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Original Scientific Article

INVESTIGATION OF TWO *SALMONELLA* SEROVAR ENTERITIDIS OUTBREAKS USING THE PULSED-FIELD GEL ELECTROPHORESIS: A GOOD EXAMPLE OF COLLABORATION AT THE NATIONAL LEVEL

Mateja Pate[1]*, Jasna Mićunović[1], Vojka Bole-Hribovšek[1], Majda Biasizzo[1], Maja Bajt[2], Andreja Krt Lah[3], Mateja Ravnik[3], Marta Košir[4], Tatjana Harlander[4], Tjaša Žohar Čretnik[5]

[1]National Veterinary Institute / Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana; [2]Veterinary Administration of the Republic of Slovenia, Ministry of Agriculture, Forestry and Food, Dunajska c. 22, 1000 Ljubljana; [3]Institute of Public Health Kranj, Gosposvetska ul. 2, 4000 Kranj; [4]Institute of Public Health Novo mesto, Mej vrti 5, 8000 Novo mesto; [5]Institute of Public Health Celje, Ipavčeva ul. 18, 3000 Celje, Slovenia

*Corresponding author, E-mail: mateja.pate@vf.uni-lj.si

Summary: *Salmonella* is an important zoonotic pathogen in animals and humans. In the European Union, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (serovar Enteritidis) is one of the serovars most frequently associated with human illness. The most important food vehicles responsible for the infection are eggs and egg products. We describe two serovar Enteritidis outbreaks on account of consumption of contaminated eggs. The first outbreak due to vanilla cream served as dessert in a restaurant involved 36 persons. As the eggs used in preparing the vanilla cream were no longer available for examination, an indirect epidemiological link between the infected laying hen flock and humans was demonstrated by testing the faeces and dust samples from the relevant laying hen flock. In the second outbreak, two persons developed a severe form of salmonellosis after having consumed fried eggs. A sample of eggs taken from the same laying hen flock as the eggs consumed by the two patients tested positive for serovar Enteritidis.

Isolates from both the outbreaks were subjected to molecular typing for the assessment of genetic relatedness. Pulsed-field gel electrophoresis (PFGE) revealed that the profiles of the majority of isolates from the same outbreak were indistinguishable and should therefore be considered to represent the same strain.

This is the first molecular epidemiological investigation of serovar Enteritidis outbreaks in Slovenia that involved the public health and veterinary authorities and as such set a good example of collaboration of different national services.

Key words: *Salmonella* serovar Enteritidis; salmonellosis; outbreak; genotyping; laying hens; eggs; humans

Introduction

Salmonella has long been recognised as an important zoonotic pathogen of economic significance in animals and humans. A common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which results in a variety of food of animal and plant origin as sources of infections. Transmission often occurs when organisms are introduced in food preparation areas and allowed to multiply in food, either due to inadequate storage temperatures, inadequate cooking or cross-contamination of ready-to-eat food (1).

In humans, the symptoms of salmonellosis are often mild and most infections are self-limiting, lasting a few days. However, in patients with primary and secondary disorders of the immune response and in patients with severe underlying diseases, the infection may be more serious. In animals, sub-clin-

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ical infections are common. *Salmonella* may easily spread undetected between animals which may become intermittent or persistent carriers (1).

In 2009, *Salmonella* was the predominant causative agent of reported food-borne outbreaks in the European Union (EU). The most important food vehicles in the outbreaks with known causative agent were eggs and egg products, mostly associated with *Salmonella enterica* subsp. *enterica* serovar Enteritidis (serovar Enteritidis) contamination. Serovar Enteritidis was the most frequently reported serovar (52.3% of all known serovars in human cases) (1).

For efficient preventive and control measures, it is of utmost importance to determine the sources of infection and routes of transmission. Pulsed-field gel electrophoresis (PFGE) has been shown to be appropriate for epidemiological studies of *Salmonella* (2-6). It has been used as the principal method for subtyping salmonellas in the USA since 1996 (7) and successfully applied to outbreak investigations due to serovar Enteritidis (8-10). The method enables the assessment of genetic relatedness among the isolates and, together with supporting epidemiological data, provides the possibility to detect links between isolates from different sources.

The objective of this paper is to report on the investigation of two serovar Enteritidis-related outbreaks recorded in 2009 and to demonstrate the importance of good collaboration between the public health and veterinary professionals.

