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MULTILOCUS VARIABLE NUMBER OF TANDEM REPEATS ANALYSIS (MLVA) - A RELIABLE TOOL FOR RAPID INVESTIGATION OF SALMONELLA TYPHIMURIUM OUTBREAKS

M Heck (Max.Heck@rivm.nl)¹

1. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM), Bilthoven, the Netherlands

Salmonella enterica subsp. *enterica* serovar Typhimurium is a frequently occurring foodborne pathogen which causes many sporadic cases worldwide and is frequently the responsible agent in outbreaks of gastroenteritis. In elucidating outbreaks involving consumption of contaminated food, source tracing in a timely manner is imperative. Furthermore, it is important for risk managers to be able to accurately attribute sporadic cases to specific animal host species and to understand transmission routes of *S. Typhimurium*. Epidemiological meaningful subdivision of this serotype is therefore indispensable.

Phage-typing and pulsed field gel electrophoresis (PFGE) are among the methods most frequently applied. Both have been used successfully but have the disadvantage that reading the typing results is difficult to standardise, which hampers the exchange of typing results between laboratories and the construction of international reference databases. Source tracing or attribution using these methods fails when a frequently occurring important phagetype like DT104 (or PT 4 within *Salmonella* Enteritidis) that may have different sources, cannot be further subdivided.

Unambiguous typing results are critical in both detecting outbreaks and determining their source. Multilocus variable number of tandem repeats analysis (MLVA) is a PCR-based method that has recently become a widely used highly discriminatory molecular method for typing *S. Typhimurium*. It is based on amplification and fragment analysis of five repeat loci. MLVA has the advantages of typing methods based on PCR (low cost, short time, and easy to perform) that are independent of equipment and yield unambiguous typing data. For the latter purpose, the authors of the article published in today's issue of Eurosurveillance [1] developed a set of reference strains that can be used for easy normalisation of fragment sizes in each laboratory. According to the authors MLVA turned out to have a discriminatory power similar to that of phage typing and PFGE. Their results suggest that MLVA are reliable in epidemiological studies, including analyses of outbreaks and transmission routes. The authors propose a simple and definitive universal nomenclature based on the fragment size of tandem repeat loci allowing the comparison of MLVA profiles between laboratories. A further advantage of this nomenclature is that it allows easy recognition of related but slightly different MLVA-profiles that may be epidemiologically linked.

We strongly believe that molecular typing is the way forward and MLVA is a step in that direction. Nevertheless, at present it cannot fully replace the older typing techniques irrespective of all its advantages. Still faster methods are necessary for timely intervention both in outbreaks and during quality control along the food chain. Furthermore, epidemiological significance of related strains would be greater if molecular methods more fully exploited the phylogenetic information in the DNA of *Salmonella*.

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OUTBREAK OF HEPATITIS A AMONG MEN WHO HAVE SEX WITH MEN IN BARCELONA, SPAIN, SEPTEMBER 2008 – MARCH 2009

C Tortajada (ctortaja@aspb.cat)¹, P G de Olalla¹, R M Pinto², A Bosch², J Caylà¹

1. Public Health Agency of Barcelona, Barcelona, Spain

2. Enteric Virus Laboratory of the Department of Microbiology of the University of Barcelona, Barcelona, Spain

Between 1 September 2008 and 9 March 2009, 150 cases of hepatitis A were reported in Barcelona, representing a threefold increase compared with the same period in the previous two years. The majority of the cases occurred in adult men, including 87 who reported having sex with men. This indicated the possibility of an outbreak ongoing in the population of men who have sex with men (MSM) and emphasised the need to target this community with more effective vaccination programmes.

Introduction

In Spain, hepatitis A is a reportable disease defined by acute hepatitis symptoms combined with the presence of immunoglobulin M antibodies to hepatitis A virus (IgM anti-HAV) [1]. Physicians and laboratories report cases to the local public health agencies. The Public Health Agency of Barcelona is the relevant office for the city of Barcelona, covering a population of 1,600,000 inhabitants. The Health Department of the Government of Catalonia collects cases from all the regional agencies of Catalonia and reports them to the National Centre of Epidemiology in Madrid.

Since September 2008, an increase in the number of reported cases of hepatitis A in the municipality of Barcelona has been observed. Between 1 September 2008 and 9 March 2009, a total of 150 confirmed cases of hepatitis A were reported from the area. In the same period in 2006-7 and 2007-8 the numbers of notified cases were 54 and 55 respectively.

The notification data indicated that the increase may affect predominantly men who have sex with men (MSM). An outbreak alert was raised after five cases had been notified in one day, including four men aged 23-25 years of whom three were known to be MSM. For comparison, in the previous two years, the average number of notifications ranged from 0 to 12 cases per month. This prompted us to undertake a survey among the reported adult male cases, to determine whether they belonged to the group of MSM and whether they engaged in activities associated with an increased risk of hepatitis A infection [2-5].

The outbreak is still ongoing and notifications occur at a frequency of one case per day.

Methods

For the purpose of the outbreak investigation, a case was defined as a man over 18 years old who had sex with men, was resident in Barcelona city and had symptoms of acute hepatitis with onset from 1 September 2008 and positive result of IgM anti-HAV test.

To identify cases according to the above definition, all reported hepatitis A patients who were male and older than 18 years, resident in Barcelona city and had symptoms onset from September 2008 were interviewed with a modified questionnaire based on the standard questionnaire for hepatitis A of the Health Department of the Government of Catalonia but with additional questions on sexual behaviour. The interviews were done by telephone or e-mail. Cases that had been reported before the outbreak alert but could fulfill the case definition criteria were re-interviewed retrospectively, using the modified questionnaire.

Questions included having sex with men, number of sexual partners, visiting bathhouses, bars and discos, use of the internet to look for sexual partners, having group sex, and working as sex worker during the two months before symptoms onset, as well as hepatitis A immunisation status and infection with human immunodeficiency virus (HIV).

Contact-tracing was performed according to standard procedures, as done routinely by the local Public Health Agency for every case of hepatitis A reported. During the interview, the patient is asked to identify close contacts. These people are then contacted directly by the Agency and informed about the risk of infection and offered vaccination or postexposure prophylaxis. Vaccination and immunoglobuline is provided free of charge in the Agency offices or, in some cases, administered by healthcare workers visiting the contacts.

Sera from 14 cases who fulfilled the case definition were sent to the Enteric Virus Laboratory of the Department of Microbiology of the University of Barcelona for genetic analysis.

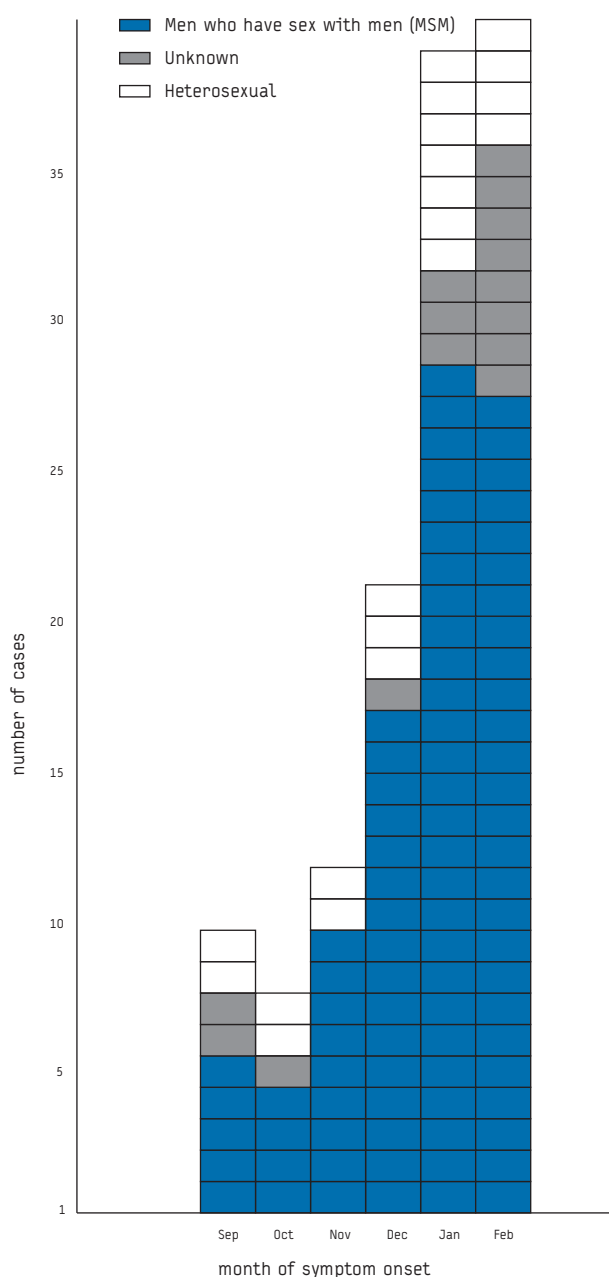
Results

From 1 September 2008 to 9 March 2009, a total of 150 laboratory-confirmed hepatitis A cases were reported. Of the 150 cases, 137 (91%) were older than 18 years, and of these, 126

