numbers of *Salmonella eastbourne* in chocolate (2 to 3 CFU/g) were sufficient to cause illness during an outbreak in the United States and Canada in 1973 and 1974, when 199 cases were recorded (2, 4). Likewise, epidemiological evidence and retrospective laboratory analysis of contaminated chocolate from an outbreak of *Salmonella napoli* infection in England and Wales in 1982 indicated that large numbers of salmonellae may not have been a prerequisite to human infection (7, 8). According to D'Aoust (3), it is conceivable that ingredients present in chocolate protect salmonellae against the action of gastric acid. The few salmonellae present in the final product could then colonize the lower gastrointestinal tract and produce clinical symptoms. On the other hand, we cannot exclude the possibility that the chocolate may have been unevenly contaminated with large particles containing many viable *Salmonella* cells, which may have caused infections via large doses not small doses. Such large particles may have led to underestimation of the contamination level in the quantitative analysis which we employed (most-probable-number method). However, this possibility seems less likely since

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the chocolate was subjected to thorough mixing both at the factory and at the laboratory. Moreover, the finding of equally low contamination levels in a great majority of the samples examined by two independent laboratories may support our suggestion that a small dose was involved.

The low contamination levels detected in the outbreak may explain the dominance of children among the cases, since a greater infective dose would probably be required to cause illness in adults. The age distribution does not necessarily reflect a high rate of consumption among children, since the incriminated chocolate products are popular in all age groups. A predominance of children among cases was also reported during the chocolate-related outbreaks in 1973 and 1974 and in 1982 (2, 7). The age distribution reported in the outbreak of *S. eastbourne* infection in 1973 and 1974 was at least partly attributable to a high rate of consumption of Christmas chocolate among children.

Another factor which might explain the unusual low infective dose estimated in the outbreak in Norway and Finland is the possible greater virulence of the epidemic strain compared with the virulence of other strains and serovars previously studied. In an investigation which included 60 antibiotic-susceptible *S. typhimurium* isolates from 25 countries, Helmuth et al. (11) found that 88% of the strains carried a 60-MDa plasmid. The presence of the plasmid was correlated with mouse virulence, colonization of the liver, and increased resistance against bactericidal activity of serum. The importance of this plasmid for the virulence of *S. typhimurium* has been further substantiated by several recent investigations (9, 20). It is interesting, however, that the epidemic strain lacked the 60-MDa plasmid and was nonlethal for mice, even though the mice were challenged with a dose which was 100,000 times larger than the 50% lethal dose of the control strain (11). Moreover, hybridization experiments indicated that the strain did not harbor any genetic determinants from the virulence plasmid. These observations do not support the hypothesis that the epidemic strain had unusually high virulence. The question arises, however, whether the mouse virulence assays used in this and other studies provide an adequate means to measure virulence for humans, since many of the patients developed serious, hemorrhagic diarrhea which required hospitalization.

In a comprehensive review of salmonellae and the chocolate industry, D'Aoust (3) pointed out that chocolate confectioners are faced with a rather unusual situation, in which the low moisture content and high sugar content of chocolate do not favor bacterial proliferation, but significantly increase the thermal resistance of bacteria. Fatty materials in milk chocolate further increase the thermal resistance. Thus, heat treatment, such as dry roasting of cocoa beans, heating of cocoa liquor, and conching of chocolate (a process in which the chocolate is swirled and heat-dried to the proper consistency), cannot be expected to efficiently eliminate salmonellae (3). This problem is serious as several of the ingredients used in chocolate production have been shown to contain salmonellae (3). Although isolation of salmonellae from raw and roasted cocoa beans has not been reported, salmonellae have been recovered from cocoa powder on several occasions (3). Both cocoa beans and cocoa powder have been incriminated as sources of outbreaks (3). In this study, however, the presence of the outbreak strain in raw ingredients could not be documented retrospectively despite comprehensive bacteriological examinations.

In a previous investigation (15), we compared isolates from the outbreak with five groups of control isolates in order to evaluate the discriminatory power of six epidemiological marker methods. Whereas the outbreak strain was easily distinguishable from a majority of the controls, we detected seven isolates which could not be differentiated from the outbreak strain by any of the methods employed. These seven isolates, six of which were recovered over a period of years from dead passerine birds in Norway and Finland, exhibited the same unique plasmid profile and the same rare phage lysis pattern that were involved in the outbreak. Three of these isolates were obtained in 1987 and 1988 from dead bullfinches (*Pyrrhula pyrrhula*) from the same county where the incriminated chocolate factory is located. Although we cannot exclude the possibility that the outbreak was caused by contaminated raw ingredients imported from a foreign country, it is tempting to speculate that the outbreak strain may have been derived from an avian wildlife reservoir in Norway. Although the chocolate factory was relatively modern, the possibility exists that birds may have gained access to the plant and introduced contamination somewhere along the production line. Indeed, birds were observed in the factory before the outbreak, an observation which is not unusual in the food industry. Inspection of the factory revealed several opportunities for contamination from various sources, especially during transport on open conveyor belts.

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