# Salmonellosis outbreak due to *Salmonella* Enteritidis phage type 14b resistant to nalidixic acid, Austria, September 2010

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We report on a salmonellosis-outbreak due to *Salmonella* Enteritidis phage type 14b resistant to nalidixic acid (*S.* Enteritidis PT14b Nx) among residents and employees of a student residence in Austria, September 2010. The outbreak was described and analysed by a retrospective cohort study, and microbiological environmental investigations were conducted to identify the outbreak source(s) and the reservoir of the outbreak strain. A total of 66 persons fulfilled the outbreak case definition including 14 laboratory-confirmed cases. Food specific cohort-analyses by day revealed that consumption of potato salad (RR: 1.65, 95%CI: 1.35–2.01, p=0.001) and a cheese-sausage cold plate (RR: 2.24, 95%CI: 1.29–3.88, p=0.002) on 14 September was associated with being an outbreak case. We hypothesised that cross-contamination with *S.* Enteritidis PT14b Nx positive eggs had occurred during preparation of the potato salad and cold plate as a result of preparing in parallel egg-containing breaded cutlets on 14 September. A traced laying hen holding in eastern Austria was identified as the sole source of the consumable eggs in the student residence. By applying the legally mandated sampling method for epidemiological-related laying hen farms (one pooled dust sample à 150g, two paired boot swabs cultured separately), the outbreak strain could not be detected. Our findings, that legally required sampling methods for laying hen farms failed to detect the causative pathogen in a laying hen holding, despite an epidemiological link, underline the request stated by the European Food Safety Authority Panel on Biological Hazards for a more sensitive sampling plan in epidemiologically-associated laying hen flocks.

## Introduction

In the European Union (EU), food-borne outbreaks are mandatorily reported since 2003 [1]. Since then, between 30% and 60% of the reported outbreaks have been caused by *Salmonella* [2]. In Austria, among the 1,255 *Salmonella* outbreaks reported within the past five years, the most frequent serovar was *Salmonella* Enteritidis, accounting for more than 82% (unpublished data).

Eggs from *Salmonella*-positive laying hen flocks and products made from such eggs were the most frequently associated food vehicles [2].

Before 2001, *S.* Enteritidis phage type (PT) 14b was considered a rare causative agent of human salmonellosis in the EU [3]. In 2001, Norway, Sweden and Finland reported increased numbers of cases of infection with *S.* Enteritidis PT14b in patients who had travelled to Greece [3]. In 2002, an outbreak with continuing exposure to a source of infection, associated with eating bakery products, was observed in the United Kingdom and was caused by *S.* Enteritidis PT14b susceptible to nalidixic acid [4]. In Austria, from 2001 to 2003, only 1.8% of the total 21,247 *S.* Enteritidis cases registered, were of *S.* Enteritidis PT14b and all were susceptible to nalidixic acid. In these cases, there was no history of travel.

In 2009, an upsurge in the number of non-travel associated cases of infection with *S.* Enteritidis PT14b resistant to nalidixic acid (*S.* Enteritidis PT14b Nx) was observed in the United Kingdom and linked to the consumption of egg-containing food [5]. In Austria, cases of infection with *S.* Enteritidis PT14b Nx were first reported in 2005 and accounted for 0.3% of the 4,669 registered *S.* Enteritidis cases. From 2006 onwards the proportion of *S.* Enteritidis PT14b Nx among *S.* Enteritidis cases increased continuously: 0.3% in 2006, 0.9% in 2007 and 1.5% in 2008. In 2009, none of the isolates from 20 cases of *S.* Enteritidis PT14b, among the registered 1,829 cases of *S.* Enteritidis, were resistant to nalidixic acid, according to the data of the Austrian reference laboratory for *Salmonella*.

We report on the first documented food-borne outbreak due to *S.* Enteritidis PT14b Nx, which occurred in September 2010 in Austria, and discuss different

environmental sampling methods with respect to the sensitivity of detecting the outbreak strain in epidemiologically-linked poultry flocks.

