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An outbreak of multidrug-resistant *Salmonella enterica* subsp. *enterica* serotype Typhimurium, DT104L linked to dried anchovy in Singapore

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SUMMARY

Multidrug-resistant *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104L was first reported in Singapore from mid-July to mid-October 2000. Salmonella strains isolated from clinical laboratories were submitted to a reference laboratory for serotyping, phage-typing and pulsed-field gel electrophoresis (PFGE) using *Xba*I restriction endonuclease. An epidemiological investigation was conducted to determine the source of infection and mode of transmission using a structured questionnaire. A total of 33 cases involving mainly infants and toddlers were detected in the 3-month long outbreak. The outbreak strain was of the R-type ACGSTSu, i.e. resistant to ampicillin, chloramphenicol, gentamicin, streptomycin, tetracycline and sulphonamide. PFGE showed all isolates had an indistinguishable pattern, indicating a common source of infection. Consumption of imported dried anchovy was found to be the vehicle of transmission after adjusting for all confounding variables in the case-control study using stepwise logistic regression (OR 25·6; 95% CI 3·9–167·9; $P = 0{\cdot}001$). Imported dried seafood should be properly processed, packed, labelled, and thoroughly cooked to prevent transmission of multidrug-resistant *S.* Typhimurium.

INTRODUCTION

The emergence of salmonella strains which are resistant to commonly used antimicrobials is of importance to clinicians, microbiologists and those responsible for the control of foodborne infectious diseases. *Salmonella enterica* subsp. *enterica* serotype Typhimurium definitive type 104 (DT104) with resistance to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline (ACSSuT) was first reported in the United Kingdom in 1984 [1, 2]. Since then, it has been isolated with increasing frequency in other European countries, Japan and the United States [3–6]. In the United Kingdom, it is the second most prevalent type of salmonella isolated from humans [7]. Outbreaks related to this strain have also been reported in Ireland, Northern California and Denmark [8–10] and the vehicles linked with foodborne transmission include chicken, pork sausage and meat paste, contaminated beef, turkey and raw-milk cheese [11–14].

In July 2000, an unusual increase in the isolation of *S.* Typhimurium DT104L with resistance to ampicillin, chloramphenicol, gentamicin, streptomycin, tetracycline and sulphonamide or R-type ACGSTSu, was noted. The isolates came mainly from the stool cultures of children under 18 months of age. We report here the findings of our microbiological and epidemiological investigations.

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METHODS

Microbiological investigations

S. Typhimurium was identified by serotyping using slide and/or tube agglutination following the

Kauffman–White scheme and phage typed by the method of Ward et al. [15]. The isolates were tested for susceptibility to 14 antimicrobial agents by agar disk diffusion tests on Mueller–Hinton agar (BBL Microbiology Systems, Cockeysville, MD) according to the National Committee for Clinical Laboratory Standards [16]. The agents were ampicillin (10 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), co-trimoxazole (1·25/23·75 μg), gentamicin (10 μg), amikacin (30 μg), nalidixic acid (30 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), neomycin (30 μg), sulphonamide (0·25 mg) and trimethoprim (5 μg).

Isolates were subcultured on Tryptic Soy Agar (TSA) containing 5% sheep blood overnight at 37 °C and colonies were inoculated into 10 ml of Tryptic Soy Broth (TSB) medium. After overnight incubation at 37 °C, the cells were pelleted by centrifugation and resuspended in sterile saline equal to the weight of the cell pellet. This suspension (20 μl) was added to 300 μl of TEN buffer (0·1 M Tris–HCL, 0·15 M NaCl, 0·1 M EDTA [pH 7·5]). 340 μl of 2% low-melting-point agarose (FMC BioProducts, Rockland, ME) was added to the TEN buffer mixture and pipetted into plug moulds. The agarose plugs were incubated for 5 h at 37 °C in 2 ml EC buffer (6 mM Tris, 1 M NaCl, 0·01 M EDTA, 0·5% Brij 58, 0·2% sodium deoxycholate, 0·5% Sarkosyl [pH 7·5] with 200 μl lysozyme (20 mg/ml)), washed twice with 5 ml 1× CHEF TE buffer (10 mM Tris–HCL, 1 mM EDTA), deproteinated in 2 ml ES buffer (400 mM EDTA, 1% Sarkosyl) with 100 μl proteinase K (20 mg/ml) and incubated overnight at 50 °C. The plugs were washed four times with 5 ml 1× CHEF TE buffer and genomic DNA was digested with restriction endonuclease *Xba*I (New England Biolabs). Electrophoresis was carried out with a GenePath apparatus (Bio-Rad Laboratories, Richmond, CA) in gels of 1% agarose in 0·5× Tris-borate-EDTA buffer for 25 h at 200 V at a temperature of 14 °C with ramped times of 2–40 s. The gels were stained with ethidium bromide (0·2 μg/ml) and photographed under UV transillumination. The DNA size standards used were a lambda ladder PFG marker of size range 50–1000 kb (New England, Biolabs).

