Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips

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SUMMARY

Between April and September 1993, a nationwide outbreak of salmonellosis occurred in Germany which was traced to contaminated paprika and paprika-powdered potato chips. Of the estimated 1000 cases, children below 14 years were principally affected. Levels of 0·04–0·45 organisms per gram were found in the snacks. The infective dose was estimated at 4–45 organisms with an attack rate of 1 in 10000 exposed persons. The unique feature of the outbreak was the variety of serovars involved. *S. saintpaul*, *S. rubislaw* and *S. javiana* were isolated during the same time period from paprika powder, spice mixtures, snacks and patients. Their clonal identity was confirmed by molecular typing methods. Furthermore, monophasic and non-motile strains of rare salmonella O-groups were isolated from both paprika products and patients. This is the largest documented outbreak due to contaminated spices which proved that even extremely low numbers of salmonellae adapted to the dry state were able to cause illness.

INTRODUCTION

In Germany, as in most industrialized countries, human salmonellosis is a fundamental public health concern. Thus, in 1992, an incidence rate of 241 per 100000 inhabitants has been calculated from the 195000 notifications [1]. This figure probably reflects the 'tip of the iceberg'; according to the German Federal Health Office a tenfold higher incidence might be more realistic [2]. Human salmonellosis is caused mainly by the serovars *enteritidis* and *typhimurium* with food of animal origin as the main vehicles. In such food the organisms are usually present in a state of active metabolism and propagation.

Between April and September 1993, a nationwide outbreak of human salmonellosis was observed in Germany which was characterized by infections with a great number of different serovars of rare or monophasic salmonella strains. Investigation by several State Food Inspection Offices revealed that paprika powder of one German producer was repeatedly positive with serovars identified during the outbreak. Finally, paprika-powdered potato chips produced with this powder were thought to be the main vehicle of transmission.

In this paper we describe the features of the outbreak and show that human

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disease was caused by extremely low numbers of culturable salmonellae adapted to the dry state.

METHODS

Background to the outbreak

In the period April to July 1993, an increased number of rare salmonella serovars was sent to the National Reference Centre for Enteric Pathogens which in former years had been identified only exceptionally. At the end of May and in June, several State Food Inspection Offices all over Germany reported the isolation of the same serovars from paprika powder and paprika-flavoured potato chips.

Epidemiological investigations revealed that a lot of paprika powder, originating from South America, had been released in February and March 1993 by a leading German producer which, apart from a smaller amount destined for ready-to-use packs, was delivered to a company producing different kinds of snacks.

Spice mixtures containing different quantities of this paprika powder were produced and used for flavouring potato chips. The spice mixtures were applied to the roasted chips at the end of the production line in a rotating cylinder where the temperature of the chips had dropped to about 60 °C. The bulk of the spice mixtures containing the contaminated paprika powder was used in April and May.

By the end of June 1993, the public was warned and the contaminated lots of paprika-powdered potato chips were recalled.

Bacteriological examination

Qualitative examination of paprika powder, spice mixtures, and powdered potato chips was performed by overnight pre-enrichment of 25 g samples in 225 ml of phosphate-buffered peptone broth (pH 7·2), followed by enrichment of 10 ml aliquots in 90 ml of Preuss' modification of tetrathionate broth (Merck, Darmstadt, Germany). After 18–24 h enrichment at 37 °C subcultures were made on xylose-lysine-deoxycholate (XLD) and Salmonella-Shigella agar (SS), respectively (Merck, Darmstadt, Germany).

For the quantitative examination of salmonella a most probable number (MPN) method was used. Quantities of 10, 1 and 0·1 g of the specimens were cultured in triplicate in, respectively, 90, 10, and 10 ml of phosphate-buffered peptone broth. After overnight incubation at 37 °C aliquots of 1 ml each of the pre-enrichment cultures were transferred into 9 ml of Preuss' tetrathionate broth, followed by subculture on XLD and SS agar as described above. Suspected colonies were isolated and serotyped according to standard methods [3].

Molecular typing

A total of 13 strains each of *S. saintpaul*, *S. javiana*, and *S. rubislaw* from patients and foodstuff was submitted to molecular typing. Strains of the three serovars unrelated to the outbreak were included for comparison. They were obtained from the culture collections of the WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris (M. Y. Popoff), the Institute of Hygiene and Microbiology, University of Würzburg (H. Karch), and

the National Reference Centre for Enteric Pathogens, Institute of Hygiene, Hamburg.