## Outbreak description

At the end of September 2010, the Austrian Agency for Health and Food Safety (AGES) was informed by the Austrian reference laboratory for *Salmonella* of a cluster of 14 cases of gastroenteritis due to infection with *S.* Enteritidis PT14b Nx in a western province of Austria. These cases had occurred in a student residence after its re-opening, following the summer break, on 12 September. Another 30 cases of gastroenteritis among the residents of the student residence were reported by the public health authority by 30 September 2010. The student residence hosted 142 male students and 19 staff, which included five kitchen workers, seven tutors and seven administrative staff.

In the previous five years, from 2005 to 2009, a total of seven cases of 40 *S.* Enteritidis PT14b that had been registered in this western province showed antimicrobial resistance to nalidixic acid, according to the data of the Austrian reference laboratory for *Salmonella*.

AGES was mandated by the competent public health authority to investigate the student residence outbreak on 30 September. The aim of the investigation was to describe the outbreak epidemiologically to identify the outbreak source(s) and the reservoir of the causative pathogen in order to set appropriate control and preventive measures.

## Case definition

The following outbreak case definition was applied: A probable outbreak case was defined as a person who (i) was a resident or working as kitchen staff or tutor in the particular student residence from 12 September onwards, and (ii) fell sick with symptoms of gastroenteritis (at least three loose stools per day or vomiting) on 13 September at the earliest. A confirmed outbreak case was defined as a person who fulfilled criteria (i) and (ii) and tested positive for *S.* Enteritidis PT14b Nx. In personal interviews, cases were asked about demographics, disease onset, symptoms, hospitalisation and duration of disease, and whether they had stool samples handed in for testing.

A total of 66 persons fulfilled the outbreak case definition including 52 probable (only student-cases) and 14 confirmed outbreak cases (involving 13 student-cases and one tutor-case). The duration of diarrhoea ranged from one to 10 days with a mean of 4.4 days. The median age of the cases was 16.8 years (range: 14.1–21.1); cases were all male and diarrhoea was the dominant symptom (62/66, 94%).

## Outbreak characteristics

The outbreak occurred from 14 September to 21 September and peaked with 29 cases on 16 September. The pattern indicated a point source active on 14

September followed by a continuous common source (Figure).

## Methods
### Retrospective cohort study

It was hypothesised that food offered by the residence kitchen was the most likely source of the outbreak. An analytical epidemiological investigation was performed using a retrospective cohort study in order to identify the food item(s) most likely associated with the risk of infection with the outbreak strain *S.* Enteritidis PT14b Nx and to generate a hypothesis on the reservoirs of the outbreak strain.

### Cohort of interest and food exposure history

The student residence hosted students aged between 14 and 18 years from Sunday evening until Friday midday. According to information provided by the local public health authorities, the cohort of interest comprised 161 persons with 142 student residents and 19 employees (five kitchen workers, seven tutors and seven administrative staff), present from 12 to 17 September. Meals were offered three times a day, made in the kitchen, served and consumed in the residence refectory. The menus for 12 September, evening until 17 September, lunch (no dinner was offered on 17 September), were provided by the kitchen chef. A continental breakfast was served daily and included muesli containing wheat, oats and corn eaten with milk or yogurt. On Sunday evening, when the student residence kitchen re-opened after the summer break, a ham and cheese toast was offered for dinner, on Monday spaghetti with salad were served for lunch and frankfurters with baked roll for dinner, on

