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Mini Review

Genotyping of selected bacterial enteropathogens in Norway

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ABSTRACT

In Norway the Norwegian Institute of Public Health (NIPH) is the primary facility for nationwide surveillance of foodborne infections, and it is vital that we can perform rapid and high resolution identification of foodborne bacteria at the strain level. During the last decade a rapid introduction of DNA-based methods has been introduced, which show promise in enhancing the speed and discriminatory capability of the typing laboratory. The laboratory responsible for genotyping enteropathogens at NIPH is limited in staff, thus methods demanding reduced labour, high degree of automation and increased ease of interpretation is essential. We found that this could be achieved by focusing on MLVA for some of the most predominant enteropathogenic species. Bacterial genotyping is performed by several laboratories in Norway, however this review will address the use of routine genotyping by MLVA of common foodborne bacteria at NIPH. The emphasis will be on *Escherichia coli*, *Salmonella typhimurium*, *Shigella* spp. *Yersinia enterocolitica* and *Listeria monocytogenes*. This review is based on an oral presentation given at the 9th International Meeting on Microbial Epidemiological Markers in Wernigerode Germany on September 1st 2010.

Introduction

Typing systems for discriminating between bacteria from a single species have traditionally been based on phenotypic criteria, such as serotype, biotype, phage typing, or antibiogram. These methods are usually quite labour intensive, and in many cases do neither have the optimal resolution nor speed needed during an outbreak. However, based on the amount of data collected using these methods they are still widely used, and often it is the only way to compare with historical data. Prior to 1987 the typing of bacterial enteropathogens at NIPH relied solely on phenotypic methods. It was realised in the late 80s that the institute needed experience with the newer DNA-based technologies, and Dr. Kapperud was given the responsibility of introducing genotyping methods for enteropathogenic bacteria at NIPH. The earlier methods introduced were, among others, plasmid profiling, DNA-hybridization and restriction endonuclease analysis of chromosomal DNA (REAC) (Caugant et al., 1989; Kapperud et al., 1989, 1990a,b; Kapperud and Nesbakken, 1987), which were proven methods internationally at that time. These methods became a great asset for the institute and during the following years the laboratory incorporated methods such as PFGE and AFLP (Heir et al., 2000, 2002, 2004; Lindstedt et al., 2000). The laboratory has in its recent years focused on developing and implementing MLVA as well as other novel bacterial genotyping methods, which will be reviewed here.

Salmonella typhimurium genotyping

S. typhimurium has been genotyped using the MLVA method published by our laboratory in 2003 and 2004 (Lindstedt et al., 2003a, 2004a). This method has recently been improved by introducing a reference strain set for standardising runs across different CE machines and dye chemistries as well as a new nomenclature system (Larsson et al., 2009). The standardised approach makes it very easy to share genotype information between laboratories by use of online resources. From January 2009 to September 2010, a total of 489 isolates of *S. typhimurium* was MLVA genotyped by NIPH; 88 (18%) of these 489 isolates displayed a monophasic serotype. The monophasic isolates were fully typeable and grouped together with the *S. typhimurium* isolates, indicating that these isolates most likely originated from *S. typhimurium*. This view is supported by several studies (Ido et al., 2010; Laorden et al., 2010; Switt et al., 2009; Trupschuch et al., 2010). Our MLVA method displayed 170 (34.8%) of 489 isolates where all five loci were present and typeable. The majority of isolates thus display a genotype with one or more loci showing no PCR amplification (NA). The three most common genotypes during the selected period are: 3-11-9-NA-2/11 with 33 (6.7%) isolates, 3-12-9-NA-2/11 with 27 (5.5%) isolates and 3-15-NA-NA-3/11 with 20 (4.1%) isolates. The first two MLVA genotypes are associated with a multiresistant monophasic *Salmonella enterica* serovar, which has shown a marked increase in prevalence in Europe (Hopkins et al., 2010); and our data confirm that these two genotypes are the most commonly isolated also in Norway during our study period. The first two genotypes are highly related as there is only one repeat unit difference at the strongly

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polymorphic STTR5 locus. The 3-15-NA-NA-3/11 genotype is a recurrent infection every autumn almost exclusive to the western parts of Norway, and has earlier been associated with hedgehogs, and has previously been monitored by PFGE (Handeland et al., 2002; Heir et al., 2002). Excluding the above mentioned genotypes, the routine MLVA typing during this study period revealed 13 clusters of identical genotypes comprising five or more isolates. Some of these were single-locus variants (SLV) of the three most common clusters, usually showing polymorphism in the STTR5 or STTR6 locus. Other clusters with a previously investigated source were 2-11-3-NA-2/12 and its STTR5 SLV 2-10-3-NA-2/12. These two genotypes are in Norway associated with small birds as a source, and usually infect children (Heir et al., 2002; Refsum et al., 2002a,b). An interesting cluster (4-16-9-9-2/11) was ascribed to an outbreak of imported minced meat where phage typing in different countries displayed different phage types, while MLVA displayed a single genotype (Bruun et al., 2009). Thus, in this case it would have been misleading if only phage type information had been exchanged between the countries (Bruun et al., 2009).

Escherichia coli O157:H7 genotyping

E. coli O157:H7 has been genotyped using the MLVA method published by our laboratory in 2003 and 2004 (Lindstedt et al., 2003b, 2004b). *E. coli* O157:H7 is rare in Norway and from January 2009 to October 2010, a total of 21 isolates were MLVA genotyped by NIPH displaying 15 distinct genotypes. No major clusters of O157:H7 were detected during the study period.

