should therefore be given to identifying target groups and to find appropriate ways to reach them by additional immunisation initiatives. This includes assessment of vaccination coverage at an earlier age.

Generally, coverage of the second dose of measles vaccine still needs to be improved at all local, regional and nationwide levels.

The outbreaks provide evidence that, despite the decline in measles incidence in Germany due to increased vaccination coverage and improved measles surveillance in recent years, the potential for local outbreaks is still present, and measles control and vaccination awareness should be continued and improved at all levels.

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ORIGINAL ARTICLES

Outbreak report

A REGIONAL OUTBREAK OF *S.* TYPHIMURIUM IN DENMARK AND IDENTIFICATION OF THE SOURCE USING MLVA TYPING

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In Denmark, as part of the national laboratory-based surveillance system of human enteric infections, all *S.* Typhimurium isolates are currently sub-typed using phage typing, antibiogram typing, and pulsed-field gel electrophoresis (PFGE). However, the discriminatory ability of PFGE is not always high enough to discriminate within certain phage types, and it is not always possible to separate unrelated and related isolates. We have therefore applied multiple locus variable number of tandem repeats analysis (MLVA) for surveillance typing of *S.* Typhimurium since 2004. In May and June 2005, an outbreak with 26 cases of *S.* Typhimurium infection was identified by MLVA. The isolates were fully sensitive and had one of the most frequently occurring Danish phage types (DT12) and PFGE types. *S.* Typhimurium DT12 isolates from routine surveillance of animals and food were typed using MLVA and PFGE for comparison with the human isolates. The typing results revealed that an isolate from a pig herd and its corresponding slaughterhouse located in the same geographic region as the outbreak had the same PFGE and MLVA type as the human isolates. In contrast, all other DT12 isolates investigated, which had the same PFGE profile, had different MLVA types. The conclusion that the pig herd was the source of the human infections was supported by patient information, and pork from the herd stopped entering the market on 29 June. MLVA may contribute significantly to both surveillance and outbreak investigations of *S.* Typhimurium, as without MLVA typing this outbreak would not have been found nor its origin traced.

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Introduction

In Denmark there is a large and coordinated surveillance of salmonella infections in food-production animals. *Salmonella enterica* subspecies *enterica* serotype Typhimurium (*S.* Typhimurium) is the second most frequent serotype causing infections in humans after *S.* Enteritidis [1].

Typing is an important tool for surveillance as well as for investigating outbreaks of human *S.* Typhimurium infections, and as part of surveillance in Denmark, all *S.* Typhimurium isolates are routinely typed for resistance, phage, and pulsed-field gel electrophoresis (PFGE). PFGE has been shown to be useful in investigations of *S.* Typhimurium outbreaks [2,3] and is widely used in local, national and international surveillance [1,4,5]. Unfortunately the discriminatory ability of both PFGE and phage typing is not always high enough within *S.* Typhimurium when trying to link outbreak isolates. The discriminatory ability of PFGE is particularly low within DT12 and DT104 (two of the most frequent phage types in Denmark) where 80%-90% of all human infections are caused by the same PFGE type. Multiple locus variable number of tandem repeats analysis (MLVA) is a new and promising typing method [6] that has been shown to have good discriminatory power within

S. Typhimurium and within the uniform phage type DT104 [7]. We have therefore begun using MLVA for routine surveillance of human *S.* Typhimurium infections.

An outbreak was discovered based on an increased level of a specific MLVA type between 8 May and 23 June 2005. The outbreak included 26 case-patients, four were children under 5 years, three were adults over 70 years, 15 were females, and one case died. Sixteen of the patients lived in the same county.

Methods

Bacterial isolates and phenotypic characterisation

Isolates were cultured and serotyped using antisera from Statens Serum Institut in accordance with the Kaufman-White scheme [8]. *S.* Typhimurium isolates were further phage typed at the Danish Institute for Food and Veterinary Research in accordance with international standards [9].

PFGE procedure

Isolates were grown overnight on blood plates and PFGE was performed using the PulseNet USA protocol developed for salmonella [5]. The gels were analysed and interpreted using BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). All bands between 33 and 1135 Kb were included in the interpretation of PFGE patterns and isolates differing at one band were assigned a new PFGE type.

MLVA

MLVA was performed using the same primers and a modified version of the method previously described [6]. Isolates were grown overnight on blood plates and a small loophole of cells was taken directly into the PCR mix. PCR was performed using a multiplex kit from Qiagen (Hilden, Germany) in a total of 25 _l and including 2.50 pmol of each of the primers STTR3-F, STTR3-R, STTR6-F and STTR6-R and 1.25 pmol of each of the primers STTR5-F, STTR5-R, STTR9-F, STTR9-R, STTR10pl-F and STTR10pl-R. Amplification was performed using a GeneAmp9700 (Applied Biosystems, Foster City, USA), starting with 15 min at 94ºC, followed by 25 cycles of 30 s at 94ºC, 1 min at 60ºC and 1.5 min at 72 ºC and ending with an extension step for 10 min at 72ºC. Fragment sizes for all loci were imported to BioNumerics 4.0 and allele numbers were assigned for each strain. Unique allelic combinations were assigned a new MLVA type.