Outbreak cases of *Salmonella* Enteritidis phage type 14b resistant to nalidixic acid, by day of symptom onset, Austria, 14–21 September 2010 (n=66)
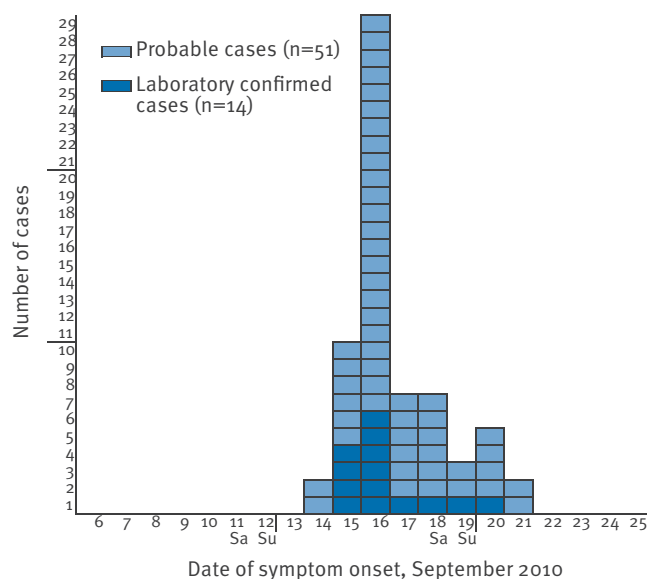


Date of symptom onset is unknown for one outbreak case.

Tuesday breaded cutlets (i.e. traditional Austrian dish "Wiener Schnitzel") and potato salad were offered for lunch and a cold plate for dinner, on Wednesday pizza with salad for lunch and noodles with ham for dinner, on Thursday fish sticks with vegetable rice for lunch and fried chicken with mixed salad for dinner, and on Friday, pancakes with cheese or with jam were served for lunch.

### Data collection and analysis
Data on food exposure for the days from 12 to 17 September were ascertained by a self-administered questionnaire. Food-specific attack rates (AR) and relative risks (RR) were calculated for a total of 27 dishes and food items regardless of the day on which a specific food item had been served. The data were entered into Epi Info version 3.5.1 and STATA version 11 was used for univariate and stratified analyses. Differences in food-specific AR between exposed and unexposed groups were tested by chi-square or Fisher's exact test yielding the RR with a 95% confidence interval. In a second approach, food-specific cohort analyses were performed for each relevant day (12 September to 17 September). A specific study cohort was defined for each day including disease free members only, i.e. outbreak cases occurring the days prior to or on the day under study were excluded from the respective day-specific study cohort. A diseased person was defined as a member of the day-specific study cohort who had fallen sick with symptoms of gastroenteritis within three days following exposure to the food item of the specific day under study (considering a maximum incubation period of 72 hours). Exposure to cereals was defined as consumption of muesli at breakfast on any day from 13 until 19 September, because the day-specific consumption of muesli could not be recalled by the cohort members.

### Microbiological and environmental investigation
As initiated by the outbreak investigators on 30 September, stool samples from five kitchen workers and six tutors, who had all remained asymptomatic throughout the outbreak, were tested for *Salmonella*. Isolates were serotyped according to the Kauffmann-White scheme, and phage-typed as described elsewhere [6,7]. Samples obtained from the kitchen environment were tested as described previously [8]. As part of the environmental investigation, the laying hen holding identified as the source of the eggs used in the relevant period by the student residence was sampled for *Salmonella* testing, by one sample of pooled dust à 150 g and two paired boot swabs per flock (cultured separately). This method was according to the official sampling conducted once a year, which is done in addition to the 15 week sampling within the regulatory monitoring program [9]. Microbiological workup of these environmental samples was performed as described elsewhere [10]. In addition to microbiological testing within the outbreak investigation, we reviewed the results of *Salmonella* testing within the regulatory monitoring program.

No food samples were available for testing when the outbreak investigation was performed.

Multiple-locus variable number tandem repeat analyses and pulsed-field gel electrophoresis
Pulsed-field gel electrophoresis (PFGE) by use of the restriction enzyme *Xba*I and multiple-locus variable number tandem repeat analyses (MLVA) were performed with the human isolates of *S.* Enteritidis PT14b Nx obtained from student-cases and with the environmental isolates [11,12].