(84% of the total) were men and 11 (7% of the total) were women. In the equivalent period in 2006-7, of the 54 hepatitis A cases reported, 29 (54%) were older than 18 years, including 21 (39%) men. Similarly, in 2007-8, there were 55 cases in total, 24 (43%) of whom were over 18 years old, including 13 (23%) men.

FIGURE

Number of cases of hepatitis A among men older than 18 years, by month of onset of symptoms and sexual behaviour, Barcelona, 1 September 2008 - 9 March 2009 (n=122, preliminary data)



Source of data: Public Health Agency of Barcelona, Spain

Of the 126 adult male patients, 107 were interviewed using the modified questionnaire. In response, 87 (69%) declared to have had sex with men and 20 (16%) defined themselves as heterosexual. For the remaining 19 notified cases (15%) this information was not available (Figure).

As a result, 87 persons fulfilled the case definition criteria. The median age of these cases was 33 (IC 95%: 31-34) years. Ten (11%) were HIV-positive. Only one had been vaccinated against hepatitis A and another one had received only one dose of the vaccine.

A considerable proportion of MSM cases reported engaging in activities that may be associated with increased risk of infection. The mean number of sexual partners was four (IC 95%: 3-6), 14 cases (16%) used the internet to look for sexual partners, 26 (30%) frequented discos or bars and 19 (22%) visited bathhouses.

The virological analysis showed HAV genotype IA in sera obtained from 14 patients. The results of phylogenetic analysis are not available yet.

Control measures

Vaccination against hepatitis A of all cases' contacts and postexposure prophylaxis of close contacts and sexual contacts within 15 days of the last exposure has been recommended. Vaccination and immunoglobuline is offered free of charge in the Public Health Agency of Barcelona.

We performed contact-tracing and offered vaccination and immunoglobuline to those identified. In cases when patients did not have or did not want to give this information (address or telephone), we advised them to inform their partners and close contacts to get the vaccination or immunoglobuline.

In addition, we have also strengthened the existing recommendations for vaccination of MSM by distributing fliers and posters in collaboration with the Spanish "Coordinadora Gai-Lesbiana" a federation which coordinates the activity of gay non-governmental organisations (NGO) and other associations.

The vaccination program for hepatitis A and B in gay bathhouses, which has been in place in Barcelona since 2004, has been reinforced, as well, by increasing the number of visits of healthcare workers and by covering more establishments.

To raise awareness about the possible outbreak, e-mail alerts were sent to microbiology laboratories, local practitioners and hospitals to enhance notification.

Gay organisations were informed about the hepatitis A outbreak affecting MSM, and information about the outbreak was published on some gay websites.

Discussion

An increase in the number of reported hepatitis A cases in Barcelona has been observed since September 2008. Of the 150 cases reported between 1 September 2008 and 9 March 2009, 87 were identified as MSM.

An increase in the number of notifications has recently been observed in other regions of Spain, as well. The data available are from the period between week 36 of 2008 and week 4 of

2009. Andalusia has reported an increase from 175 and 125 cases for that period in 2006-7 and 2007-8, respectively, to 350 in 2008-9; Madrid has reported an increase from 95 and 75 to 230 and Castilla – La Mancha has registered an increase from 15 and 20 cases to 60 [6]. It is not clear whether these increases are due to outbreaks and whether they affect a particular risk group but investigations are ongoing.

In Spain vaccination for hepatitis A is not included in the routine immunisation schedule, but is recommended for certain risk groups, including MSM [7].

In recent years, 2002-3 and 2004, two outbreaks of hepatitis A among MSM, affecting 48 and 60 people respectively, were detected in Barcelona. Most of them (80%) were bathhouse users [data from the Public Health Agency of Barcelona, not published]. Similar venues have also been associated with hepatitis A outbreaks elsewhere in Europe [2-5]. The strain identified in the current outbreak is different from the one detected in the MSM outbreaks in 2002-3 and 2004.

Since 2004 a special vaccination programme for hepatitis A and B has been targeted at those who frequent gay bathhouses. Healthcare workers from the Public Health Agency of Barcelona visit these venues and offer information about hepatitis A, B, C and sexually transmitted infections (STI), perform rapid tests for HIV and administer vaccinations for hepatitis A and B. To date, 3,000 bathhouse guests have used this opportunity [data from the Public Health Agency of Barcelona, unpublished].

The scenario in the present outbreak seems to be different from the previous two outbreaks since only 22% of the cases identified as MSM were bathhouse users.

Interventions aimed at the sexual contacts of the cases were difficult to carry out since in a considerable proportion of the cases the partners could not be identified in the course of contact-tracing process.

All but two cases among MSM were unvaccinated. Vaccination of MSM could help to control this outbreak and is crucial in preventing future ones. Thus information campaigns and immunisation programmes which effectively reach the MSM community are needed.

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DEVELOPMENT OF A NEW NOMENCLATURE FOR *SALMONELLA* TYPHIMURIUM MULTILOCUS VARIABLE NUMBER OF TANDEM REPEATS ANALYSIS (MLVA)

J T Larsson¹, M Torpdahl¹, R F Petersen¹, G Sørensen², B A Lindstedt³, E M Nielsen (emn@ssi.dk)¹

1. Statens Serum Institut, Copenhagen, Denmark

2. National Food Institute, Technical University of Denmark, Copenhagen, Denmark

3. Norwegian Institute of Public Health, Oslo, Norway

Multilocus variable number of tandem repeats analysis (MLVA) has recently become a widely used highly discriminatory molecular method for typing of the foodborne pathogen *Salmonella* Typhimurium. This method is based on amplification and fragment size analysis of five repeat loci. To be able to easily compare MLVA results between laboratories there is a need for a simple and definitive nomenclature for MLVA profiles. Based on MLVA results for all human *S. Typhimurium* isolates in Denmark from the last five years and sequence analysis of a selection of these isolates, we propose a MLVA nomenclature that indicates the actual number of repeat units in each locus. This nomenclature is independent of the equipment used for fragment analysis and, in principle, independent of the primers used. A set of reference strains is developed that can be used for easy normalisation of fragment sizes in each laboratory.

Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium is one of the most important foodborne pathogens in industrialised countries. This *Salmonella* serovar often causes foodborne outbreaks, and there is a need for highly discriminatory typing of isolates to be able to detect and investigate outbreaks. Multilocus variable number of tandem repeats analysis (MLVA), especially the method described by Lindstedt *et al.* [1], has been increasingly used for typing of human, animal and food isolates in several countries. This method has shown to provide the high discrimination necessary for surveillance and outbreak investigations of *S. Typhimurium* [2-7]. The fairly simple procedure of MLVA and the possibility of converting the results into a simple text string with discrete numbers are some of the advantages of MLVA as compared to pulsed-field gel electrophoresis (PFGE) and other typing methods based on band patterns.

Many food products are distributed internationally and are thereby posing a risk of causing foodborne disease outbreaks affecting more than one country. Several recent examples of such international foodborne outbreaks [8-11] have highlighted the need for comparability of typing results between laboratories in order to be able to perform effective case finding and source tracing.