Genomic DNA used in the pulsed-field gel electrophoresis (PFGE) was isolated by a modified procedure of Grothues and Tümmler [4]. Briefly, 10 ml of fresh double yeast-tryptone medium was inoculated with $100 \mu l$ of a salmonella overnight broth culture, followed by incubation at 37 °C to an optical density of 0·6 at 600 nm. The cells of 1·5 ml suspensions were harvested by centrifugation. The pelleted bacteria were washed twice with 75 mM-NaCl–25 mM EDTA (SE; pH 7·4) and resuspended in 100 μl SE. The bacterial suspension was mixed with an equal volume of 2% low melting agarose (Bio-Rad, München, Germany). Immediately, the mixture was dispensed into plug molds (Bio-Rad). Cell lysis was performed overnight at 50 °C by incubation of the solidified plugs in 400 μl of 50 mM Tris–50 mM EDTA-1% N-laurolysarcosine (pH 8·0), containing 1 mg/ml proteinase K. On the next day, the plugs were washed four times with 10 mM Tris–5 mM EDTA (pH 7·5). After equilibration with the restriction buffer, the agarose-embedded DNA was digested overnight with 30 U *Xba* I (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol. PFGE was performed according to the manufacturer's instructions using the CHEF DR III apparatus (Bio-Rad) and a 1% agarose gel. The conditions of the run were as follows: a constant voltage of 200 V, pulse times of 5–50 s with linear ramping, an electrical field angle of 120°, and a temperature of 14 °C. The run was stopped after 22 h and the gel was stained with ethidium bromide. Yeast chromosomes (Pharmacia) were used as molecular size markers.

For ribotyping, salmonella chromosomal DNA was purified according to Wilson [5]. 10 μg of genomic DNA was digested for 2 h with 30 U *EcoR* I (Pharmacia), supplemented with 0·4 μg of RNAse, according to the manufacturer's instructions. After electrophoresis in a 0·8% agarose gel and staining with ethidium bromide, the DNA fragments were transferred to a nylon membrane (Pall, Dreieich, Germany) as described by Southern [6]. A mixture of 16S and 23S rRNA from *E. coli* (Boehringer, Mannheim, Germany) was labeled by reverse transcription with digoxigenin-labeled dUTP as described by Popovic and colleagues [7]. Hybridization with the transferred DNA fragments from salmonella strains and detection of the digoxigenin-labeled probe was performed as recommended by the manufacturer (Boehringer). Therefore, chemiluminescent probes, binding to the blotted DNA fragments, were exposed for 1 h at room temperature to a TRIMAX 100 NIF film (Scotch, Neuss, Germany). The hybridization patterns were analysed in comparison with a digoxigenin-labeled *EcoR* I–*Hin*d III-digested λ-DNA as molecular size marker (Boehringer).

Isolation of plasmids was performed by the alkaline denaturation method described by Birnboim and Doly [8]. Plasmid DNA was separated in 0·7% agarose with λ-DNA and *EcoR* I–*Hin*d III-digested λ-DNA as molecular size markers (Boehringer).

For random amplified polymorphic DNA assay (RAPD), DNA was set free by boiling the salmonella suspensions for 10 min. Three units of Replitherm DNA polymerase (Biozym, Hessisch Oldendorf, Germany) amplified a ladder of DNA fragments from the boiled suspension using 50 pmol of the arbitrary S3 primer with the sequence 5′-TCACGATGCA-3′, 200 μM dNTPs and the Replitherm

reaction buffer containing 1·5 mM-MgCl$_2$. PCR was conducted in 45 cycles with denaturation for 1 min at 94 °C, annealing for 1 min at 25 °C, and polymerization for 2 min at 72 °C. The resulting PCR products were analysed by electrophoresis in 1·8% agarose gels.