## Results
### Retrospective cohort study
Completed questionnaires from 144 of 161 persons of the cohort of interest were provided (response rate 90%) giving a study cohort of 141 students (including 65 student-cases), two tutors (one tutor case) and one kitchen worker. The food-specific cohort-analyses yielded consumption of breaded cutlet (RR: 3.97, 95%CI: 1.37–11.50, p<0.0001), potato salad (RR: 3.58, 95%CI: 1.43–8.95, p=0.000), spaghetti (RR: 2.31, 95%CI: 1.05–5.08, p=0.010), frankfurters (RR: 2.22, 95%CI: 1.23–4.01, p=0.001), baked roll (RR: 2.12, 95%CI: 1.21–3.70, p=0.002), meat and cheese cold plate (RR: 2.06, 95%CI: 1.34–3.19, p=0.000) and bread slices (RR: 2.00, 95%CI: 1.30–3.09, p=0.08) as factors significantly associated with the infection risk. Of these seven dishes, the following food items were re-identified as risk associated by the food-specific analyses by day: cold plate (RR: 2.24, 95%CI: 1.29–3.88, p=0.002), bread slices (RR: 2.17, 95%CI: 1.25–3.76, p=0.002), breaded cutlet (RR: 1.69, 95%CI: 1.41–2.02, p=0.001) and potato salad (RR: 1.65, 95%CI: 1.35–2.01, p=0.001) served at lunch on 14 September, served at dinner on 14 September (Table 1). After stratifying the effect of breaded cutlet and bread consumption by the exposure status to potato salad, and the effect of bread consumption by the exposure status to the cold plate, eating breaded cutlet and eating bread slices became insignificant.

A total of 52 (96%) of the 54 outbreak cases having occurred from the evening of 14 September, until the morning of 18 September had consumed the potato salad at lunch on 14 September, and 40 (77%) of the 52 outbreak cases having occurred from 15 to 18 of September, ate the cold plate at dinner on 14 September.

Eating muesli at any day at breakfast from 13 until 17 September revealed a RR of being a case of 1.51 (95%CI: 1.01–2.28, p=0.037).

### Microbiological and environmental investigation
One of the five kitchen workers and one of five tutors without symptoms of gastroenteritis tested positive for

S. Enteritidis PT14b Nx. All 20 environmental samples taken from the kitchen tested negative for *Salmonella*.

A laying hen holding in eastern Austria was identified as the sole source of consumable eggs for the residence. This laying hen holding comprised four business premises (A, B, C and D) dispersed across three districts. Premise C was identified to have provided eggs to a local retailer in the outbreak-province, September week 1 (on 7 September), from which the residence manager subsequently purchased the eggs for September week 2, for the re-opening after the summer break. The review of the results of the regulatory operator monitoring revealed that in premise C, one of the 12 flocks (premise C flock I, involving 18,000 laying hens) had tested positive for *S.* Infantis in June and positive for *S.* Enteritidis PT8 on 14 September. The marketing ban applied on 28 September had been continued, after *S.* Enteritidis PT8 was also found among 4,000 eggs (1/99 pools of 40 eggs was positive)

from premise C flock I. A neighbouring flock, premise C flock II, consisting of 19,500 laying hens - tested positive for *S.* Enteritidis PT19 at the beginning of December (Table 2).