Case definition and case-control study

Cases were defined as *S.* Typhimurium positive with a distinct MLVA type with onset of disease prior to the intervention at 29 June 2005. Based on initial hypothesis-generating patient interviews a case-control study was conducted, beginning on 21 June. Controls were selected from the Danish population register, matched by municipality, sex, and week of birth. Participants were interviewed by phone using a questionnaire focusing on consumption of a number of varieties of pork and beef, besides other types of meat, fruit, vegetables, places where food was bought, and other exposures.

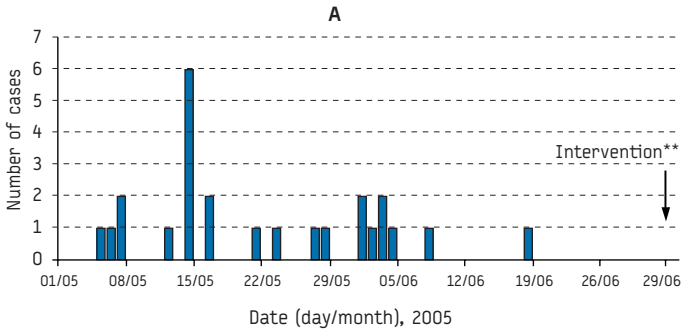
Results

Since June 2004, MLVA typing has been used for routine surveillance of human *S.* Typhimurium infections in Denmark. In the beginning of June 2005, a cluster of isolates with the same MLVA type (JPX.0216.DK) was found. The isolates were phage typed to DT12 and all isolates also had identical PFGE types. During the time of the outbreak 26 isolates with this particular MLVA type were found in humans over a period of seven weeks [FIGURE 1B]. Figure 2 shows the distribution of human MLVA types within DT12 isolates with the most frequently seen PFGE type (PFGE22) of all Danish human isolates from June 2004 to June 2005. Most MLVA types contained between one and three isolates and only three major clusters of MLVA types, JPX.0216.DK, JPX.0052.DK and JPX.0056.DK were found within the period. Two of the MLVA types, JPX.0052.DK and JPX.0056.DK resulted in human outbreaks in the summer of 2004 and the new cluster, JPX.0216.DK, therefore also seemed to be caused by a common source [FIGURE 2].

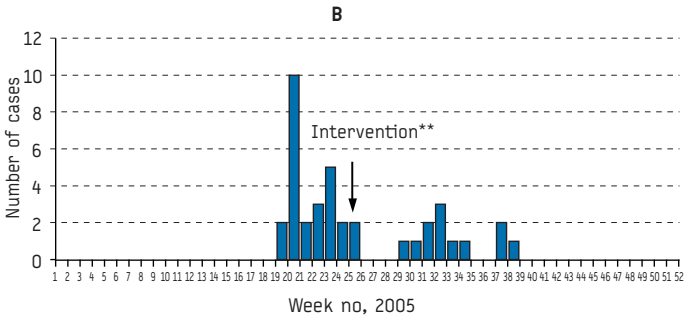
FIGURE 1

S. Typhimurium infections with the epitype, Denmark, 2005

1A: *S.* Typhimurium outbreak cases by the date of onset of symptoms (n=26*)



1B: All human cases with the epitype in Denmark in 2005, by week of sample receipt at the laboratory

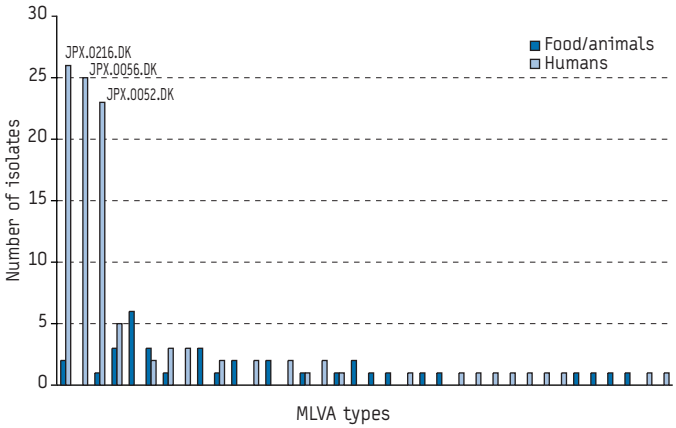


* For one case, date of onset was not available

** The arrows shown in both panels point to the date when pigs from contaminated herd were withdrawn from the market and a press release made public

FIGURE 2

Distribution of MLVA types within *S.* Typhimurium DT12 isolates with the most common PFGE type, Denmark



Note: Distribution of MLVA types in human isolates that were typed as part of national surveillance from June 2004- June 2005. Distribution of MLVA types in food and animal isolates that were typed as part of the outbreak investigation. MLVA types are shown for three clusters containing an increased number of human isolates