Materials and methods

Investigation of Outbreak No 1

In March 2009, an anonymous report of food-borne intoxication was received by the Public Health Inspectorate of the Republic of Slovenia. At the same time, the Clinical Microbiology Laboratory of the Institute of Public Health of Novo mesto (IPH–NM) reported cases of serovar Enteritidis isolation to the epidemiological services. This was the basis for conducting an epidemiological investigation in order to determine the source of infection. Inquiries revealed that intoxication had presumably occurred on 15 March 2009 in a restaurant in the Dolenjska region. Relevant meal was consumed by 30 restaurant customers, six restaurant employees and their family members. Sanitary examination of the kitchen took place and the staff was instructed to follow the guidelines for prevention of transmission of enteric contagious disease and to carry out the cleaning and disinfection measures. Surface swabs were taken and microbiological examination of stool samples of the diseased customers, kitchen staff and family members was conducted.

Two stool samples of patients were examined at the beginning of the outbreak and both tested positive for serovar Enteritidis. After the reported outbreak, 17 stool samples of persons connected with the outbreak (restaurant employees, customers and contact persons) were examined. Serovar Enteritidis was detected in three persons, including two restaurant employees. Only one patient was hospitalised, and there were no fatalities. Surface swabs were all negative for serovar Enteritidis. Five serovar Enteritidis isolates were sent to the Institute of Public Health of Celje (IPH–CE) for PFGE typing. PFGE profiles of all the isolates were identical.

Interviews with patients showed that vanilla cream (made of fresh eggs and used as topping on canned fruit) was the possible source of infection. The dessert was consumed by all the persons that later developed the enteric disease. Vanilla cream was not subjected to testing as it was no longer available. However, the Public Health Inspectorate managed to detect the origin of the eggs and notified the competent Regional Office (RO) of the Veterinary Administration of the Republic of Slovenia (VARS) of a suspected outbreak of salmonellosis caused by food of animal origin, indicating all the particulars of the relevant food business operator (FBO). The competent VARS RO conducted official control in the establishment of the FBO involved in egg production, taking two faeces samples and a single dust sample in the relevant laying hen flock, as required by the applicable regulations.

The National Veterinary Institute (NVI) detected serovar Enteritidis in the dust and faeces samples. The competent VARS RO instituted the required measures in the relevant FBO's establishment, including the ban on placing table eggs on the market and on any movements of the animals, except for killing or slaughter.

Investigation of Outbreak No 2

In the first five months of 2009, an unusually high number of persons (n=51) suffering from enteric disease caused by serovar Enteritidis were detected by the Institute of Public Health of Kranj (IPH–KR), as compared to a 10-year average of 15 cases within a period between January and May. Three family outbreaks of salmonellosis were investigated in the first

half of 2009. Epidemiological investigation revealed that infections were predominantly linked to consumption of raw or insufficiently heat-treated eggs or inadequately prepared egg dishes in the patients' home environment. Due to delayed notification of the two family outbreaks, microbiological examination of food samples was no longer possible and, due to a small number of affected persons, analytical epidemiological studies could not be performed.

This case describes a family outbreak due to consumption of fried eggs, which caused a severe form of salmonellosis in a 13-month-old child and his 36-year-old mother in May 2009. Both the patients were admitted to hospital. Serovar Enteritidis was detected in their stool samples. The isolates were PFGE typed at IPH–CE, and shared a common PFGE profile.

IPH–KR notified VARS of a salmonellosis outbreak and of a suspected source of infection. A single egg originating from the same batch as table eggs involved in the outbreak was brought to VARS RO by a member of the affected family. VARS was able to trace back the laying hen house by the producer's identification imprinted on the egg. The competent VARS RO conducted official control in the establishment of the FBO involved in egg production, taking two faeces samples and a single dust sample in the relevant laying hen flock, as required by the applicable regulations, and submitting a sample of eggs for *Salmonella* testing to the NVI. Serovar Enteritidis was identified in all the samples. The competent VARS RO instituted the required measures in the FBO's establishment as referred to above. As a precautionary measure, the media were requested by IPH–KR to disseminate information among the general public on the safety of use, preparation and consumption of raw eggs and egg dishes.

In June 2009, NVI received a request from VARS to compare the PFGE profiles of isolates involved in the two outbreaks. PFGE profiles of human isolates were obtained from IPH–CE and compared with the PFGE profiles of non-human isolates in the NVI database.