As premise C was assumed to be the most likely source of the eggs for the student residence, in the relevant time period, and without knowing the egg-producing flock(s), the two flocks that had already tested positive for *Salmonella* in the previous months (premise C flocks I, II) were sampled and re-tested for *Salmonella* within the outbreak investigation end of December. The sampling method included a single sample of pooled dust and two pairs of boot swabs (cultured separately) from each flock. In premise C flock I *S.* Enteritidis PT8 was detected in two samples (one dust sample, one boot swab) and *S.* Enteritidis PT19 in one sample (one boot swab) of five (one dust sample, four boot swabs). In none of these five environmental samples was *S.* Enteritis PT14b detected. In premise C flock II, one

**TABLE 1**

Day-specific cohort analysis by food exposure, *Salmonella* outbreak, Austria, 14–21 September 2010

| Date | Day-specific cohort | Meal | Food items | Food exposed | | | Food unexposed | | | Univariable analyses | | |
|------|---------------------|------|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | | | | Cases | Total Number of exposed cohort members | Attack rate, as % | Cases | Total Number of unexposed cohort members | Attack rate, as % | Relative risk | 95% C.I. | p-value |
| Sep 12 | 144 | Dinner | Ham and cheese toast | 6 | 71 | 8 | 6 | 73 | 8 | 1.03 | 0.35–3.04 | 0.960 |
| Sep 13 | 144 | Lunch | Soup | 8 | 22 | 36 | 33 | 122 | 27 | 1.34 | 0.72–2.51 | 0.373 |
| | | | Spaghetti | 38 | 122 | 31 | 3 | 22 | 14 | 2.28 | 0.77–6.75 | 0.094 |
| | | | Green Salad | 20 | 64 | 31 | 21 | 80 | 26 | 1.19 | 0.71–2.00 | 0.509 |
| | | Dinner | Frankfurters, mustard | 34 | 108 | 31 | 7 | 36 | 19 | 1.62 | 0.79–3.33 | 0.166 |
| | | | Baked roll | 33 | 106 | 31 | 8 | 38 | 21 | 1.48 | 0.75–2.91 | 0.237 |
| Sep 14 | 144 | Lunch | Soup | 7 | 16 | 44 | 47 | 128 | 37 | 1.12 | 0.71–1.76 | 0.784 |
| | | | Breaded cutlet | 53 | 122 | 43 | 1 | 22 | 4 | 1.69 | 1.41–2.02 | 0.001 |
| | | | Potato salad | 52 | 118 | 44 | 2 | 26 | 8 | 1.65 | 1.35–2.01 | 0.001 |
| | 142 | Dinner | Cold plate | 40 | 85 | 47 | 12 | 57 | 21 | 2.24 | 1.29–3.88 | 0.002 |
| | | | Bread slices | 40 | 86 | 47 | 12 | 56 | 21 | 2.17 | 1.25–3.76 | 0.002 |
| Sep 15 | 132 | Lunch | Soup | 4 | 15 | 27 | 39 | 117 | 33 | 0.80 | 0.33–1.92 | 0.604 |
| | | | Pizza | 43 | 125 | 34 | 0 | 7 | 0 | NA | NA | 0.059 |
| | | | Green Salad | 19 | 65 | 29 | 24 | 67 | 36 | 0.82 | 0.50–1.34 | 0.419 |
| | | Dinner | Noodles with ham | 35 | 107 | 33 | 8 | 25 | 32 | 1.02 | 0.54–1.92 | 0.946 |
| | | | Salad | 16 | 49 | 33 | 27 | 83 | 33 | 1.00 | 0.60–1.67 | 0.988 |
| Sep 16 | 103 | Lunch | Soup | 3 | 17 | 18 | 14 | 86 | 16 | 1.08 | 0.35–3.37 | 0.890 |
| | | | Fish sticks | 11 | 75 | 15 | 6 | 28 | 21 | 0.68 | 0.28–1.68 | 0.411 |
| | | | Dip | 10 | 61 | 16 | 7 | 42 | 17 | 0.98 | 0.41–2.38 | 0.971 |
| | | | Vegetable rice | 12 | 64 | 19 | 5 | 39 | 13 | 1.46 | 0.56–3.84 | 0.432 |
| | | | Green Salad | 4 | 44 | 9 | 13 | 59 | 22 | 0.41 | 0.14–1.18 | 0.080 |
| | | Dinner | Fried chicken | 17 | 88 | 19 | 0 | 15 | 0 | NA | NA | 0.062 |
| | | | Bread | 14 | 64 | 22 | 3 | 39 | 8 | 2.84 | 0.87–9.27 | 0.060 |
| | | | Mixed salad | 11 | 56 | 20 | 6 | 47 | 13 | 1.54 | 0.62–3.85 | 0.349 |
| Sep 17 | 96 | Lunch | Soup | 3 | 17 | 18 | 12 | 79 | 15 | 1.16 | 0.37–3.67 | 0.800 |
| | | | Pancake | 11 | 53 | 21 | 4 | 43 | 9 | 2.23 | 0.76–6.51 | 0.124 |
| | | | Milk | 3 | 15 | 20 | 12 | 81 | 15 | 1.35 | 0.43–4.22 | 0.611 |

