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Research paper

Time to review the gold standard for genotyping vancomycin-resistant enterococci in epidemiology: Comparing whole-genome sequencing with PFGE and MLST in three suspected outbreaks in Sweden during 2013–2015

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ABSTRACT

Vancomycin-resistant enterococci (VRE) are a challenge to the health-care system regarding transmission rate and treatment of infections. VRE outbreaks have to be controlled from the first cases which means that appropriate and sensitive genotyping methods are needed.

The aim of this study was to investigate the applicability of whole genome sequencing based analysis compared to Pulsed-Field Gel Electrophoresis (PFGE) and Multi-Locus Sequence Typing (MLST) in epidemiological investigations as well as the development of a user friendly method for daily laboratory use.

Out of 14,000 VRE - screening samples, a total of 60 isolates positive for either *vanA* or *vanB* gene were isolated of which 38 were from patients with epidemiological links from three suspected outbreaks at Uppsala University Hospital. The isolates were genotypically characterised with PFGE, MLST, and WGS based core genome Average Nucleotide Identity analysis (cgANI). PFGE was compared to WGS and MLST regarding reliability, resolution, and applicability capacity.

The PFGE analysis of the 38 isolates confirmed the epidemiological investigation that three outbreaks had occurred but gave an unclear picture for the largest cluster. The WGS analysis could clearly distinguish six ANI clusters for those 38 isolates.

As result of the comparison of the investigated methods, we recommend WGS-ANI analysis for epidemiological issues with VRE. The recommended threshold for *Enterococcus faecium* VRE outbreak strain delineation with core genome based ANI is 98.5%.

All referred sequences of this study are available from the NCBI BioProject number PRJNA301929.

1. Introduction

Since 2008, Sweden has experienced four major nosocomial outbreaks of VRE of *vanB* genotype of which the largest outbreak occurred between 2013 and 2014 in Gavle County with over 300 patients involved. PFGE was the molecular method used at the Public Health Agency in Sweden for genotyping the isolates. PFGE is a stable and reproducible method and considered the "gold standard" for genotyping VRE in nosocomial outbreaks (Valdezate et al., 2009; Werner et al., 2012). PFGE is time consuming and highly qualified and experienced laboratory staff is needed for data evaluation (Tenover et al., 1995; van Belkum, 1994). The method is based on restriction of the whole bacterial genome followed by scoring the obtained size of DNA fragments (Werner, 2013). Standardisations for inter-laboratory comparisons do not exist for typing of VRE –isolates (Cookson et al., 2007). Thus, the method is applicable to compare isolates for regional surveillance in which the isolates have to be compared in one laboratory. MLST is the standard method for epidemiological investigations for large scale international comparisons (Maiden et al., 1998). MLST is not as discriminating as PFGE, however the sequence types (ST) are defined and can be exchanged between laboratories worldwide (Ruiz-Garbajosa et al., 2006). Whole Genome Sequencing (WGS) could be an alternative in the molecular epidemiological investigation of VRE (Kao et al., 2014). In addition to providing the same genetic data as MLST, many other genetic loci can be used in single nucleotide polymorphism (SNP) analysis or Core Genome MLST (cgMLST) (de Been et al., 2015).

The genetic distance between two whole genomes can be calculated by the average nucleotide identity (ANI). Results of ANI analysis correlates strongly with DNA – DNA hybridization. A value of 70% in DNA –

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DNA reassociation corresponds to 93–94% in ANI analysis and the majority of bacterial strains with an ANI >94% belongs to the same species (Konstantinidis and Tiedje, 2005). Due to the high resolution of WGS, even strains of the same species can be discriminated. WGS is therefore a suitable tool for molecular epidemiologic analysis in outbreak investigations. The increasing number of available WGS data makes it possible to assign new outbreak related genomes to existing data.

The purpose of this study was to compare PFGE and MLST with WGS- ANI regarding reliability, discriminatory power, epidemiological concordance and convenience criteria such as software based analysis, availability of databases, and comparability of the results of different laboratories for epidemiological molecular typing during outbreak investigations involving VRE-isolates. We furthermore aimed to develop an easy to use WGS-ANI workflow and to determine the cut off criteria for outbreak isolate assignment to use in a clinical microbiology laboratory setting.

2. Material and methods

2.1. Epidemiological investigation

According to the national recommendation of the Public Health Agency in Sweden, an epidemiological investigation should be carried out whenever VRE is isolated in a clinical culture from a patient admitted to a hospital or a nursing home in order to detect outbreaks at an early stage. The patient should be isolated in a single room with an en-suite bathroom and maximal contact precautions should be undertaken to prevent transmission. Active surveillance samples should be undertaken repeatedly in order to find all cases. Every patient admitted to the same ward as a patient with VRE should be screened for VRE in faeces, wounds and urine. Screening for VRE should be done once weekly and when patients are discharged from the ward for as long as there is a known VRE-positive patient present in the ward.