The MLVA procedure specifically developed for *S. Typhimurium* is based on PCR amplification of five variable number of tandem

repeats (VNTR) loci followed by detection of the fragment sizes using capillary electrophoresis with an internal size standard in each sample [1]. In principle, the five fragment sizes should be easily comparable between laboratories; however, the fragment analysis is not fully comparable when using different sequencers, polymers, fluorescent labels, etc. [12]. With the precision needed for MLVA methods based on these relatively short repeat units (6 bp and up), the designation of allele numbers is therefore not as uncomplicated as first expected.

In this study, we analyse the VNTR regions of the five loci used in the widely accepted MLVA method for *S. Typhimurium* [1]. The exact fragment sizes and the actual number of repeat units of different alleles are determined by sequencing. On the basis of these results, we suggest a simple and rational nomenclature for naming of MLVA patterns. This nomenclature is independent of the equipment and materials used for fragment analysis, theoretically independent of the primers used, and in accordance with the principles agreed on by a group of scientists from European reference laboratories participating in a MLVA workshop held in Copenhagen in May 2008.

Methods

Bacterial isolates

Isolates were selected from a collection of approximately 4,000 MLVA-typed, primarily human *Salmonella* Typhimurium isolates collected at the Statens Serum Institut in Copenhagen and at the National Food Institute, Technical University of Denmark. The MLVA profiles are stored in a BioNumerics database. Eighty-one isolates were selected in order to cover most of the alleles for each locus that are registered in the database. One or more of the five VNTR loci were sequenced for these isolates.

Among these 81 isolates, 31 were selected as a set of reference strains. Together, these reference strains (Table 1) cover most of the size range reported by a number of European reference laboratories and the collection covers alleles well spread over the size range for each of the five MLVA loci.

MLVA

MLVA was performed using the same primers as previously described [1] but with a changed dye set from DS-34 to DS-30 for primer labelling. STTR9 and STTR6 were labelled with 6-FAM™,

TABLE 1

Reference strains of *Salmonella* Typhimurium sequenced at the Statens Serum Institut in Denmark (n=31)

| Strain | MLVA fragment sizes | MLVA profile |
|-----------|---------------------|----------------|
| STm-SSI01 | 198-235-342-371-490 | 6-9-13-10-211 |
| STm-SSI02 | 207-271-336-383-517 | 7-15-12-12-311 |
| STm-SSI03 | 216-247-NA-NA-490 | 8-11-NA-NA-211 |
| STm-SSI04 | 225-265-NA-NA-490 | 9-14-NA-NA-211 |
| STm-SSI05 | 171-253-330-437-517 | 3-12-11-21-311 |
| STm-SSI06 | 171-277-342-455-517 | 3-16-13-24-311 |
| STm-SSI07 | 171-295-324-NA-490 | 3-19-10-NA-211 |
| STm-SSI08 | 171-307-330-NA-490 | 3-21-11-NA-211 |
| STm-SSI09 | 162-319-396-389-523 | 2-23-22-13-212 |
| STm-SSI10 | 162-325-NA-NA-463 | 2-24-NA-NA-111 |
| STm-SSI11 | 162-337-306-359-523 | 2-26-7-8-212 |
| STm-SSI12 | 162-247-342-365-523 | 2-11-13-9-212 |
| STm-SSI13 | 171-271-348-377-517 | 3-15-14-11-311 |
| STm-SSI14 | 171-265-354-449-517 | 3-14-15-23-311 |
| STm-SSI15 | 162-253-408-359-523 | 2-12-24-8-212 |
| STm-SSI16 | 162-241-414-359-550 | 2-10-25-8-312 |
| STm-SSI17 | 171-265-438-NA-517 | 3-14-29-NA-311 |
| STm-SSI18 | 162-247-342-335-523 | 2-11-13-4-212 |
| STm-SSI19 | 162-235-336-341-523 | 2-9-12-5-212 |
| STm-SSI20 | 171-277-342-485-517 | 3-16-13-29-311 |
| STm-SSI21 | 180-235-300-359-616 | 4-9-6-8-314 |
| STm-SSI22 | 162-301-342-377-469 | 2-20-13-11-12 |
| STm-SSI23 | 162-277-318-395-484 | 2-16-9-14-310 |
| STm-SSI24 | 180-283-312-347-265 | 4-17-8-6-105 |
| STm-SSI25 | 162-253-342-347-298 | 2-12-13-6-106 |
| STm-SSI26 | 171-283-378-407-517 | 3-17-19-16-311 |
| STm-SSI27 | 189-253-312-371-436 | 5-12-8-10-11 |
| STm-SSI28 | 189-259-300-353-337 | 5-13-6-7-8 |
| STm-SSI29 | 171-223-360-497-517 | 3-7-16-31-311 |
| STm-SSI30 | 162-211-288-389-370 | 2-5-4-13-9 |
| STm-SSI31 | 171-253-306-NA-571 | 3-12-7-NA-511 |

“NA” designates a locus not present. The fragment sizes are the true size according to sequence results. The MLVA profile is based on the number of repeated units as described in Tables 3 and 4.

TABLE 2

Sequencing primers used in the study of *Salmonella* Typhimurium isolates at the Statens Serum Institut in Denmark

| | |
|------------|---------------------------------------|
| STTR9-F | 5'-AGA GGC GCT GCG ATT GAC GAT A-3' |
| STTR9-R | 5'-CAT TTT CCA CAG CGG CAG TTT TTC-3' |
| STTR5-seqF | 5'-TTA TTA TTC TGA GCA CCG C-3' |
| STTR5-seqR | 5'-TGA TAC GCT TTT GAC GTT GC-3' |
| STTR6-F | 5'-TCG GGC ATG CGT TGA AAA-3' |
| STTR6-R | 5'-CTG GTG GGG AGA ATG ACT GG-3' |
| STTR10-F | 5'-CGG GCG CGG CTG GAG TAT TTG-3' |
| STTR10-R | 5'-GAA GGG GCC GGG CAG AGA CAG C-3' |
| STTR3-seqF | 5'-GAA AAA CGC GCA AAA CTC TC-3' |
| STTR3-seqR | 5'-GCC ACT GGT TGT CCT GTT CT-3' |

STTR5 and STTR3 with HEX™ and finally STTR10 was labelled with NED™. The size marker was the same GenFlo-625 as in [1] but with a label change from TAMRA to ROX. The primers were used in a single multiplex PCR followed by detection on an ABI310 [6].

Sequencing

For sequencing of the VNTR loci, genomic DNA was isolated from bacterial isolates using the PrepMan Ultra kit (Applied Biosystems). For sequencing of STTR3 and STTR5, new primers were designed to include a larger part of the flanking region than what is obtained with the primers used for MLVA. The primers used for the initial PCR and for sequencing are listed in Table 2. Capillary electrophoresis was performed using an ABI3130xl (Applied Biosystems).

Data analysis

Sequencing data were imported, corrected and analysed with BioNumerics (Applied Maths NV). Sequence alignment and visual analysis of the corrected data were performed using Jalview [13].

Results

The DNA sequences of the repeat region as well as the flanking regions of the VNTR loci were determined for the 81 *S. Typhimurium* isolates selected from our collection of Danish isolates. For each locus, between 50 and 80 sequences were analysed. Sequence results confirmed that the loci STTR5, SSTR6 and STTR10 have 6-bp repeat units and that STTR9 has 9-bp repeat units. STTR3 has a combination of two repeat units measuring 27 bp and 33 bp, respectively.

For each locus, the repeated unit was determined by comparing up to 80 sequences and manually assigning the correct start and stop (Table 3). In STTR9, STTR6 and STTR10, the repeat units were identical in all strains and repeats. In STTR5 and STTR3, some ambiguity was seen in the repeat unit, and in the case of STTR5 there was also an ambiguous base in the 5' flanking region (Table 3, Figure). For these two loci, the VNTR region is located inside a coding DNA sequence, and therefore the repeat unit was also analysed on the translated level with the requirement that the repeat unit must be located in the correct reading frame. This gave a much clearer overview of where the repeat starts or stops.