NA: not applicable.

dust sample tested positive for *S*. Enteritidis PT8, and one of the four boot swabs for *S*. Enteritidis PT8 and PT19, but negative for *S*. Enteritidis PT14b (Table 2). A marketing ban was imposed on the eggs from premise C flock II at the end of December 2010. The other 10 flocks of premise C were not tested within the outbreak investigation.

## Multiple-locus variable number tandem repeat analyses typing results

Six *S*. Enteritidis PT14b isolates, arbitrarily chosen from the 13 laboratory-confirmed student-outbreak cases, were further characterised by PFGE and MLVA and found to be indistinguishable from each other (MLVA-pattern: 9−6−5), but different from the four *S*. Enteritidis PT8 and the two PT19 isolates found in the environment of the premise C-flock I and flock II (PFGE patterns not shown; MLVA-results listed in Table 2). All environmental isolates were susceptible to nalidixic acid.

## Discussion

We report the first food-borne outbreak due to *S*. Enteritidis PT14b Nx documented in Austria, which occurred in 2010, after no case of *S*. Enteritidis PT14b Nx had been identified in 2009. The food-specific cohort analyses revealed eight dishes as significantly associated with infection risk. Of these, cooked frank-furters, baked rolls or refined spaghetti appeared non-plausible as sources of infection considering the mode of their preparation. After an analysis of the food-specific AR by day, potato salad and cheese-sausage cold plate remained the most plausible outbreak sources. 96 percent of the cases that occurred from the evening of 14 September, until the morning of 18 September, could be explained by eating potato salad served at lunch on 14 September and consumption of foods on the cold plate, served at dinner on 14 September

explained 77 percent of cases from 15 to 18 September. The outbreak pattern indicates a point source outbreak with 14 September as the most likely day on which common exposure occurred.

With respect to the maximum incubation period of approximately three days for *S*. Enteritidis, the 11 cases which occurred from the evening of 18 September until 21 September cannot be explained by consumption of the potato salad or cold plate. Even though the risk analysis of eating muesli any day at breakfast during the week of 13 until 17 September revealed only a weak association with the infection risk indicated by a RR of 1.51 (95%CI: 1.01−2.28, p=0.037), at least 50 of the 66 (76%) outbreak cases could also be explained by this food item. The muesli being a possible continuous common source of *Salmonella* in this outbreak is also biologically plausible, as the left-over muesli on the buffet table were returned into the same storage bowl to be served the next day. If contamination had occurred in the kitchen, where the muesli was arranged, then *Salmonella* growth would be enabled in the muesli left in un-refrigerated storage as described in documented salmonellosis outbreaks linked to breakfast cereals [13,14]. There was no muesli left for microbiological testing.