RESULTS

Course of the outbreak

Beginning in April 1993, the National Reference Centre for Enteric Pathogens noticed an increase of rare salmonella serovars of human origin isolated by diagnostic laboratories all over Germany. Beside *S. saintpaul, S. javiana*, and *S. rubislaw* which prevailed and were therefore taken as indicator organisms for the following epidemiological studies, *S. loenga, S. florian, S. inganda, S. lille* and others were repeatedly found together with a great number of different monophasic or non-motile strains of salmonella subspecies I. Among the latter, a considerable number was characterized by resistance to potassium cyanide [9]. Altogether, 94 different serovars or non-motile strains were isolated from both patients and paprika-containing food items [Aleksic and colleagues, submitted]. Figure 1 shows the course of the outbreak exemplified by the three prevailing serovars. After warning of the public and a recall of the contaminated lots of paprika-powdered snacks at the end of June, the incidence dropped, and by the end of September the outbreak had ended.

About 1000 cases were identified in the authors' laboratory which were associated with rare serovars present in different samples of paprika powder and paprika-flavoured products during the outbreak period.

Age distribution of cases

As shown in Fig. 2, illness occurred mainly in children, with 14 cases in those below 1 year of age. This unusual observation was verified in some instances by telephone interviews of the parents. The age distribution of the outbreak was in contrast to the general situation in Germany where salmonellosis is frequently seen in the adult population [1].

Molecular typing of outbreak strains

Thirteen outbreak strains each of the prevailing serovars *S. saintpaul, S. rubislaw* and *S. javiana* demonstrated a remarkable clonal identity regarding the patterns of PFGE macrorestriction, ribotyping, plasmids, and RAPD. For ribotyping, the best discrimination was achieved by digestion with *Eco*R I as compared to seven other restriction endonucleases (*Acc*I, *Cla* I, *Hinc* II, *Nsp* I, *Sph* I, *Pst* I, *Xho* II); for RAPD, an arbitrary 10mer containing 50% G+C gave the best results of five tested random primers.

The four molecular typing techniques distinguished unambiguously between outbreak strains and unrelated isolates of the corresponding serovars. Figs 3–6 show representative results obtained for *S. javiana* by the four typing methods. In this context it is noteworthy that a strain of *S. javiana* isolated from a lot of paprika powder in November 1992, 5 months before onset of the outbreak, differed by five *Xba* I-generated fragments in PFGE, two *Eco*R I fragments of 2·4 and 5·0 kbp size from *rrn* gene clusters and by one amplicon of 290 bp in RAPD from
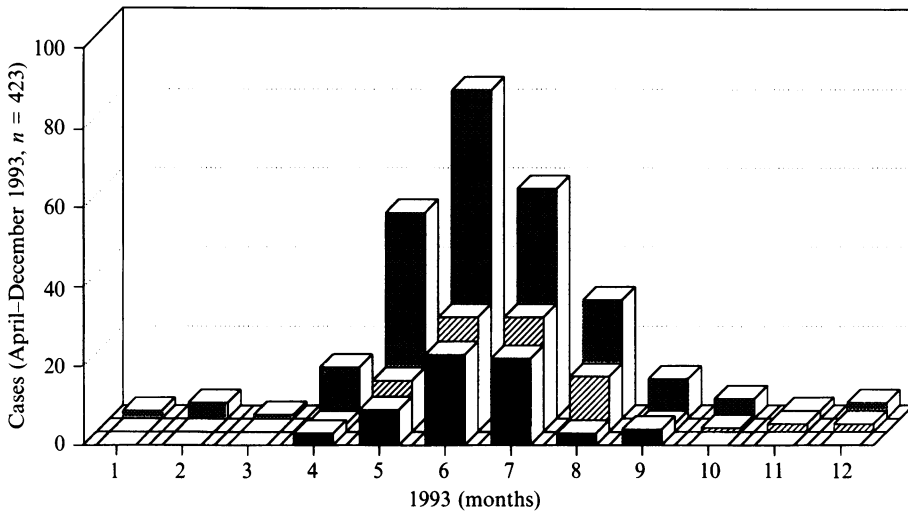
Fig. 1. Paprika-associated infections caused by *S. javiana*, *S. rubislaw*, and *S. saintpaul*. ■, *S. rubislaw*; ▨, *S. javiana*; ■, *S. saintpaul*.
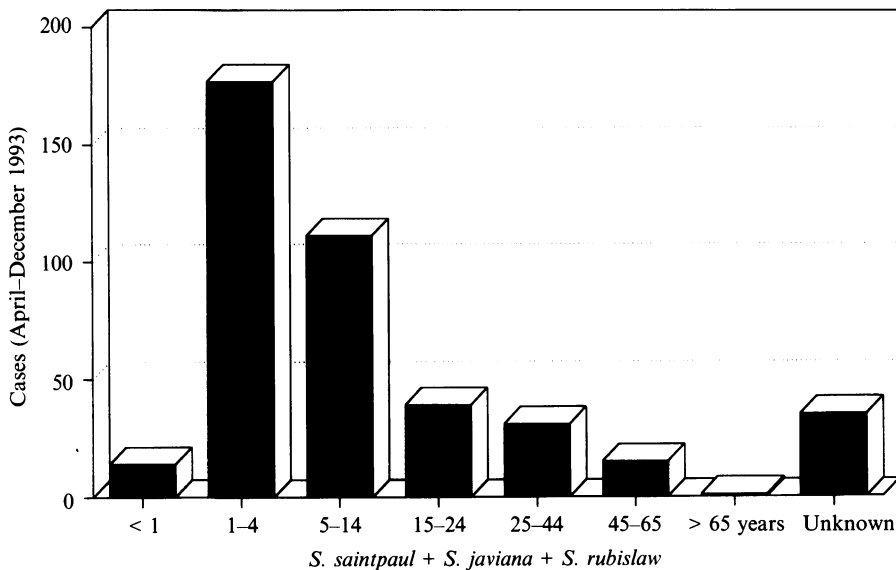