The flanking regions of VNTRs contain various amounts of 'partial repeats' - bases that are the same as the first or last part of the repeat unit. If the repeat is located in non-coding regions there is no assistance to what should be the 'real' repeat. As an example, the STTR6 repeat unit could be any of gcaagg/caaggg/aagggc/agggca/gggcaa. With no help from translation the first one in sequence was consequently chosen. This approach was also taken for STTR10 and STTR9.

Discussion

There is a long tradition of international standardisation of phenotypic typing methods, e.g. serotyping. With the current shift towards molecular typing methods there is also a need for standardisation of these, and the standardisation of pulsed-field gel electrophoresis (PFGE) for foodborne pathogens by PulseNet [14,15] is a successful example of such an international standard. MLVA generates reproducible and unambiguous data and is generally a faster and cheaper method than PFGE. MLVA discriminates better than PFGE within most phagetypes of *S. Typhimurium*, especially the highly clonal phagetype DT104 [16,17]. Therefore, MLVA is

a very strong tool in outbreak investigations. MLVA methods are already in use as a supplement and sometimes a replacement of PFGE as the most important highly discriminatory typing method for foodborne pathogens. In Europe, the 5-locus MLVA for *S. Typhimurium* is widely used in public health and veterinary/food laboratories. The MLVA profile of strains related to outbreaks is commonly reported in the "urgent inquiries" sent out by the public health laboratories via the European Centre for Disease Prevention and Control (ECDC). Thus, this MLVA method has the potential to

become a new standard typing method if a clear and exchangeable nomenclature of the MLVA profiles is agreed on. To obtain this, a way of normalising raw data obtained in different laboratories should be developed and laboratories should agree on a definitive way of naming profiles.

The raw data obtained by fragment analysis by capillary electrophoresis have systematic deviations from the actual size of the fragment. This depends on the DNA composition, the

TABLE 3

Analysis of MLVA data for *Salmonella Typhimurium*. Repeat sequences and part of the flanking sequences of the variable number of tandem repeats (VNTR) regions. Formula for calculating the allele number on the basis of the fragment size.

| Locus | Length of 5' flank | 5' flank | Repeat ^b | 3' flank | Length of 3' flank | Allele number ^c |
|--------------------|--------------------|------------|---|------------|--------------------|----------------------------|
| STTR9 | 81 | TCGCRTCGTT | TGCGATGTC | TGCGGTGGAT | 63 | (X-144)/9 |
| STTR5 ^a | 40 | AAACCAYCAT | CACRAC | CATCATGGTC | 141 | (X-181)/6 |
| STTR6 | 146 | GACATCAATA | GCAAGG | GCAATCTGAG | 118 | (X-264)/6 |
| STTR10 | 193 | TAATACGCTG | CCTGTT | CATTCTGCTG | 118 | (X-311)/6 |
| STTR3 ^b | 27 | TGGCGGCGAC | 27 bp: GTYACCCRCYACGATGGCGGCAAC 33 bp: GTVRYCCVCCYGAYGATRGHGGYATGRYRAY | GACACGCCCC | 46 | See Table 4 |

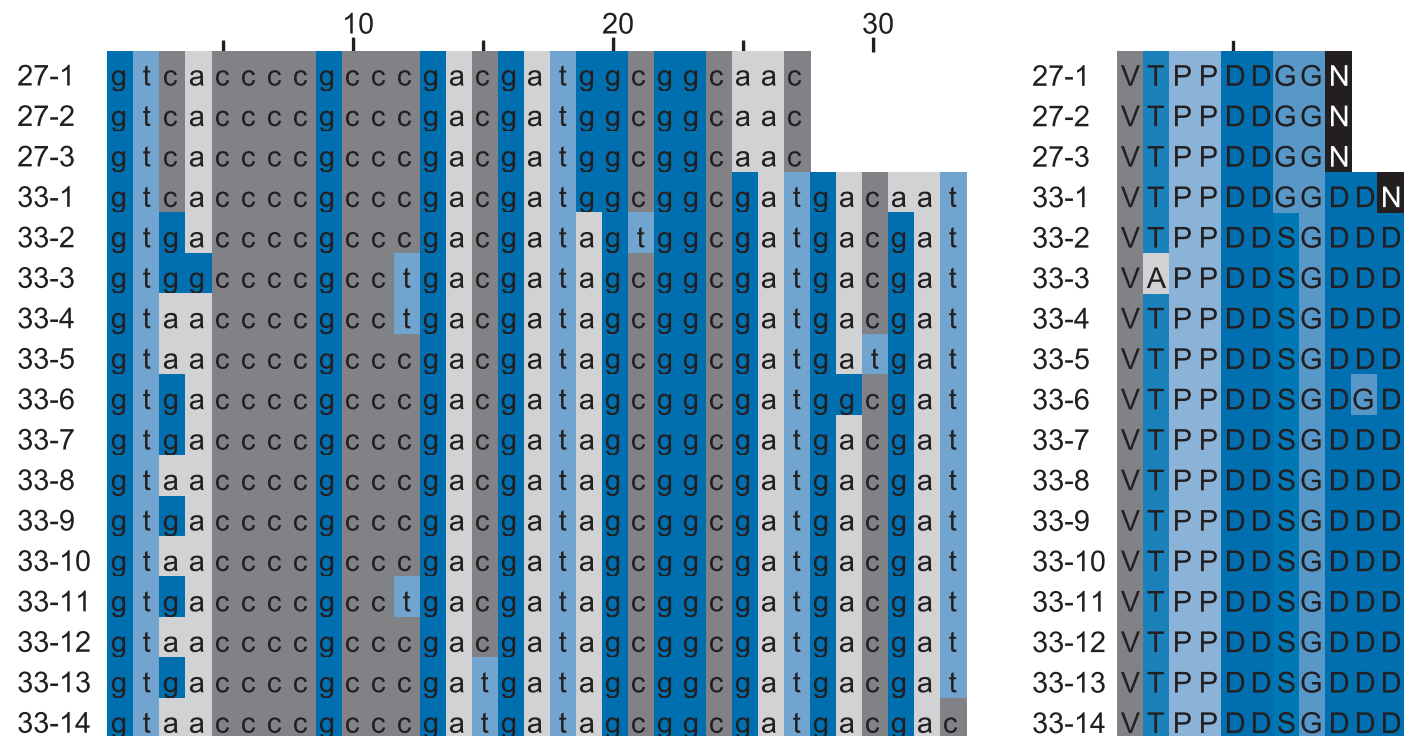
^aThe repeat unit in STTR5 has a polymorphism in the very first repeated unit; The fourth base is shifted from a G to an A in 7 of the 71 sequenced strains. In the 5' flanking region 9 of the 71 strains show a C→T transition.

^bThe two repeat sizes in STTR3 show polymorphism on the nucleotide level but much less on a functional amino acid level. See Figure.

^cX designates the real length of the analysed fragment. This is not necessary the same as the length measured from the capillary electrophoresis.

FIGURE

An example of the STTR3 locus (STm-SSI21, allele number "314"). Analysis of *Salmonella Typhimurium* isolates at the Statens Serum Institut in Denmark.



The translated sequence shows that the large majority of base exchanges are synonymous substitutions. Amino acids are coloured according to physicochemical properties. Noteworthy is the final cytosine in the last 33 bp repeat. This sequence variation is present in all the 76 sequenced strains but does not bear any functional meaning due to being a synonymous exchange.

applied instrument, polymers used, etc. Therefore, the measured fragment sizes should be normalised to the actual size to ensure the comparability between laboratories. A set of reference strains with verified fragment sizes which covers the range of the most common alleles for each locus is presented in Table 1. This set offers the possibility for each laboratory to normalise their raw data to the actual fragment sizes.