Microbiological examination

At suspected epidemic of an enteric contagious disease, samples are investigated for the presence of most common bacterial, viral and parasitic agents.

Accordingly, stool samples were inoculated both into enrichment medium for enterobacteria (selenite broth) and onto selective media for other intestinal pathogens (Karmali agar, sMaC agar, Drigalski agar, blood agar). After 24 hours, they were transferred from enrichment onto *Salmonella* selective medium (XLD), which was checked for suspect colonies. These were subsequently identified to the species/serotype level by biochemical tests and agglutination.

Dust and animal faeces were processed according to EN/ISO 6579-2002/Amd1:2007. After 18 ± 2 hours' enrichment in buffered peptone water, they were inoculated onto modified semisolid Rappaport-Vassiliadis agar and, in case of swarming, the culture was inoculated onto XLD and Rambach agar. *Salmonella*–suspect colonies were identified by biochemical tests and agglutination. The sample of eggs was tested according to ISO 6579:2002.

PFGE typing

Human isolates were sub-typed by PFGE at IPH–CE that performs the nation-wide PFGE typing of isolates from human outbreaks. Isolates from dust, eggs and hen faeces were sub-typed at the NVI. Details on isolates subjected to analysis of genetic relatedness are given in Table 1. A standardised protocol was used for PFGE typing, using the restriction endonuclease *Xba*I (11). Fragments generated were separated by electrophoresis for 20 hours at 6 V/cm and 14 °C, and with pulse times from 2 s to 64 s in a CHEF-DR II system (BioRad, Hercules, CA, USA). PFGE profiles were subjected to computer-assisted analysis with BioNumerics software (version 5.0, Applied Maths, Sint-Martens-Latem, Belgium). Serovar Braenderup strain H9812 was used for normalisation. Dendrograms were created using an UPGMA (Dice coefficient) algorithm.

Table 1: Origin of serovar Enteritidis isolates involved in PFGE typing

Event	Origin and number of isolates			
	Man	Laying hen	Dust	Egg
Outbreak 1	5	1	1	0
Outbreak 2	2	2	1	1[a]

[a] pooled sample of 10 eggs

Results

PFGE typing

Dendrogram generated from seven isolates related to the first outbreak revealed identical PFGE pro-

files for six isolates, whilst the profile of one isolate from dust differed in one band.

Similar results were seen when comparing genotyping results of isolates involved in the second outbreak. All isolates shared a common profile, with the exception of the one obtained from a laying hen that exhibited a one-band difference (Figure 1).

Discussion

Different *Salmonella* serovars have been related specifically with some foods. Serovar Enteritidis is particularly related to eggs (12, 13). This is linked to the ability of this serovar to persistently colonise the avian reproductive tract, resulting in internally contaminated eggs, and in egg shell contamination (14). According to the European Food Safety Authority (EFSA) report for 2009, *Salmonella* was only found in a very a low proportion of table eggs and egg products, at levels of 0.5 % and 0.6 %, respectively. However, *Salmonella* was still the most frequently reported cause of food-borne outbreaks and the main food vehicles were eggs and egg products (1).

A key factor enabling the egg to be an efficient vehicle for human infection is the manner in which people handle and eat eggs. Eggs are one of the few animal products that are frequently eaten raw or undercooked. Hedberg et al. (15) found that patients with sporadic serovar Enteritidis were over five times more likely to have eaten raw or undercooked eggs in the three days before their illness, compared with healthy control subjects. The extent to which eggs were not cooked was directly associated with illness. In the second outbreak described herein,