We hypothesised that eggs were the most likely source of contamination with *S*. Enteritidis PT14b Nx of the potato salad and cold plate based on the knowledge gained from the investigation of a large increase in non-travel-associated cases of *S*. Enteritidis PT14b Nx observed in 2009, in England and Wales. This upsurge in non-travel associated cases included at least 16 outbreaks and was epidemiologically and microbiologically traced back to imported Spanish eggs [5]. Breaded cutlet a traditional Viennese dish is dunked

| Flock | Date of sampling | Type of sample | Serovar Phage Type | MLVA pattern | Measures |
|---|---|---|---|---|---|
| Testing within regularly operator monitoring | | | | | |
| Flock I[a] | 29 Jun 2010 | Dust sample | *S*. Infantis | nd | Not mandatory[b] |
| Flock I[a] | 14 Sep 2010 | Boot swab | *S*. Enteritidis PT8 | PT8: 10−5−7 | From 28 Sep 2010 marketing ban on fresh eggs |
| Flock I[a] | 29 Sep 2010 | eggs (4,000) | *S*. Enteritidis PT8 | PT8: 10−5−7 | Culling of the flock in Oct 2010 |
| Flock II | 06 Dec 2010 | Boot swab | *S*. Enteritidis PT19 | nd | Measures taken in concert with the outbreak investigation |
| Testing within the outbreak investigation | | | | | |
| Flock I[a] | 20 Dec 2010 | Dust sample | *S*. Enteritidis PT8 | PT8: 10−5−7 | From 20 Dec 2010 marketing ban on fresh eggs; culling of the flocks in Jan 2011 |
| Flock I[a] | 20 Dec 2010 | Boot swab | *S*. Enteritidis PT8, PT19 | PT19: 9−5−7 | |
| Flock II | 20 Dec 2010 | Dust sample | *S*. Enteritidis PT8 | PT8: 9−5−7 | |
| Flock II | 20 Dec 2010 | Boot swab | *S*. Enteritidis PT8, PT19 | PT8: 9−5−7 PT19: 10−5−7 | |

MLVA: multiple-locus variable number tandem repeat analyses; nd: not done; PT: Phage type.

[a] Hens in Flock I were replaced after culling in Oct 2010.

[b] Mandated control measures only for *S*. Enteritidis and *S*. Typhimurium in Austria.

in flour, raw eggs and breadcrumb before being fried. The breaded cutlet was offered at lunch the day on which the potato salad and the cold plate were served. It is possible that cross-contamination occurred with *S*. Enteritidis PT14b Nx-positive eggs during pealing and cutting the boiled potatoes for the salad before adding the vinegar marinade, and during arranging the cold plate as a result of the parallel preparation of the breaded cutlets. Potato salad accompanying breaded cutlets is a well documented dish associated with outbreaks of salmonellosis in Austria [15].

A number of measures were taken to control and prevent further spreading of the outbreak. In the student residence, the kitchen was thoroughly cleaned and hand washing was reinforced on order of the school superior. Based on the assumption that eggs were the most likely source of contamination for the two risk-associated dishes, the egg-producer was traced. A large laying hen holding including four premises dispersed across two districts in eastern Austria turned out to have been the single egg-source for the student residence prior to the outbreak. The eggs consumed in the residence in September week 2 originated from the largest premise including 12 flocks. The two flocks of this premise, which had already tested positive for *S*. Enteritidis PT8 in September and for PT19 at the beginning of December, 2010 within the regulatory 15 week operator monitoring program, were re-investigated within the outbreak investigation end of December 2010 by testing a sample of 150 g pooled dust and two pairs of boot swabs per flock. This legally mandatory sampling method for epidemiologically-associated laying hen farms is in accordance to the sampling method employed once a year within the regulatory monitoring program, in addition to the 15 week sampling [9]. Even though this yearly sampling scheme is more sensitive compared to the 15 week sampling scheme (two paired boot swabs only cultured as one sample) [16], the outbreak strain *S*. Enteritidis PT14b Nx could not be detected. The isolates of *S*. Enteritidis PT8 and PT19 found in the two tested flocks of the related laying hen farm were susceptible to nalidixic acid and also distinguishable from the outbreak strain by the PFGE and MLVA pattern.