Hitherto, the naming of profiles has been based on a string of arbitrary allele numbers that do not directly reflect the numbers of repeat units in the loci [1]. Rather, the fragment sizes are binned into allele size categories and then assigned an allele number. There are several advantages of naming the MLVA profiles as the string of five numbers showing the actual number of repeat units in each of the five loci. This way, the MLVA profile can be deduced without looking it up in a table of allele numbers, e.g. maintained on a website. When comparing different MLVA profiles, the difference in number of repeat units in a specific locus can be seen directly. In particular, this is important in outbreak situations where it is relevant to assess whether isolates with deviations in the MLVA profile should be considered part of the outbreak. With a similar MLVA for *E. coli* O157, it has been suggested that loss or gain of one repeat unit is more likely to occur in epidemiologically related isolates [18]. Furthermore, this definitive nomenclature is independent of the primers used for amplification of the fragments. In principle, this means that the allele numbers obtained in laboratories that use other MLVA protocols that also include these VNTR loci will be identical. For example, PulseNet US has developed 7-locus MLVA protocol for *S. Typhimurium* that includes the five loci in the European method, but using different primers and therefore obtaining different fragment sizes [Eija Hyytia-Trees, personal communication]. However, in case of polymorphisms in the primer regions a difference in MLVA profile can be obtained, e.g. a fragment can be obtained using one primer pair whereas no product might be obtained by another primer pair (i.e. assigned as a null allele). However, this should not be of major concern as the sequence analysis of this study shows that the flanking regions are highly conserved.

For loci STTR9, STTR6 and STTR10, our definitions of the size of the flanking regions are in full agreement with those suggested by Gilbert [19]. Our analysis shows that the flanking region for STTR5 is 6 bp longer while in the case of STTR3 the flanking region is 33 bp shorter. The analysis of STTR3 sequences showed that the final nucleotide of the 33 bp repeat units were thymidine for all repeat units except for the very last repeat in each VNTR region, where a T→C transition was present (Figure). This sequence variation was seen in all the 76 sequenced STTR3 loci. This could warrant exclusion of the final 33 bp repeat, but after analysing the sequences we find that although there is an extended polymorphism in the STTR3 repeat units (an example is seen in Figure) the translated sequence is well preserved. The transition in the last repeat unit is also a synonymous mutation (Figure) and we find that this unit should be part of the VNTR region.

The suggested definition of the VNTR region in these five loci is that the region should only contain whole number of repeats. This results in a simple integer designating the number of complete repeat units in each locus. After sequencing up to 80 strains in each loci, it is clear that the flanking region is almost totally conserved (Table 3). 'Half repeats' might indeed be active in a mechanism that changes the repeat number, but from a surveillance perspective these fractions of repeats just add complexity without additional informational value.

For the VNTR loci with 6 bp and 9 bp repeat units, the proposed nomenclature is straightforward as the allele numbers can be assigned by a simple calculation based on the analysed fragment size (Table 3). Furthermore, these allele numbers can be translated into the commonly used and previously described system of arbitrary allele numbers. STTR3 pose a more complicated situation as this locus can possess both 27 bp and 33 bp repeat units. The original assignment of allele numbers came around this problem by making large bins for each allele. Thereby, different combinations of the two repeat units were assigned the same allele number (Table 4). This means a loss of discriminatory power. There are several possibilities for assigning allele numbers to the STTR3

TABLE 4

Frequent alleles in the STTR3 locus of Danish *Salmonella* Typhimurium isolates and the assignment of allele number.

| Fragment size | 27bp repeats | 33bp repeats | Allele number | Previous allele number* |
|---------------|--------------|--------------|---------------|-------------------------|
| 337 | 0 | 8 | 8 | 08 |
| 370 | 0 | 9 | 9 | 07 |
| 436 | 0 | 11 | 11 | 05 |
| 451 | 3 | 9 | 309 | 01 |
| 463 | 1 | 11 | 111 | 01 |
| 469 | 0 | 12 | 12 | 01 |
| 490 | 2 | 11 | 211 | 02 |
| 496 | 1 | 12 | 112 | 02 |
| 517 | 3 | 11 | 311 | 03 |
| 523 | 2 | 12 | 212 | 03 |
| 544 | 4 | 11 | 411 | 04 |
| 550 | 3 | 12 | 312 | 04 |
| 572 | 5 | 11 | 511 | 04 |
| 616 | 3 | 14 | 314 | Not assigned |

*According to the allele number system previously described [1].

locus that more accurately reflect the composition of the alleles seen in this locus. For example, the locus can be treated as two separate loci with 27 bp and 33 bp repeat units, respectively, so that the total MLVA type is a string of six numbers. However, this would give more weight to the STTR3 locus, e.g. when constructing dendrograms, and complicate the transition from the previously used profile assignments. Another possibility is to simply use the fragment size in basepairs as has been decided for comparison between Australian laboratories [19]. This is a simple solution, but only practical if some kind of bins are established as the accuracy of determining the fragment size is at least +/- 1 bp when using the same instrument [20]. Analysis of the fragment sizes found in around 4,000 MLVA typed isolates and the sequence analysis of STTR3 in almost 80 isolates have shown a general pattern for STTR3: STTR3 mainly consists of between 0 and 5 27-bp repeat units and between 8 and 14 33-bp repeat units.

Furthermore, not all combinations of these seem to occur. In our reference set we have included some rare variants with even fewer 33 bp repeats. These short variants make up for around 0.1% of our total *S. Typhimurium* database and should mainly be considered useful for machine calibration purposes and not for creating bins. Considering these restrictions, it is possible to predict the number of repeat units of each size based on the fragment size even if an inaccuracy of up to +/- 2 bp is allowed. For STTR3, we therefore propose that the allele number is a combination of the number of repeat units of each size, either as a four digit number, e.g. 0114 or simply 114 (1 27-bp repeat and 14 33-bp repeats) (Table 4). Omission of the leading zeros is suggested for more easy data handling using software such as BioNumerics or Excel.

Theoretically, the number of repeat units can be zero even though the VNTR locus is present, i.e. a PCR product is obtained as the flanking region is present. We have not been able to verify the presence of such alleles among our *S. Typhimurium* strains, but we have seen this for other serotypes. We propose that such alleles should be assigned 0. Additionally, it is fairly common that a PCR product is not obtained for one or more loci. The naming of such absent loci should be distinguished from loci with 0 repeats, and therefore, we suggest that these are assigned NA.

The suggested nomenclature presents a rational and scientifically based way of assigning names to MLVA profiles in a standardised manner. A collection of reference strains with MLVA fragment sizes determined by DNA sequencing offers a possibility of normalising the raw data obtained by each laboratory. A number of laboratories in Europe and North America have agreed to test this approach.

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THE EMERGENCE OF *CLOSTRIDIUM DIFFICILE* PCR RIBOTYPE 027 IN DENMARK – A POSSIBLE LINK WITH THE INCREASED CONSUMPTION OF FLUOROQUINOLONES AND CEPHALOSPORINS?

L Søes (LMS@ssi.dk)¹, K Mølbak², S Strøbæk³, K Truberg Jensen⁴, M Torpdahl¹, S Persson¹, M Kemp¹, K E Olsen¹

1. Department of Bacteriology, Mycology and Parasitology, Statens Serum Institut, Copenhagen, Denmark

2. Department of Epidemiology, Statens Serum Institut, Copenhagen, Denmark

3. Department of Infection Control, Sydvestjysk Hospital, Esbjerg, Denmark

4. Department of Clinical Microbiology, Sydvestjysk Hospital, Esbjerg, Denmark

Increasing rates of *Clostridium difficile* infection (CDI) with an unusual, severe course have been reported in several countries; this rise has partly been ascribed to the emergence of a virulent strain, *C. difficile* PCR ribotype 027 (CD027). An intriguing question is whether this could be related to increasing consumption of broadspectrum antibiotics. From 1997 to 2007, the number of hospital discharges in Denmark with the diagnosis enterocolitis caused by *C. difficile* increased from eight to 23 per 100,000 hospital discharges. This increase was proportional to a concomitant rise in the consumption of fluoroquinolones and cephalosporins. The first outbreak of CD027 in Denmark occurred from October 2006 to August 2007 and included 13 patients, most of them elderly, admitted to three hospitals in the same region. Most of the patients had overlapping periods of admission. All patients had been treated with broadspectrum antibiotics, in particular cephalosporins and fluoroquinolones, prior to positive culture of CD027. Thirty days after confirmation of diagnosis, three of the 13 patients had died. Taken together, the data support the hypothesis that the increasing use of certain broadspectrum antibiotics may be related to a possible increase of *C. difficile* infection, and show that the specific contribution by CD027 in its emergence needs to be determined.