Sorbitol-fermenting E. coli O157

Sorbitol-fermenting (SF) *E. coli* O157:H-is a non-motile variant of *E. coli* O157, which has been associated with an increased risk of contracting haemolytic-uremic syndrome (HUS) (Alpers et al., 2009; Ammon et al., 1999; Bielaszewska et al., 2007; Pollock et al., 2010; Werber et al., 2011). When SF *E. coli* O157:H-isolates are run in our *E. coli* O157:H7 MLVA assay (Lindstedt et al., 2004b) a number of NA loci are displayed. Thus, for SF *E. coli* O157:H-the O157:H7 method was not optimal, and we are in the progress of developing an MLVA assay specifically directed at SF strains by substituting the three NA loci (Vhec2, Vhec4 and Vhec7) with three new VNTR loci. From January 2009 to December 2010, a total of 42 isolates were MLVA genotyped by NIPH, using the SF-MLVA method (unpublished), displaying 13 distinct genotypes. However, six of these genotypes may possibly be considered highly related as they are SLVs at the hypervariable Vhec1 (TR2/ O157-10) locus (Lindstedt et al., 2004b; Noller et al., 2006; Vogler et al., 2006), which is retained in the SF-O157 assay. The predominant genotype includes 20 isolates and is related to an, as yet, unresolved national outbreak starting in January 2009. No source for this outbreak has at present been identified.

E. coli generic genotyping

In Norway the number of submitted pathogenic *E. coli* other than serotype O157 have been increasing, thus a genotyping method for all serotypes of *E. coli* was needed. In 2007 we published a first version of a generic *E. coli* MLVA assay (Lindstedt et al., 2007). This assay proved to be very helpful and immediately in its implementing period before publication contributed to solve a large and serious outbreak of shiga-toxin-producing *E. coli* (STEC) O103:H25 (Schimmer et al., 2008; Sekse et al., 2009). The first published version was based on a combination of 7 DNA-repeat loci with varying degrees of polymorphisms (Lindstedt et al., 2007). During the recent years we wanted to improve this method by increasing the typing resolution for several serotypes including *E. coli* O26:H11. It was found that while version-1 of the generic MLVA assay was fast and typed *E. coli* O26:H11 in correspondence with major PFGE groups (Miko et al., 2010), it still showed a reduced number of genotypes compared to PFGE for O26:H11. This has been addressed in the newly developed version 2 of the generic MLVA assay (unpublished). The new assay also displayed improved typing resolution for a number of *E. coli* serotypes other than O26:H11. The new assay employs a combination of 10 repeat loci for genotyping, and the dye colours used for marking the primers have been changed to accommodate the newer versions of CE machines from Applied Biosystems. In a sample set of 583 *E. coli* isolates typed by both versions, version 1 gave 246 distinct genotypes while version 2 produced 334 distinct genotypes (an increase of 88 genotypes) (unpublished). A total of 311 (53%) isolates presented unique (seen-once) genotypes. This is a major improvement in resolution, while the assay is still easy to perform, and the 10-loci assay will hopefully contribute to increase the speed and precision of outbreak detection and investigation when published. In the sample set, which is representative for the isolates submitted to NIPH for routine genotyping, we detected 19 clusters of five or more *E. coli* isolates. The largest two clusters each contained 13 isolates, where one was a cluster of atypical enteropathogenic *E. coli* (aEPEC) strains of unknown serotype isolated in 2010 from January to December in Norway, and the other was a cluster of O103:H2 STEC strains isolated from September 2009 until November of 2010. The 10-loci *E. coli* MLVA scheme is now the routine genotyping tool for all non-O157 *E. coli* isolates at NIPH, and all genotypes are stored digitally for use in outbreak detection, surveillance and further research as e.g. comparing MLVA genotypes with virulence profiles, pathotypes, source and site of infection. All *E. coli* isolates submitted to NIPH are additionally tested for selected virulence factors to determine their pathotype using a multiplex PCR assay (Brandal et al., 2007). When this 'pathotype PCR' was run on the MLVA sample set described here, the result was an astonishing high number of isolates which were negative for any pathotype-determining PCR profile and were thus labelled as 'non-enteropathogenic *E. coli* (NON) isolates'. This NON group comprised 247 (42%) of the 583 studied isolates, and in some cases, clustered away from the enteropathogenic *E. coli* strains (unpublished). When a small number of confirmed enteropathogenic ($n = 25$) and NON isolates ($n = 23$) were tested for additional virulence markers, it was discovered that both groups frequently contained an array of additional virulence markers (Fig. 1) (unpublished). Some of these additional factors are well known in the literature as e.g. the cytotoxic necrotizing factor 1 gene (*cnf1*) coding for the CNF1 toxin (Boquet, 1998, 2001) and the brain microvascular endothelial cells invasion gene (*ibeA*) (Che et al., 2010; Ewers et al., 2007). This confirms that *E. coli* isolates carrying virulence factors typically associated with extraintestinal pathogenic *E. coli* (ExPEC) are frequently found in *E. coli* isolates from faeces (Johnson and Russo, 2005). It has recently additionally been shown that pathogenicity-associated islands, on which the majority of ExPEC-related virulence genes are situated, are fitness elements involved in intestinal colonization (Diard et al., 2010). Thus, our observation of the massive presence of ExPEC factors in human faeces isolates makes sense.

Shigella spp. genotyping

Even though the generic *E. coli* MLVA scheme is capable of genotyping *Shigella* species (Lindstedt et al., 2007) the recent full genome sequencing of all *Shigella* species, and several publications displaying VNTR loci suitable for genotyping (Chiou et al., 2009; Gorge et al., 2008; Liang et al., 2007; Wang et al., 2009), prompted us to develop an MLVA genotyping assay specifically targeted at *Shigella* spp. The assay was published in late 2010 and used seven VNTR