Fig. 2. Paprika-associated infections caused by *S. javiana*, *S. rubislaw*, and *S. saintpaul*, according to the age of the patients.

that of the outbreak strains (Figs. 3, 4, 6). Thereby, the best discrimination was achieved by PFGE and the least by RAPD which was calculated from the Dice-coefficients of similarity with 0·73 for PFGE, 0·89 for ribotyping and 0·97 in the case of RAPD. *S. javiana* was further characterized by the presence of four plasmids of, respectively, 25, 15, 4·6, and 4 kbp, whereas strains unrelated to the outbreak harboured only one plasmid of high molecular weight (Fig. 5). On the other hand, single strains of *S. saintpaul* and *S. javiana* isolated during the outbreak from surface water and a sewage effluent, respectively, were indistinguishable from patient and snack isolates.
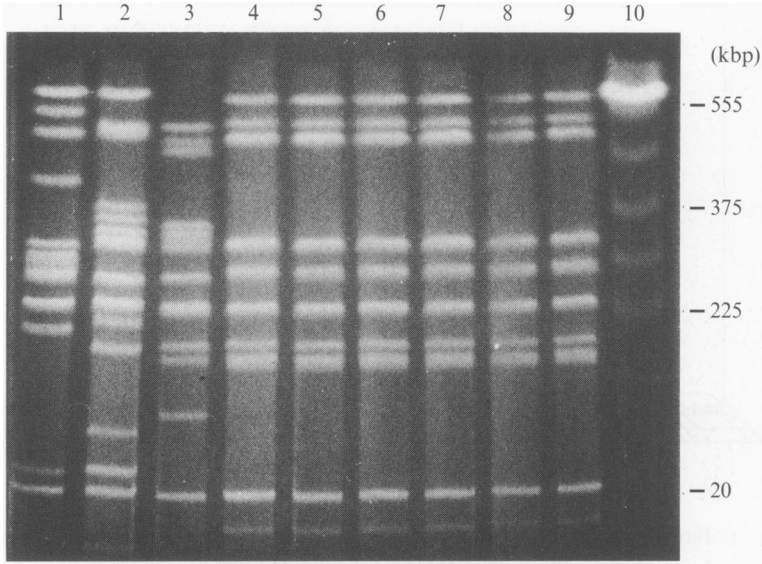
Fig. 3. PFGE *Xba* I-macrorestriction analysis of *S. javiana* strains. Lanes 1 and 2, isolates from unrelated outbreaks; lane 3, *S. javiana* from contaminated paprika powder, November 1992; lane 4, strain from a lot of paprika-powder used since March 1993 for the production of paprika-flavoured snacks; lane 5, *S. javiana* strain isolated from a spice mixture used in the production during the outbreak; lane 6, isolate from an incriminated lot of paprika-powdered potato chips; lanes 7 and 8, strains from stool specimens of patients; lane 9, *S. javiana* from a sewage effluent in Hamburg; lane 10, yeast chromosomes (225–1900 kbp) obtained from Pharmacia (Uppsala, Sweden) as DNA size markers.
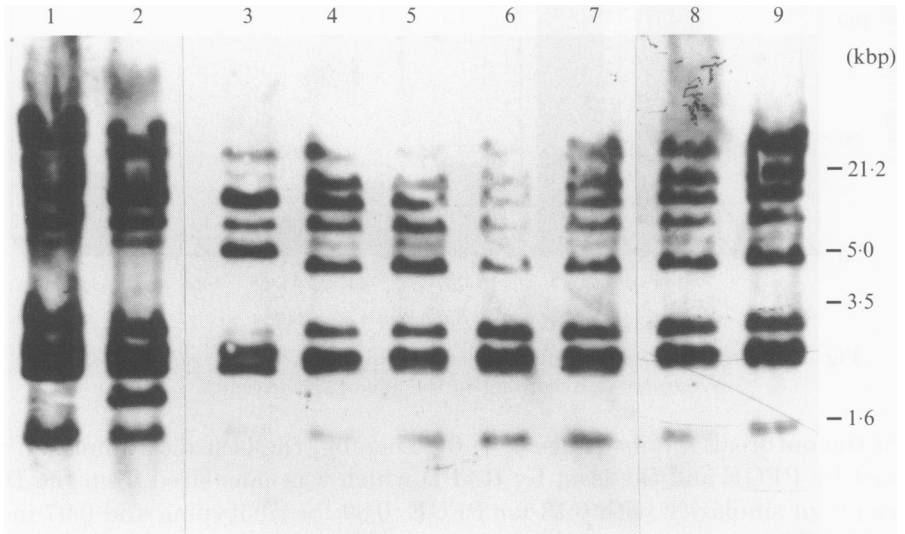