Introduction

Infection with toxin-producing strains of *Clostridium difficile* is a common cause of diarrhoea and varies from mild to severe cases of diarrhoea. Cases are frequently antibiotic-associated and occur mostly in hospitals. Pseudomembranous colitis in already impaired patients e.g. with an underlying condition is a serious manifestation of *C. difficile* infection (CDI) and can result in death.

Reports from North America, Europe and Japan have drawn attention to a recently discovered strain of *C. difficile* that is characterised as PCR ribotype 027, toxinotype III (CD027) [1-4]. This strain has an increased pathogenic capacity, possibly a higher infectious potential and a particular resistance profile. The increased pathogenicity is thought to be associated with an enhanced production of toxin A and toxin B caused by mutations

in a regulatory gene, but the fact that this strain in addition produces a binary toxin CDT may also contribute to increased pathogenicity. This strain has caused severe outbreaks of CDI in hospital environments, but has also been described as the cause of outbreaks and sporadic cases outside hospitals [2-4].

The aims of the present report are to summarise national hospital data with a discharge diagnosis of CDI and to describe the first outbreak of CD027 in Denmark.

Methods

Because of the international emergence of CD027 and the subsequent recommendations from ECDC [5], we obtained hospital discharge data on CDI in Denmark from 1997 to 2007 and conducted a retrospective characterisation of *C. difficile* isolates from November 2006 to March 2007. In addition, Statens Serum Institut (SSI) asked Danish departments of clinical microbiology to continuously report *C. difficile* findings and to forward isolates for typing on suspicion of an outbreak or severe disease.

The hospital discharge data were obtained from the statistics of the Danish National Board of Health (<http://sundhedsdata.sst.dk>). Specifically, we obtained the annual aggregated number of discharges with the ICD10 diagnosis code DA04.7 ("enterocolitis caused by *C. difficile*", i.e. enterocolitis independent of PCR ribotype) as well as the annual number of all discharges from somatic hospitals, i.e. hospitals treating only somatic and not psychiatric diseases. Data about consumption of fluoroquinolones and cephalosporins were obtained from The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) [6].

Isolates of *C. difficile* were characterised by PCR ribotyping, toxin gene profiles, and deletion studies undertaken by the National Reference Laboratory for Enteropathogenic Bacteria at SSI.

Stool samples were cultured on cycloserine cefoxitin fructose agar (CCFA) (SSI Diagnostica, Hillerød, Denmark) in an atmosphere composed of 86% N₂, 7% H₂ and 7% CO₂ at 37°C for 48 hours.

Colonies with typical morphology and distinctive odour were identified. The colonies were analysed by 5-plex PCR directed towards *tcdA*, *tcdB*, *cdtA*, *cdtB* and 16S rDNA and by sequencing of the 5'-end of *tcdC* in order to search for premature stop codons and internal deletions [7]. PCR ribotyping was performed according to Bidet *et al.* [8].

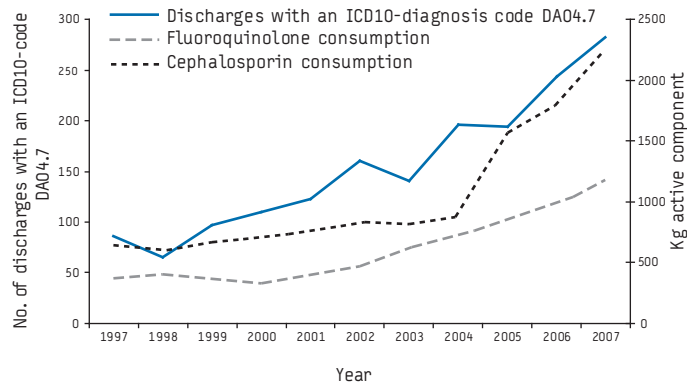
Results

Hospital discharges of CDI in Denmark

The aggregated number of discharges of enterocolitis caused by *C. difficile* increased from 86 (eight per 100,000 discharges) in 1997 to 282 (23 per 100,000 discharges) in 2007. In the same period, the consumption of fluoroquinolones and cephalosporins used in primary healthcare and hospitals taken together, increased

FIGURE 1

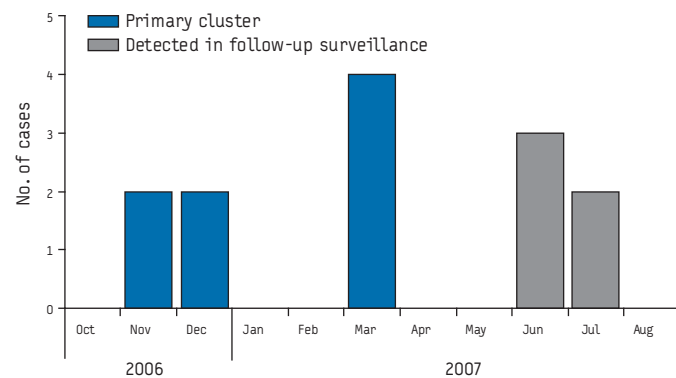
Annual number of hospital discharges with enterocolitis caused by *Clostridium difficile* (ICD10 diagnosis code DA04.7) and annual consumption of fluoroquinolones and cephalosporins for human use, Denmark, 1997-2007



Source: [6]

FIGURE 2

Number of patients with *Clostridium difficile* infection caused by CD027, Denmark, October 2006-August 2007 (n=13)



from 384 to 1,162 kg and from 626 to 2,285 kg active component per annum, respectively (see Figure 1) [6].

Detection of CD027 in Denmark

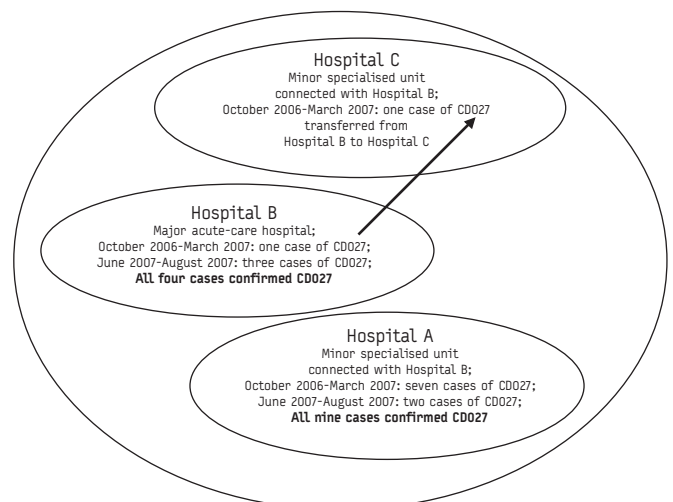
In the retrospective survey, isolates obtained between November 2006 and March 2007 were characterised; eight CD027 cases were found (Figure 2). The isolates came from eight hospitalised patients from the Region of Southern Denmark (the former Ribe County). Seven of the patients had been admitted to the same small hospital A, while the last case was a patient in another hospital in the same local area. Prompted by this cluster, active surveillance for CD027 was established in the area, and an additional 22 isolates of *C. difficile* were received between June and August 2007, of which five were CD027 (see Figure 2).

Thus, a total of 13 patients with CD027 were identified. Mean age was 79 years (age range 64 to 96 years), and 10 cases were women. The patients were admitted to hospital in the period October 2006 to July 2007. Nine of the patients were admitted to the same medical ward at the small hospital A, which consisted of only the one ward and a surgical day clinic. Most of these patients had overlapping periods of admission. The CD027-positive stool sample from one of these patients was requested by the general practitioner 13 days after the patient's discharge from hospital. The other eight were obtained during admission. The remaining four patients were admitted to three different medical departments at the larger hospital B. Two of these patients had overlapping periods of admission at the same ward. One of these four patients was moved to another medical ward at another small hospital C (see Figure 3).

The isolates were all PCR ribotype 027, carried the binary toxin gene, had an 18 bp deletion in the regulatory gene *tcdC*, and a 1 bp deletion at position 117 of *tcdC*. They were all resistant to fluoroquinolones (including moxifloxacin), but susceptible to erythromycin and clindamycin. Interestingly, at the same time and in the same geographical area, but unrelated to the outbreak

FIGURE 3

Distribution of cases of CD027 in the three hospitals, Denmark, October 2006-August 2007



another isolate was found that also carried the binary toxin gene, had the 18 bp and the 1 bp deletion at position 117 in the regulatory gene *tcdC*, but was not PCR ribotype O27. In contrast to the CD027 strains it was sensitive to moxifloxacin.