Geographical assessment of the cases showed that the majority lived within the same region of the county of Funen and the investigation focused on a local source. The regional veterinary and food control authorities were notified and a local slaughterhouse, from which a sample positive for DT12 had recently been obtained, was identified. Pigs from a local pig herd with a history of clinical illness were slaughtered on the same day that the isolate was found positive for DT12, and isolates from both the slaughterhouse and the pig herd were typed with MLVA. To get an idea of the diversity of MLVA types from different animal and food sources, 13 other isolates originating mostly from pork sampled at slaughterhouses during the outbreak period were included in the analyses. Furthermore, 21 isolates that

had previously been typed were also included. The distribution of MLVA types within DT12 isolates with the most frequently seen PFGE type from animal and food sources is shown in figure 2. From the total number of 36 food and animal isolates, only two were found to have an MLVA type identical to the outbreak type, namely the isolate from the abovementioned local pig herd and the isolate from the slaughterhouse where pigs from this herd had been slaughtered The diversity within the rest of the isolates was high and the isolates were separated into 20 different MLVA types (Figure 2). An isolate from the sow herd that delivered pigs to the local pig herd was also included in the investigation and the isolate differed from the outbreak type by one PFGE band, however the MLVA profile was identical.

Concomitant with the microbiological investigation, a case-control study was conducted. It comprised 21 patients and 82 controls. No specific type of food, nor any shop or supermarket was particularly prevalent among cases or found to be associated with disease in matched or unmatched analyses. However, 19 patients reported possible consumption of pork prior to falling ill and 20 patients reported consumption of beef. Almost all cases appeared to have been infected locally.

After the discovery, on 29 June, that the specific herd was the suspected source of the outbreak, pork from this herd was taken off the market and a press statement was released by the Danish Veterinary and Food Administration. No further patients were identified during a three week period following this intervention, but this was followed by a second cluster of nine patients in a six week period (Figure 1B), the majority of whom also lived in the same geographical region. Patient interviews indicated that these patients were not infected via the pig herd that had been identified, and a continued investigation by typing was undertaken under the hypothesis that these cases were also infected via locally produced pork. Another 17 animal and food isolates were PFGE and MLVA typed in order to investigate the distribution of the animal and food isolates from this later period. One isolate from a different pig herd in the same region was found to have the outbreak profile. This pig herd had received pigs from the same sow herd as the original infected pig herd, but the pork originating from this pig herd was distributed nationwide and not just locally.

Discussion

In 2004, DT12 was the most common phage type within *S.* Typhimurium accounting for 18% of human *S.* Typhimurium infections in Denmark [1]. PFGE has been used for surveillance of *S.* Typhimurium isolates and several clusters of PFGE types as well as tracking of common source outbreaks have successfully been done. Unfortunately, discrimination within DT12 and therefore cluster detection is difficult with PFGE; in Denmark we find that 80% of all DT12 isolates have the same PFGE type. MLVA [6] is currently used for routine surveillance of human *S.* Typhimurium infections in Norway and has been shown useful in outbreak situations [10]. We therefore started using MLVA for routine surveillance of human *S.* Typhimurium infections.

An outbreak including 26 patients with *S.* Typhimurium DT12 was detected by MLVA. The majority of patients lived in a confined geographic region. Isolates from a local pig herd and a local slaughterhouse were also typed and had the same PFGE and MLVA types. PFGE and MLVA typing of other food and animal isolates revealed a high diversity of MLVA types within DT12, whereas all isolates were assigned to the same PFGE type. On this basis, it was concluded that the increase of human infections was caused by pork that originated from a local pig herd processed at the local slaughterhouse. The case-control study was inconclusive, but patient interviews support the conclusion reached by the typing. We suspect that the contaminated pork was used to make a large number of different pork- products, giving the case-control study insufficient power. Eating pork is a very common exposure in Denmark. A second cluster of human isolates with the same PFGE and MLVA type was found three weeks after intervention. It is possible that the continued occurrence of the outbreak type was due to other pig herds receiving pigs from the sow-herd where Salmonella with a different PFGE profile but a identical MLVA profile was isolated. This would allow further spread of the outbreak type, although on a smaller scale.

The increase of human *S.* Typhimurium isolates might possibly have been discovered using phage typing and PFGE typing, but neither of the two typing methods would have been useful for separating outbreak related and non-related human cases or tracking the source of the outbreak and thus MLVA was the best method for the current outbreak investigation. There were several other advantages of MLVA for routine surveillance when compared with PFGE. Data were acquired faster and at a lower cost and MLVA data were also easier to analyse and interpret. The standardisation of MLVA makes it possible to exchange data between laboratories and we routinely exchange data between Denmark and Norway either as fragment sizes or allelic combinations. We also found that MLVA is a highly discriminatory method and we were clearly able to discriminate between DT12 isolates with the most common PFGE type [FIGURE 1]. In conclusion, we found that MLVA is a highly useful method for surveillance and outbreak investigations of *S.* Typhimurium.

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