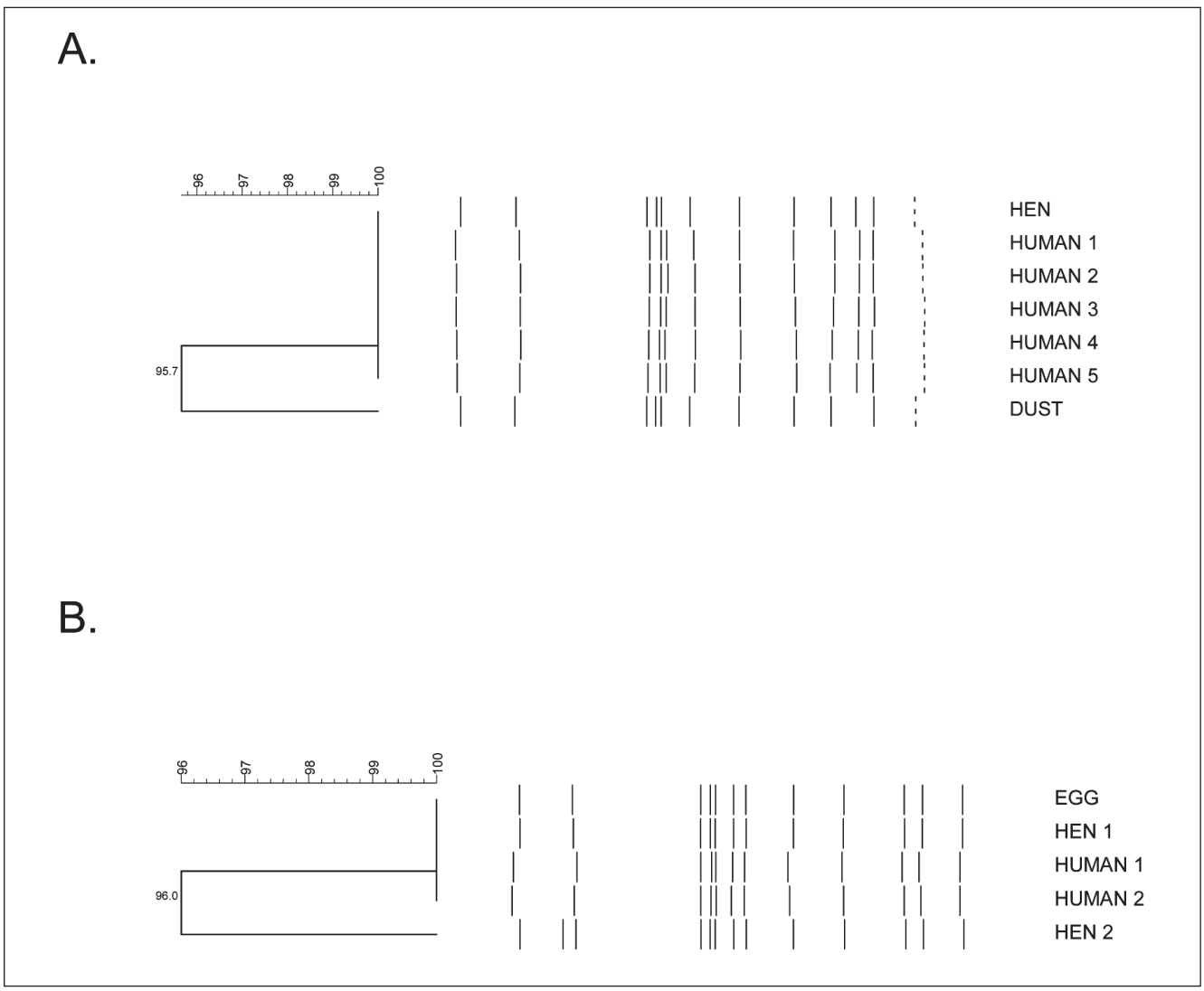

Figure 1: Dendrograms showing genetic relatedness of serovar Enteritidis isolates related to the first (A) and the second (B) outbreak

two persons suffered from severe form of salmonellosis due to consumption of fried eggs. It has been reported before that fried eggs may be a source of infection with serovar Enteritidis (16), especially if when eaten, the egg white and yolk are still soft or runny. Infection with serovar Enteritidis is also associated with eating outside the home (17). One reason that commercial or other large-scale food preparation settings may be more frequently associated with illness is the practice of pooling large numbers of eggs for use in egg dishes (18). Thus, one egg can contaminate a large amount of food and endanger a large number of consumers. The first outbreak described in this paper was an example of infection acquired in a restaurant. Eight out of 36 exposed persons developed symptoms of enteric disease and serovar Enteritidis was isolated from five patients.

The numbers of human salmonellosis cases reported in the EU continued to decline in 2009 as a part of a statistically significant trend since 2005. The reduction was particularly substantial for the most frequently reported serovar Enteritidis. It is assumed that the observed reduction of salmonellosis cases is mainly due to successful *Salmonella* control programmes in fowl populations (1). However, the risk of infection with serovar Enteritidis still persists and should not be neglected or underestimated due to potential complications of the disease. Early epidemiological and microbiological investigation is of utmost importance for preventing the transmission of infection and molecular typing is essential for detection of *Salmonella* source.