As this cluster of 13 cases was detected in a setting with ample possibilities of transmission and at the time represented the only detection of CD027 in Denmark, it is reasonable to assume that an outbreak with CD027 occurred during this period. Multilocus variable-number tandem-repeat analysis (MLVA) or restriction endonuclease analysis (REA) [9,10] will be performed in order to elucidate the connection between the isolates.

All of the 13 patients were treated with broadspectrum antibiotics prior to positive culture of CD027. Eleven patients received cephalosporins and nine fluoroquinolones; seven received both cephalosporins and fluoroquinolones, either simultaneously or consecutively. Thirty days after confirmation of diagnosis, three of the 13 patients had died. It is unknown if the deaths were directly attributable to *C. difficile*.

Discussion

It is not known with certainty why the number of patients discharged after an episode of enterocolitis caused by *C. difficile* is increasing. However, it is certain that the patients with a discharge diagnosis of ICD10 code DA04.7 only comprise a modest fraction of the true number of cases. In 2007, 1,342 culture-confirmed cases of *C. difficile* infections were reported to the national surveillance system in Denmark (25 per 100,000 population). Surveillance was established in 2007. Data before this is therefore not available. Although increased diagnostic activity and awareness may play a role, it is also likely that changes in the strains' pathogenicity are important contributing factors to the emergence of CDI. This includes the appearance of CD027 and possibly other hypervirulent strains. Several factors may be of importance to understand the emergence of *C. difficile* and in particular of CD027. The CD027 strain is resistant to the newer fluoroquinolones, including moxifloxacin, and it has been suggested that this may be the main reason for its wide dissemination [2,3]. This hypothesis is supported by the almost parallel increase in CDI discharge diagnoses and the consumption of fluoroquinolones as illustrated in Figure 1. However it should be emphasised that resistance to moxifloxacin and several other fluoroquinolones is also seen in other *C. difficile* PCR ribotypes [11,12]. Furthermore, increased use of other broadspectrum antibiotics including cephalosporins may also be related to the emergence of *C. difficile* since the same almost parallel increase is observed in CDI discharge diagnoses and consumption of cephalosporins (Figure 1).

However, these possible relations should be interpreted with caution. Other circumstances may also be of considerable importance, such as the increasing challenges in the area of hospital hygiene. For example, increased virulence of *C. difficile* resulting in pronounced diarrhoeal symptoms may have promoted spread and cross-infection within healthcare institutions, possibly because of dissemination of spores by incontinent patients [3]. The emergence of *C. difficile* and CD027 in particular is likely to be a result of environmental as well as person-to-person transmission in healthcare facilities rather than solely a result of increased antibiotic pressure. Finally, demographic changes such as an age distribution with an increasing proportion of elderly people and

changes in the patterns of hospitalisation towards increased "turn-over" of patients may also contribute.

The recognition of the outbreak of CD027 in this particular geographical area of Denmark may not be an isolated observation. The initial cluster was detected in a convenience sample of stool specimens from diarrhoeal patients as part of a project including molecular characterisation of *C. difficile* isolates. Hence, it is conceivable that the cases discovered only represent the tip of the iceberg. On a voluntary basis, strains from all different geographical areas of Denmark are now being submitted for surveillance to the National Reference Laboratory for Enteropathogenic Bacteria to identify CD027.

Although we cannot conclude a cause-and-effect relation between the increase in fluoroquinolone and cephalosporin consumption and the increase in CDI discharge diagnoses, we consider it important to present these data to stimulate additional research. Studies are needed to determine the burden of disease associated with CD027 and other hypervirulent *C. difficile* strains, while integrated public health and microbiological surveillance should be established to determine trends, detect clusters in healthcare institutions, and facilitate more focused infection control. To prevent spread, it is essential to focus on hospital hygiene and promote prudent antibiotic policies, including the limitation of unnecessary use of broadspectrum antibiotics, including fluoroquinolones and cephalosporins.

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Meeting reports

IMPACT OF IMMIGRATION ON HIV AND TUBERCULOSIS EPIDEMIOLOGY IN THE EURO-MEDITERRANEAN AREA

R EL Aouad (rajaeelaouad@yahoo.fr)¹, M Diez², I Cherkaoui¹

1. National Institute of Hygiene, Ministry of Health, Morocco

2. Secretariat of the National Plan on AIDS, Directorate of Public Health and Border Health / National Epidemiology Centre, Instituto de Salud Carlos III, Spain

The Institut National d'Hygiène (Morocco) coordinates a consortium with the Instituto de Salud Carlos III (Spain) which is part of a project called "Impact of migration on HIV and TB epidemiology in the Mediterranean area", funded by the Sixth Framework Programme for Research of the European Commission. The project was launched in May 2007 and is intended as a specific support action to improve the capacity of the countries in the Euro-Mediterranean area for obtaining quality epidemiological information on human immunodeficiency virus (HIV) and tuberculosis (TB) among migrants, while taking into consideration ethical and legal issues related to health in migrant populations. To this end, the project proposed to hold two workshops [1] to bring together all the relevant stakeholders: delegates of international and national non-governmental organisations (NGOs) concerned with the process, experts and health professionals, researchers, representatives of the United Nations Agencies and other decision makers (Ministries of Health, Interior and Justice).

Some 30 participants from Morocco, 11 participants from Spain and 17 other international participants attended the first workshop held in Rabat (Morocco) on 5-7 November 2007. It was organised around four main topics:

- 1) Demographical data on immigrant populations;
- 2) Epidemiological data and risk data analysis of HIV and tuberculosis in overall populations;
- 3) Epidemiological data and risk data analysis of HIV and tuberculosis in migrant populations;
- 4) HIV and Tuberculosis laboratory strategy and capacity.

A summary of the discussions on those topics during the Rabat meeting are provided below.

Definitions of immigration

Delphine Antoine (Institut de Veille Sanitaire, France) proposed some definitions of the "migrant" and the "international migrant" as recommended by the United Nations (UN) [2] considering several indicators such as the country of origin, the migration pattern and the living conditions. However, she underlined the difficulty to use such definitions in TB surveillance systems as seen from experiences in France, England and other parts of Europe.

Demographic data

Monserrat Lopez Cobo (Permanent Observatory of Immigration, Ministry of Labour and Social Affairs, Spain) presented the socio-

demographic characteristics of foreigners registered in the local municipalities in Spain. These may be legal migrants or not as the only requirement for registration is to provide a document proving the identity and proof of residence in the municipality in question. She underlined the increase in the numbers of registered foreigners from 542,314 in 1996 to 4,482,568 by the end of 2006 a figure referring to both the legal migrant population as well as parts of the illegal migrant population.

Aziz Jilali Sghir (Directorate of Migration, Ministry of the Interior, Morocco) underlined the important decrease in the number of migrants without documents arrested in Morocco since 2005 as a consequence of the strategy set up by the Ministry of the Interior on the institutional and legislative level.

Epidemiologic data in general population HIV/AIDS

Mercedes Diez (Secretariat of the National Plan on Acquired Immune Deficiency Syndrome (AIDS), Ministry of Health, Spain), Aziza Bennani (Directorate of Epidemiology and Diseases Control, Ministry of Health, Morocco) and further speakers gave an overview of the HIV epidemic in their countries and presented the most important statistical data: The incidence of AIDS in Spain (35 per million in 2006) is among the highest in Europe. Between 120,000 and 150,000 persons in Spain [3] and 20,000 in Morocco [4] are estimated to be living with HIV/AIDS. The sex ratio shows an excess of males for most of the countries. The main transmission mode in Spain at the beginning of the epidemic was intravenous drug use (IDU), but is currently sexual contact, both heterosexual and homosexual. Heterosexual transmission is the principal route in the southern Mediterranean countries.

Speakers from Spain, Morocco and Mauritania presented results of HIV sentinel surveillance which showed high HIV prevalence among IDU in Spain [5-8] and among sex workers in Morocco in particular in the southern region of Agadir [9].