Fig. 4. 16S and 23S rRNA gene restriction patterns of *S. javiana* DNA digested with *Eco*R I. Lanes 1–9, restricted DNA of *S. javiana* strains tested in the same order as in Fig. 3.
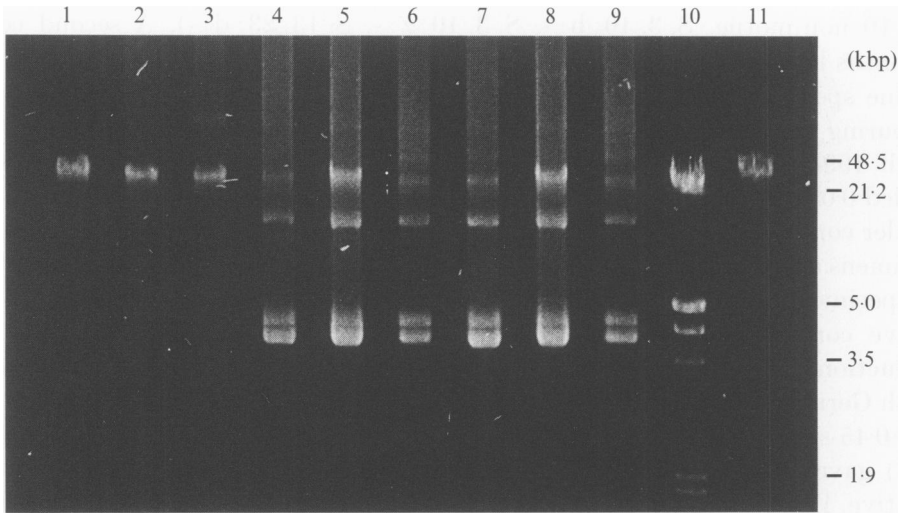
Fig. 5. Plasmid analysis of *S. javiana* strains. Lanes 1–9, undigested plasmids from *S. javiana* of the same origin and order as in Fig. 3; *Eco*R I–*Hin*d III digested λ-DNA and undigested λ-DNA, respectively, as DNA size markers.
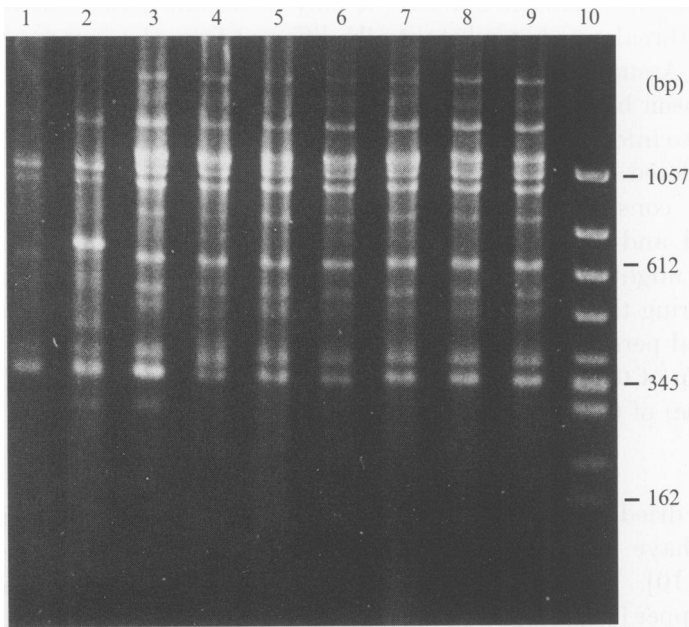