Epidemiological investigations

A case was defined as an infant or toddler aged between 1 month and 36 months whose stool cultures were positive for *S*. Typhimurium DT104L during the period July–August 2000. Each case was traced and investigated to determine the source of infection and the mode of transmission. The home of each child was visited to look for other unreported cases among contacts. The parent, guardian, or domestic maid was interviewed by two trained environmental health officers using a structured questionnaire. As well as the basic epidemiological data (age, gender, ethnic group, residential address and onset of clinical symptoms), the questionnaire included a list of food items consumed and other risk factors likely to be linked to transmission, such as food handling and personal hygiene practices in the kitchen and contact with household pets with diarrhoea. For each notified case, 2–3 controls comprising children of the same age group (within 6 months of the age of the case), gender and ethnic group living in the same neighbourhood, and with no recent travel history and gastro-intestinal symptoms during the previous 2 weeks, were selected and interviewed. The questionnaire covered 3 days preceding the onset of symptoms of the cases, and for the controls, 3 days preceding the interview. All interviews were carried out within 3 weeks of onset of illness of the reported cases.

Environmental investigation

Samples of milk powder, cereal and other food items were obtained from the homes of the cases and submitted to the Food and Water Laboratory, Singapore General Hospital, for isolation of *Salmonella* species.

Statistical analyses

Differences in proportions between cases and controls were first compared using χ^2 or Fisher's exact test and odds ratio with 95% confidence interval derived. Multiple logistic regression was used to adjust simultaneously for potentially confounding factors [17]. All calculations were performed using SPSS and Excel microcomputer based software. A *P*-value of $< 0{\cdot}05$ was considered to be statistically significant in a two-tailed test.

RESULTS

During the period 13 July–17 October 2000, 33 isolates of *S*. Typhimurium DT104L of R-type ACGSTSu were identified. Their PFGE patterns were

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Table 1. *Age-gender distribution of 33 reported cases of* S. enterica *subsp.* enterica *serotype Typhimurium DT104L, Singapore*

Age group	Male	Female	Total
< 6 months	4	2	6
6–11 months	6	4	10
1 year	3	4	7
2 years	3	0	3
3 years	1	0	1
4 years	0	0	0
5–14 years	0	2	2
15–24 years	0	0	0
25–34 years	0	1	1
35–44 years	0	1	1
55+ years	2	0	2
Total	19	14	33

indistinguishable from each other. Seventeen cases were detected in July, 10 in August, 4 in September and 2 in October. Except for 4 adults aged between 31 and 66 years, all others were children and infants as young as 2 days of age. There were 19 males and 14 females (Table 1). None of them had a recent travel history outside Singapore. Cases were not clustered in any particular locality. No secondary cases were identified in the same household.

The main presenting clinical symptoms of 19 cases investigated were watery diarrhoea (100%), fever (78·9%), vomiting (52·6%) and abdominal cramps (26·3%). About 58% had bloody stools. Seventeen of them were hospitalized and 2 were treated as outpatients. No death was reported.

Results of the case-control study based on 19 cases and 55 controls using univariate analyses, implicated porridge with 'ikan bilis' (dried anchovy) as the vehicle of transmission (OR 8·2; 95% CI 2·5–26·9; $P < 0{\cdot}0001$). No other food items such as egg, dairy products, chicken, pork, beef, fruit or raw vegetables were incriminated (Table 2). Among the risk factors analysed, there was a strong inverse relationship between handwashing before preparing food and the development of illness (OR 0·17; 95% CI 0·04–0·69; $P = 0{\cdot}02$). However, there was no significant association between illness and contact with animals or household members with diarrhoea. After adjusting for confounding variables using forward stepwise logistic regression, porridge with 'ikan bilis' remained significantly associated with the illness (OR 25·6; 95% CI 3·9–167·9; $P = 0{\cdot}001$).

None of the food samples taken from the homes of six cases, including one sample of ground dried anchovies, was positive for *Salmonella* species. However, *Salmonella* group E was isolated from 1 of the 16 samples of dried anchovies subsequently purchased from the retail outlets.

DISCUSSION

Outbreaks of salmonellosis caused by multidrug–resistant strains have occurred previously in Singapore: *S.* Typhimurium in 1971–97, *S.* Oranienburg in 1978–9, and *S.* Blockley in 1983. However, the vehicles of transmission remained undetermined. The predominant serotypes in 1999 were *S.* Enteritidis (20·3%), *S.* Stanley (12·8%), *S.* Weltevreden (10·8%), followed by *S.* Typhimurium (7·3%) [18].