Although environmental sampling is usually the most effective way to detect *Salmonella* in poultry flocks [16,17], there are several reasons for failing to detect the outbreak strain in environmental samples from laying hen flocks despite strong epidemiological indications for causal association with a salmonellosis outbreak: (i) sampling of the wrong flock(s), (ii) sampling of the flock(s) that produced the contaminated eggs for the outbreak but at a time when the flock is no longer shedding *Salmonella*, considering that most hens stop shedding the bacteria after approximately three weeks [18,19] or (iii) a too low degree of shedding in vaccinated flocks resulting in a low within-flock prevalence, which is below the detection level of either the microbiological method or the sampling procedure [20,21]. In the outbreak described here, the two flocks of the associated laying hen premise (premise C flocks I and II), which were tested within the outbreak investigation, may not have produced the eggs for the residence before the outbreak took place. But the other ten flocks of premise C, which were not tested within the outbreak investigation, may have produced the infected eggs consumed in the residence. Another reason for not detecting the outbreak strain *S*. Enteritidis PT14b Nx in the two flocks tested, may be that the hens had already stopped shedding or the number of collected samples may be too low to detect *S*. Enteritidis PT14 Nx besides the persisting strain *S*. Enteritidis PT8 and PT19. During testing of samples, competing organisms are a limiting factor in detection. Low numbers of *Salmonella* organisms, e.g. less than 10 colony forming unit /g, can be especially difficult to identify against an overwhelming background of other dominant *Salmonella* strains [22,23]. These may include dominant phage types of *S*. Enteritidis such as PT4 and PT8 or some live vaccine strains.

Our findings that microbiological tests failed to isolate the outbreak causative pathogen from a laying hen holding despite an epidemiological link to human salmonellosis cases could suggest that the currently mandated sampling plan is not sensitive enough. It is important to recommend a more sensitive sampling plan for epidemiologically-associated laying hen flocks compared to the sampling method employed in the regulatory operator monitoring program [24], given the mandatory vaccination of laying hens against *S*. Enteritidis in Austria since 2008 [9]. Vaccination reduces the risk of *Salmonella*-positive eggs, but also hampers the likelihood of detecting infected flocks as a result of lowering of the within-flock prevalence and the number of organisms shed in faeces [25-28]. Arnold et al. [16] compared three different sampling methods with respect to the sensitivity for detecting infected flocks: method I involving 10 dust and 10 faecal samples per flock, method II according to the EU baseline study on the prevalence of *Salmonella* in laying flocks, which includes two dust samples à 250 ml and five paired boot swabs (each boot swab pair represents one fifth of the flock) [29] and method III involving single samples of pooled faeces and dust (i.e. 2 specimens per flock), which is according to the monitoring method in the National Control Program across Europe. Method I was most sensitive with a 98% power to detect 0.1% prevalence. These findings indicate that culturing several samples as indicated in method I, is more sensitive in detecting infected flocks than testing one single sample representing a large proportion of the flock. As a result of the present study, the Austrian AGES advocates the stringent methodology employed in the EU baseline survey (two dust samples and five paired boot swabs per flock) as sampling procedure for an outbreak-related laying hen farm including all flocks of a laying hen farm. However, in Austria the legally mandated sampling method for epidemiologically traced laying hen farms

involves only one pooled dust sample and two paired boot swabs per flocks.

With respect to the described risk factors for failure to detect an outbreak strain, a suspected laying hen holding should not be excluded as potential reservoir of a food-borne outbreak when there is reasonable epidemiological evidence for it.

Guidance from EFSA on the appropriate sampling method for epidemiologically traced laying hen flocks in food-borne outbreaks, which guarantees sufficient sensitivity for detecting infected flocks, is highly required to improve the quality of investigation of food-borne outbreaks in Member States.

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