PFGE method has been proven to be useful for assessing the relatedness of serovar Enteritidis isolates and for investigating the outbreaks as a gold standard (2, 19). PFGE has been used for typing animal and food isolates of *Salmonella* at the NVI for several years. Previously established collaboration with IPH–CE and the use of standardised PFGE protocol undoubtedly contributed to fast and efficient work on comparison of PFGE profiles. Comparison revealed that profiles of the majority of isolates from the same outbreak were indistinguishable and should therefore be considered as a single strain. In both the outbreaks, single isolates showed a one-band difference compared to the other isolates. These isolates should be considered as closely related to the outbreak strain by the criteria for bacterial strain typing proposed by Tenover et al. (20).

Even though PFGE has been widely used for characterisation of genetic relatedness of serovar Enteritidis isolates and investigation of outbreaks, it has been suggested to have limited value in epidemiological analysis because of the high genetic homogeneity among strains of serovar Enteritidis (21, 22). On the other hand, some comparative studies indicate that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing techniques (e.g. 23). Nevertheless, it has been suggested that a single method cannot be used reliably for epidemiological analysis of unrelated and related strains of serovar Enteritidis (24-26). This vantage point should be borne in mind at future introduction of additional genotyping methods.

This is the first molecular epidemiological investigation of serovar Enteritidis outbreaks in Slovenia that involved public health and veterinary professionals, setting a good example of collaboration between different national authorities. Coordinated action resulted in successful investigation of the outbreaks. Constant high-level cooperation and introduction of new typing methods with increased discriminatory power constitute a good basis for the effective prevention and control of zoonoses in general. In the future, typing of all serovar Enteritidis isolates obtained from routine laboratory examinations would be reasonable as it would allow us to identify genotypes with a high spreading potential, to trace the source of epidemic strains, to detect large epidemics, and to follow the rapidity of spread of certain genotypes within the food chain.

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UPORABA PULZNE ELEKTROFOREZE V PREISKAVI DVEH IZBRUHOV, POVZROČENIH S SALMONELO SEROVARA ENTERITIDIS: DOBER PRIMER SODELOVANJA NA DRŽAVNI RAVNI

M. Pate, J. Mićunović, V. Bole-Hribovšek, M. Biasizzo, M. Bajt, A. Krt Lah, M. Ravnik, M. Košir, T. Harlander, T. Žohar Čretnik

Povzetek: Salmonela je pomembna patogena bakterija pri živalih in ljudeh. *Salmonella enterica* subsp. *enterica* serovar Enteritidis (serovar Enteritidis) spada med serovare, ki v Evropski uniji najpogosteje povzročajo obolenja ljudi. Najpomembnejši vir okužbe med živili predstavljajo jajca in jajčni izdelki. V prispevku opisujemo dva izbruha, povzročena s salmonelo serovara Enteritidis, zaradi zaužitja kontaminiranih jajc. Pri prvem izbruhu se je 36 ljudi okužilo z vanilijevo kremo, postreženo kot sladico v restavraciji. Ker jajca, iz katerih je bila krema pripravljena, niso bila več na voljo, je bila s preiskavo iztrebkov in prahu iz reje kokoši nesnic ugotovljena zgolj posredna epidemiološka povezava med rejo kokoši nesnic in ljudmi. Pri drugem izbruhu sta dve osebi zboleli za hujšo obliko salmoneloze, ki je bila posledica zaužitja ocvrtih jajc. Vzorec jajc iz iste reje kokoši, iz katere so izvirala zaužita jajca, je bil pozitiven na serovar Enteritidis. Z molekularno tipizacijo smo ugotavljali gensko sorodnost izolatov iz obeh izbruhov. Rezultati pulzne elektroforeze so razkrili, da so bili profili večine izolatov iz posameznega izbruha enaki in so torej predstavljali isti sev.

V Sloveniji je to prva molekularno-epidemiološka preiskava izbruhov, povzročenih s serovarom Enteritidis, pri kateri so sodelovali strokovnjaki javnega zdravstva in veterinarske medicine, in predstavlja dober primer sodelovanja med različnimi državnimi službami.

Ključne besede: *Salmonella* serovar Enteritidis; salmoneloza; izbruh; genotipizacija; kokoši nesnice; jajca; ljudje