Unlike in Western Europe and North America, where consumption of unpasteurized dairy products, poultry, pork and beef products has been associated with foodborne transmission of multidrug resistant *S.* Typhimurium, no such relationship could be established in this outbreak [11–14]. The only food vehicle linked to transmission was the consumption of ground 'ikan bilis' (dried anchovies). Risk factors identified in the United States and the United Kingdom, such as contact with farm animals and pets, were also not significantly associated with this outbreak [19]. The inverse relationship between handwashing before preparing food and the development of illness was not found to be statistically significant after adjusting for confounding variables.

Salmonella is one of the most prevalent enteric pathogens encountered in seafood in Thailand [20]. It has been isolated from ready-to-eat imported seafood such as cooked shrimp, shellfish, fish paste, smoked fish, salted dried fish and caviar in the United States [21]. Outbreaks of salmonellosis caused by ingestion of contaminated cuttlefish chips as snacks have also been reported in Japan [22].

The 'ikan bilis' are imported from Thailand, Vietnam, Malaysia and Indonesia and sold at retail outlets throughout the country. These small anchovies caught in fishing villages in these countries are lightly salted and dried in the open air. This method of processing 'ikan bilis' is subject to gross environmental contamination, including excreta of birds, rats and flies. After processing, the dried seafood is packed in gunny sacks and transported by container trucks,

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Table 2. *Results of matched case-control analysis in an outbreak of* S. enterica *subsp.* enterica *serotype Typhimurium DT104L, Jul to Oct 2000*

Food items/risk factors	Cases ($n = 19$)	Controls ($n = 55$)	OR	95% CI	*P*-value
Egg					
Uncooked	0/19	1/55	—	—	0·74
Partially cooked	4/19	12/55	0·96	0·27–3·42	0·94
Cooked	3/19	23/55	0·26	0·07–1·00	0·04
Diary products					
Powdered milk formula	18/19	48/55	2·63	0·30–22·86	0·37
Pasteurized milk	1/19	6/55	0·45	0·05–4·03	0·47
Reconstituted/UHT milk	0/19	1/55	—	—	0·74
Cereals with milk	8/19	21/55	1·18	0·41–3·40	0·76
Baby food	1/19	1/55	3·00	0·18–50·47	0·43
Cheese	9/19	13/55	2·91	0·97–8·69	0·05
Yoghurt	0/19	4/55	—	—	0·30
'Yakult'	2/19	12/55	0·42	0·09–2·09	0·28
'Vitagen'	2/19	9/55	0·60	0·12–3·07	0·54
Porridge					
Alone	6/19	10/55	2·08	0·64–6·80	0·22
With chicken	4/19	26/55	0·30	0·09–1·01	0·05
With pork	7/19	29/55	0·52	0·18–1·53	0·23
With beef	0/19	4/55	—	—	0·30
With liver	1/19	6/55	0·45	0·05–4·03	0·47
With 'ikan bilis' (dried anchovy)	14/19	14/55	8·2	2·5–26·9	0·0001
Breast fed					
With supplements	0/19	1/55	—	—	0·74
Without supplements	0/19	4/55	—	—	0·30
Fruits	11/19	21/55	2·23	0·77–6·43	0·14
Fruit juices	4/19	11/55	1·07	0·30–3·86	0·92
Raw vegetables	0/19	2/55	—	—	0·98
Animal contact	0/19	3/55	—	—	0·57
Household contact with diarrhoea	0/19	5/55	—	—	0·24
Handwashing before preparing food	13/19	51/55	0·17	0·04–0·69	0·02
Storage of 'ikan bilis' in chiller	4/19	10/55	1·2	0·33–4·40	0·78

fishing boats or lorries to Singapore. Further contamination could have occurred during transport, storage and display at retail outlets where it was sold without proper packing and labelling. The shelf life of the product is 1–2 weeks. It has a high turnover rate at the retail outlet and is sold out within a few days. The dried anchovy is a rich source of calcium and protein and is a popular ingredient for the preparation of a variety of foods, including soup stock. It is commonly deep fried and served with chilli. If used as an infant feed, the common practice is to grind it and then cook it with porridge. In the households where cases had occurred, the 'ikan bilis' was first ground and stored in a container at room temperature for subsequent use. It was either sprinkled on or added to freshly cooked porridge, instead of boiling with the porridge, and served.

The finding of a group E *Salmonella* in the dried anchovies showed that this product can be a potential food vehicle for transmission of salmonellosis caused by other serotypes. The means of prevention of salmonellosis through imported dried seafood is to implement hazard analyses and critical control points to reduce contamination. Products should be properly labelled and packed before transportation in a closed container to retail outlets. The practice of sprinkling or adding ground 'ikan bilis' on cooked porridge should be discouraged as the temperature attained is inadequate to destroy the salmonella present in the food.

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