Fig. 6. Random amplified polymorphic DNA from *S. javiana* strains. Lanes 1–9, banding pattern using DNA from *S. javiana* strains of the same origin and order as in Fig. 3; lane 10, *Hin*d III digested φX-174 RF DNA (Pharmacia, Uppsala, Sweden) as DNA size marker.

Salmonella counts in food

A sample of the paprika powder which was assumed to be the cause of the outbreak was available from the producer. A quantitative analysis performed on this sample in October yielded 2·5 salmonellae per gram; 11 strains were identified (*S. saintpaul, S. javiana, S. rubislaw, S. florian, S. loenga,* S. 6, 7 : k : -, S. 9, 12 : - : 1, 5,

S. 3, 10 non-motile, S. 3, 10: b: -, S. 3, 10: z: -, S. 13, 23: d: -). A second count 8 months later (May 1994) still revealed 0·7 salmonellae per gram.

Nine specimens of spice mixtures prepared on the 26 and 27 April 1993 for flavouring the snacks were bacteriologically examined 1 year after production (April 1994). Of these, six contained 14%paprika powder; salmonella counts yielded 0·04–0·4 organisms per gram. Three further specimens with 31% paprika powder contained, respectively, 11·0, 5·0, and 0·4 salmonellae per gram. From all specimens at least one of the serovars *saintpaul, javiana* and *rubislaw* was isolated; six specimens were contaminated with 2–5 serovars.

Five contaminated packs of paprika-powdered potato chips of which the production date is unknown, were obtained from public health laboratories in South Germany. MPN counts performed in August and September 1993 revealed 0·04–0·45 salmonellae per gram. A second count performed 8 months later (May 1994) gave 0·04–0·21 salmonella in three of the samples; the remainder were negative. From 4 of the 5 packs at least 1 of the 3 prevailing serovars was isolated; one pack was contaminated with 7 serovars.

Infective dose and attack rate

From the results mentioned above, it may be assumed that the dose ingested during the outbreak was not substantially different from the counts performed 3–4 months later. Assuming a consumption of 100 g of potato chips, the infective dose would have been between 4 and 45 culturable organisms.

According to information obtained from the snack producer, 42 million packs of paprika-powdered snacks had been produced in the period April–June 1993. Taking into consideration that probably not all packs were salmonella-contaminated and that a considerable amount of contaminated product was recalled, one might roughly estimate a total of 10 million contaminated packs consumed during the outbreak. Using these assumptions, an attack rate of 1 per 10000 exposed persons might be estimated, based on an estimated 1000 cases; a contamination of 0·04–0·45 salmonellae per gram of food corresponded to an ID (infective dose) of 0·01.

DISCUSSION

Spices and dried vegetable foods such as mushrooms, asparagus, parsley, and peppermint have long been known to be contaminated occasionally with salmonellae [10]. However, apart from one major outbreak caused by contaminated pepper [11], only sporadic cases of human disease associated with such sources have been identified so far. This is in agreement with the concept of former years that low numbers of metabolically inactive organisms might not have high infective potential.

Based on various volunteer studies, the infective dose of non-typhoid salmonellae was estimated at $> 10^5$ organisms [12, 13] but the limited number of volunteers in these studies only allowed recognition of attack rates of c. 20% (ID20). Later, outbreak investigations have shown that 10^1–10^4 salmonella present in water and various foods had been able to cause illness in a considerable number of patients [14–17].

In the present outbreak paprika powder was identified as the vehicle of

transmission. The product was said to have been imported from South American but we have not been able to determine whether it was mixed with material from other sources.