The infection prevention and control (IPC) team of Uppsala University Hospital (UUH) leads the epidemiological investigation in Uppsala County and recommends interventions for staff in the wards in order to prevent transmission. To investigate epidemiological links, a locally developed software for daily tracing of patients and their movements in the hospital wards and out-patient clinics were used for this study. Contacts found were sampled from faeces, wounds and urine according to the national policy.

2.2. Whole genome sequencing (WGS)

All screening samples from contacts, from active surveillance, and all clinical cultures collected in health-care settings in the county of Uppsala were sent for microbiological diagnostics to the clinical microbiological laboratory of UUH. All VRE isolates that were related to the outbreaks in 2013–2015 were cultured on Haematin agar plates and incubated overnight at 37 °C. Pure colonies were transferred to Brain Heart Infusion with a Vancomycin disc (5 μg; Oxoid) and incubated overnight at 37 °C. DNA extraction was performed from 400 μl of broth with MagNa Pure Compact Nucleic Acid isolation Kit I according to manufacturers' protocol version 12 for DNA extraction from bacteria. An Illumina HiSeq platform with a 2 × 100 paired end run was used for WGS. The paired reads and merging contigs were assembled by Geneious version 8.1.5. and the MIRA plugin 1.0.1 (Kearse et al., 2012). Only sequences with a coverage of >70 were proceeded. The core genome ANI was calculated using the Gegenees software version 2.2.1 with blast plugin. A threshold of 20% was chosen to make sure that only the core genomes were compared (Ågren et al., 2012). The result file was transferred as *.next file to SplitsTree4 version 4.13.1 (Huson and Bryant, 2006) to visualize the results as a phylogenetic tree. The workflow of WGS-ANI analysis is shown in Fig. 1.

2.3. Pulse-field gel electrophoresis (PFGE)

PFGE of all VRE-isolates was carried out at the clinical microbiological laboratory of the Public Health Agency of Sweden. The abbreviations of the PFGE clusters consisted of 5 to 6 sections in the locally developed nomenclature at the agency: SE = Sweden, Efm = *Enterococcus faecium*, the resistance gene *vanA* or *vanB*, the year when the cluster was detected for the first time, and a serial number for instance SE-EfmA-1410. A lowercase letter after the serial number (SE-EfmA-1410a) indicated that the band pattern was >90% but <97% similar to the base cluster (SE-EfmA-1410).

2.4. Multilocus sequence typing (MLST)

MLST was performed *in silico* using the WGS data. The online platform tool MLST 1.8 (Larsen et al., 2012) was used to determine the MLST types.

3. Results

3.1. Epidemiological investigation

The IPC team detected epidemiological links between 37 patients (38 isolates) in three separate outbreaks between 2013 and 2015 involving seven different wards. During 2013–2014 a total of 29 patients with *vanB* had epidemiological links and had been transferred between five wards. The 29 patients were suspected to have acquired VRE in one medical ward (15 patients), one surgical ward (three patients), one geriatric ward (six patients), one elderly home (two patients), and a second elderly home (three patients) (Table 1). During 2014 a total of five patients with *vanA* were suspected to have acquired VRE in a cardiologic ward and during 2015 a total of six patients with *vanB* were suspected to have acquired VRE in a medical ward.

3.2. Microbiological investigation

>14,000 screening samples were analysed at the clinical microbiological laboratory of UUH between 2013 and 2015 of which 10% resulted in positive gene detection for *vanA* or *vanB* gene. Since other species than *E. faecium* and *E. faecalis* may contain *vanB* genes, both the selective cultivation and phenotypic verification of the isolates had to be positive to define a sample as VRE positive. Out of all *vanA* or *vanB* positive samples 5% were characterised phenotypically as enterococci by Maldi-Tof. Out of 14,000 screening samples 49 isolates of *E. faecium* with *vanB* gene and 11 isolates of *E. faecium* with *vanA* gene were detected.

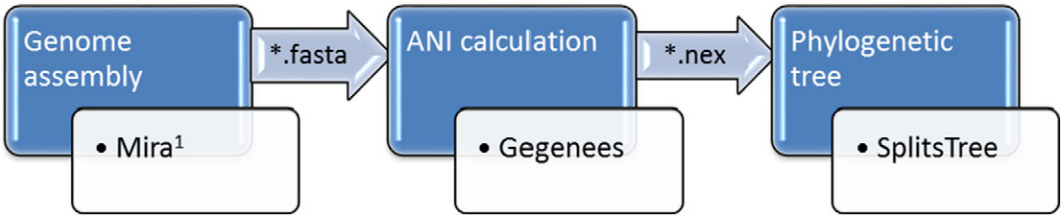

Fig. 1. Workflow for WGS-ANI determination. [1]Velvet was also tested with the same result (results not shown).