Tuberculosis

Speakers from Spain (Elena Rodriguez, Instituto Carlos III, Ministry of Health, Spain) and Morocco (Naima Bencheikh, Directorate of Epidemiology and Diseases Control, Ministry of Health) gave detailed presentations of the epidemiological situation concerning tuberculosis: the incidence rate in 2006 was higher in Morocco compared to Spain (85 per 100,000 population versus 18 per 100,000 respectively) but both countries showed regional

differences. In Spain, multidrug-resistant (MDR) TB is more prevalent in foreigners compared to Spaniards (6.9% versus 2.4% respectively in strains sent to the National Reference Laboratory (NRL) in 2006) whereas the proportion of MDR TB in Morocco is 0.6% according to results of a 2004 national study.

Speakers from other Maghrebian countries presented the specific profiles of the situation in their respective countries. As in Tunisia the incidence declined from 48.6 in 1975 to 21 per 100,000 in 2006, the country was considered to fulfil the objectives stated by the WHO to control TB. Lo Baidi (National Public Health Research Institute, Ministry of Health, Mauritania) underlined that his country has the highest TB rate in the Maghrebian region (estimated TB prevalence rate 240 per 100,000 population). Helmi Mardassi (Pasteur Institute, Tunisia) presented the results of studies on drug resistance and genetic diversity of *M. tuberculosis* in Tunisia.

Prisoners

Mercedes Diez (Secretariat of the National Plan on AIDS, Ministry of Health, Spain) presented data on prisoners in Spain. Foreign inmates represent some 30.5% of the prison population. Jawad Amar (Prison Health Department, Ministry of Justice, Morocco) presented some epidemiological features of the penitentiary population in Morocco: the HIV prevalence rate is 10 times higher in prisoners and the rate of notified cases of TB in prisoners (580 cases per 100, 000 inmates) is also higher compared to the general population.

Epidemiologic data in migrant population

HIV/AIDS

Mercedes Diez (Secretariat of the National Plan on AIDS, Ministry of Health, Spain) showed that the percentage of foreigners in newly diagnosed AIDS/HIV cases has clearly increased in the last years, although the rise in total numbers is not marked. Hence although there has been a sharp increase in the proportion of foreigners in newly diagnosed AIDS/HIV cases the overall increase in absolute numbers is not significant. This fact is a reflection of the relevant increase of the foreign population in Spain.

In Spain, foreigners with HIV/AIDS are, as a rule, younger than Spaniards and regarding HIV they reflect the epidemiological pattern of the country of origin. Alex Carballo-Diéguez (Columbia university, HIV Centre for Clinical and Behavioural Studies, New York State Psychiatric Institute, USA) [10] underlined that the overall HIV incidence for Hispanics in the US is four times greater than in Caucasians because of high risk factors such as men having sex with men, the incidence of IDU in this community and the "air bridge" between the Caribbean and the continental US. Claudia Natali (Istituto Superiore de Sanita) showed that in Italy, foreigners accounted for 21.7% of the total AIDS cases in 2006.

Tuberculosis

Elena Rodriguez (Instituto Carlos III, Ministry of Health, Spain) underlined that of the TB cases reported in Spain in 2006, 19% of the cases were in migrants. She also highlighted differences with the Spanish population: foreigners with TB are younger than Spaniards and the rate of MDR was higher (6.9% versus 2.4% respectively in strains sent to the NRL in 2006). In Italy, foreigners accounted for 43.7% of the total TB cases in 2005.

Sociocultural aspects in migrant population

Using the results of a European survey on undocumented migrants' access to healthcare carried out by the organisation Doctors of the World ("Medicos del Mundo", in Spain, or Medecins du Monde, in the French speaking world) and the Doctors of the World European Observatory in several European countries, Ramon Esteso (Medicos del Mundo) argued that carrying out field studies will help to set up new public health programmes. Miriam Navarro (Infectious Diseases Department, Hospital Al Ramon y Cajal, Madrid, Spain) presented the results of "Knowledge, Attitudes and Practices (KAP) Survey" carried out by her unit during 2006 and 2007 on sub-Saharan people from the sub-Saharan region living in Madrid, Spain. All the speakers concluded by recommending that prevention strategies should target risk factors across multiple levels (individual, community and structural factors).

Access to healthcare for migrant populations

The organisations involved in the region were represented in the workshop: the Spanish Red Cross, Medicos del Mundo (MDM), the foundation International Medical Center for Foreign Migrants (CIMME) in Spain; (Organisation Panafricaine de Lutte contre le Sida (OPALS), Association Marocaine de Lutte Contre le Sida (ALCS), Médecins sans Frontières (MSF), and Caritas in Morocco.

HIV and Tuberculosis laboratory strategy and capacity

The speakers from three laboratories (HIV National Reference Centre, National Reference TB Laboratory and Molecular Biology Laboratory) in the National Institute of Hygiene in Morocco presented the strategy of diagnosis adopted in Morocco and the techniques used: ELISA testing for HIV screening, Western Blot test for HIV confirmation, rapid HIV testing for NGOs to be confirmed by Western Blot, real-time PCR for HIV viral load titration. For TB diagnosis, microscopic examination and histopathology are used at provincial level; TB culture is used at central and regional level; susceptibility testing is reserved for the National Reference TB Laboratory.

Conclusion and recommendations of the workshop

Demographical data on migrant populations are to be completed especially for undocumented migrants in the southern countries of the Mediterranean region. In some countries, epidemiological data and risk data analysis for HIV and TB in migrants are missing and need to be documented. Although NGOs are very active, the access for migrants to healthcare for migrants still need the support of health authorities and international organisations support. All speakers concluded by recommending that carrying out field studies will help to set up health programmes targeted at migrants.

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POLIO SITUATION WORLDWIDE IN 2008 - UPDATE ON THE PROGRESS TOWARDS GLOBAL ERADICATION

Eurosurveillance editorial team (eurossurveillance@ecdc.europa.eu)¹

1. European Centre for Disease Prevention and Control, Stockholm, Sweden

According to a recent communication in the World Health Organization (WHO) Weekly epidemiological record [1], a total of 1,655 wild polio virus (WPV) cases were reported worldwide in 2008, which represents an increase of 26% compared to 2007 when 1,315 cases were reported globally. In 2008, 91% of all polio cases occurred in the four countries where polio is still endemic: Nigeria (801 cases), India (559), Pakistan (118), Afghanistan (31). The remaining 146 cases were reported from 14 countries with cases of imported WPV. The number of WPV type-1 cases increased from 321 in 2007 to 984 in 2008, whereas the number of WPV type-3 cases decreased from 994 in 2007 to 671 in 2008.

Efforts have been made to eliminate WPV transmission since 1988 when the WHO began its global eradication campaign. Since then, the incidence of polio has decreased significantly. In 1988 there were still around 350,000 cases in 125 countries worldwide whereas in the past years global figures amounted to less than 2,000. Furthermore, no circulation of WPV type-2 has been documented since October 1999 [2].

The 26% increase in WPV transmission in 2008 compared to 2007 is mainly caused by an increase in cases in Nigeria (2007: 285; 2008: 801). During the second half of 2008, WPV1 originating from northern Nigeria spread to eight neighbouring African countries, including six that were free of polio since 2005. The most recent data available show that the global vaccination rate of infants with three doses of the trivalent oral polio vaccine (OPV3) was estimated at 82% in 2007. The vaccination rates for the four polio-endemic countries are 83% in Afghanistan and Pakistan, 62% in India, and 61% in Nigeria. Nevertheless, in some parts of the countries, OPV3 coverage was below 40%.

In response to the ongoing transmission and increasing number of cases in Nigeria and the spread of the disease to countries that had been polio-free for more than a decade such as Uganda, the International Federation of Red Cross and Red Crescent Societies (IFRC) launched on 8 April 2009 an emergency appeal to support Red Cross and Red Crescent Societies from 14 countries in Africa to respond to WPV outbreaks across the continent. The related activities will support massive immunisation campaigns currently taking place or being planned in all affected countries. More than 41 million children throughout Africa will be immunised against polio in April and 42 million children from Nigeria will be immunised in May [3].

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