The identity of isolates from foods and patients was confirmed by both serotyping and molecular fingerprinting. The low moisture prevented propagation but, as shown by follow-up investigations, the organisms survived without substantial losses. Thus it may be concluded that the concentration of culturable salmonellae had not markedly changed during the 3–5 months which elapsed between consumption of the food and bacteriological examination of the remaining product. The concentration of 0·04–0·45 organisms per gram would suggest an infective dose of 4–45 salmonellas in a person who had consumed 100 g of paprika-powdered potato chips. Comparable low infective doses have been determined in previous outbreaks due to contaminated chocolate [14, 15, 18] and cheese [17, 19]. In common with paprika powder, both are characterized by a high fat content which may protect salmonellae from gastric acidity [19].

A peculiarity of the outbreak was the high incidence in children of 1–4 years of age for whom the association with consumption of paprika-powdered snacks had been confirmed by telephone interviews in a number of cases. Recently, a small outbreak due to contaminated savoury corn snacks was reported from the UK in which, the median age of those affected was 1 year [20]. The lower gastric acidity of this age group together with changing eating habits might have created a new risk group for food items formerly consumed principally by adults.

There remain, however, mysteries about the outbreak. According to our knowledge, there is no other documented outbreak in which such a variety of salmonella strains had been involved. About 42 % of the cases confirmed by our laboratory were caused by three serovars, and altogether 94 different organisms were isolated from both patients and paprika-containing products during the outbreak period. Most of the latter were incomplete serovars, i.e. they were non-motile or monophasic strains of different rare O-groups of the salmonella subspecies I [Aleksic and colleagues, submitted]. Of these, a considerable number showed the unusual ability to grow in the presence of potassium cyanide [9], and 13 isolates proved to be new salmonella serovars [21]. The considerable number of incomplete, unusual, or new organisms lead us to the hypothesis that mutagenic processes might have been induced by intrinsic factors in the paprika powder. However, using the salmonella mutagenicity test [22] as a screening method, we could not detect a mutagenic effect upon testing the powder or two of its compounds, i.e. capsaicin and oleoresin (data not shown).

The results of the outbreak suggest the need to reconsider the wide spread belief that salmonella infectivity is strictly associated with active bacterial growth. There is no doubt that the classical concept of salmonellae propagating in a suitable medium and causing illness after ingestion of metabolically active organisms is still valid, but there is sufficient evidence now that low numbers of quiescent organisms are also able to cause infection [14, 15, 18]. Using water-based assay systems it has recently been shown that stress conditions such as nutrient deprivation, oxidative stress and DNA damage are critical signals of salmonella virulence gene regulation [23]. Respective studies have not been performed on desiccated salmonella cells but similar findings appear conceivable.

Furthermore, a possible role for the viable but non-culturable (VBNC) state of salmonellae will have to be assessed; this has been shown to exist in soil [24] and in river water [25]. Previous studies with other enteropathogenic organisms have demonstrated that VBNC *V. cholerae* and enteropathogenic *E. coli* [26] as well as *Campylobacter jejuni* [27] regained culturability after animal passage. Colwell and her coworkers [28] have recently shown that human volunteers excreted culturable organisms after ingestion of VBNC *V. cholerae*. These studies have been performed in water-based assay systems but we recently induced a VBNC state of salmonella in a desiccation model, too (unpublished data). If these organisms prove to be infective in animal models further studies will have to focus on their presence and importance in naturally contaminated food items.

In conclusion, the results suggest that even a low level of salmonella contamination is not acceptable in food products ready for consumption. Contamination of snacks with as few as $\geqslant 0.04$ salmonellae per gram has caused a nationwide outbreak with estimated 1000 cases. This reflects the problem of modern mass-produced food which reaches millions of consumers within a short period and which is characterized by the fact that products with a level of contamination capable of infecting only 1 in 10000 exposed persons may achieve considerable public health importance. It becomes clear that, in order to prevent such occurrences, the traditional bacteriological inspection of end-product samples is no longer sufficient to warrant the bacteriological safety of the food. Good manufacturing practice and monitoring of the production by concepts such as the hazard analysis critical control point (HACCP) must consider the whole process including production of raw materials in their countries of origin. This is especially necessary for food items for which possibilities of decontamination are limited or effective measures (e.g. irradiation) are not generally accepted.

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