Veterinary Microbiology 177 (2015) 394–397

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Multilocus sequence typing of Australian *Streptococcus suis* type 2 by MALDI-TOF mass spectrometry analysis of PCR amplicons

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ARTICLE INFO

Article history:
Received 23 April 2014
Received in revised form 4 March 2015
Accepted 6 March 2015

Keywords:
Streptococcus suis
Pigs
Zoonosis
MLST

ABSTRACT

Streptococcus suis serotype 2 is a ubiquitous pathogen of swine and is known to cause severe disease in humans. Multilocus sequence typing (MLST) is ideal for characterising this organism because it permits isolates to be compared on a national and international scale. A novel approach to MLST using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MS-MLST) provides a more rapid alternative to dideoxy sequencing. This study used MS-MLST to define the multilocus sequence types (STs) present among a collection of Australian *S. suis* type 2, and thus, delivered a basis for comparison of Australian isolates with international strains already well characterised for virulence attributes. A collection of 45 isolates recovered from infected humans ($n = 3$) and diseased pigs ($n = 42$) was genotyped using MS-MLST and conventional MLST. Both methods were 100% concordant in their classification of sequence types (STs), although MS-MLST permitted much quicker analysis of sequence data. The collection contained ST25 ($n = 31$), ST1 ($n = 10$), ST28 ($n = 3$) and ST369 ($n = 1$). These results are consistent with the population structure of *S. suis* type 2 observed in diseased pigs and humans in Canada and the United Kingdom. MS-MLST may have utility for studying the population structure and epidemiology of *S. suis* in countries where the diversity of *S. suis* is greater and human disease is more common.

1. Introduction

Streptococcus suis serotype 2 is a prominent pathogen of swine with a ubiquitous presence in the global pig industry (Goyette-Desjardins et al., 2014). Humans that are exposed directly to infected pigs and pig carcases, or that consume contaminated pork products, can themselves become infected and develop severe and sometimes fatal disease (Goyette-Desjardins et al., 2014; Huong et al., 2014). A dramatic increase in the number of *S. suis* zoonoses has been reported globally in the last decade (Mai et al., 2008; Ye et al., 2008). Elucidating the epidemiology of this pathogen on an international scale requires assessment of *S. suis* isolates from diverse geographic origins using a typing scheme that can accurately classify isolates regardless of which laboratory performs the evaluation. A tool that eminently suits this task is multilocus sequence typing (MLST).

MLST has been widely adopted in research and disease investigation and has facilitated a quantum leap forward in the quest to define the population structure of *S. suis* serotype 2 in various countries (Fittipaldi et al., 2011; King et al., 2002; Mai et al., 2008; Schultsz et al., 2012; Ye et al.,

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http://dx.doi.org/10.1016/j.vetmic.2015.03.006
0378-1135/

2008). Notably, MLST has shown that the global population structure of *S. suis* serotype 2 is dominated by a small number of STs with greatly differing virulence capabilities (Fittipaldi et al., 2011; Schultsz et al., 2012; Ye et al., 2008). MLST thus can provide strong insight into the health risks faced by persons working in pork production and processing.

Although MLST is ideal for understanding the ecology of a pathogen across large geographic areas, application to large numbers of isolates has disadvantages. Foremost is the expense of the method which is a consequence of the onerous and time-consuming nature of analysing and interpreting dideoxy sequence data. This disadvantage can be circumvented by substituting DNA sequencing with matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) (Dunne et al., 2011; Honisch et al., 2007). MALDI-TOF MS technology streamlines the typing process by automating the analysis and interpretation of allelic data and STs (Sauer and Kliem, 2010).

A small temporal cluster of near-fatal infections due to *S. suis* serotype 2 has recently been reported in pig industry workers in Australia (Kennedy et al., 2008; Tramontana et al., 2008). Sufficient information is not available to place these isolates into an epidemiological context with strains from abroad, or to define the risks posed to public health by *S. suis* present in Australian pigs. In this study, we identified the STs present among a collection of *S. suis* type 2 recovered from infected pigs and humans in Australia, and assessed the practical utility of MALDI-TOF MS-based MLST (MS-MLST) for characterising these organisms.

2. Materials and methods

2.1. Bacterial isolates

This study focussed on a collection of 45 *S. suis* type 2 isolates recovered from clinically infected humans ($n = 3$) (Kennedy et al., 2008; Tramontana et al., 2008) and pigs ($n = 42$) between 1981 and 2011. The 42 porcine isolates originated from farms in at least 19 geographically distinct regions in Queensland ($n = 8$), New South Wales ($n = 27$), Victoria ($n = 2$) and South Australia ($n = 5$). All isolates were confirmed as serotype 2 by seroagglutination (Statens Serum Institut, Copenhagen, Denmark). Furthermore, all were tested by PCR for genes encoding the virulence-associated factors capsular polysaccharide 2 and 1/2 (*cps*2J), extracellular protein factor gene (*epf*), suilysin (*sly*), and six known allelic variants of the muramidase-released protein gene (*mrp*, mrp^S, mrp^*, mrp^{**}, mrp^{***}, mrp^{****}) (Silva et al., 2006).

2.2. MLST PCR

PCR assays were performed in 10 μL volumes, each containing ~2.5 ng of DNA template, 1× PCR buffer (Qiagen, Doncaster, Australia), 2 mM $MgCl_2$ (Qiagen), 100 nM of each forward and reverse primer, 200 μM of deoxynucleotide triphosphates (Qiagen) and 0.02 U of HotStarTaq (Qiagen). Assays were performed using the touchdown scheme described in Table S1. Primers used to amplify the seven target loci (*cpn*60, *dpr*, *recA*, *aroA*, *thrA*, *gki* and *mutS*) are shown in Table S2. Each primer featured an SP6 or T7 RNA polymerase promoter tag at the 5′ end.

2.3. MS-MLST

MLST amplicons were conditioned using a standardised protocol (Honisch et al., 2007). The entire volume of each amplicon cocktail was dephosphorylated with shrimp alkaline phosphatase (Sequenom, San Diego, CA, USA). Next, the forward and reverse strand of each amplicon was subjected to two separate in vitro transcription and base-specific cleavage reactions, with assistance from a MassARRAY Liquid Handler robot (Sequenom) (Honisch et al., 2007). In short, each fragment mixture was desalted with 6 mg of cationic resin (Sequenom) and dispensed onto a 384-element SpectroCHIP (Sequenom). The mass signal pattern of each fragment mixture was then determined using a MassARRAY MALDI-TOF mass spectrometer (Sequenom).

A reference library was constructed using allelic sequences from the *S. suis* MLST database (http://ssuis.mlst.net/). The 5′ and 3′ ends of each allele sequence were modified by incorporating a short stretch of consensus sequence, retrieved from GenBank entries NC_009443 and CP000408, and the appropriate forward or reverse primer. This was necessary as the MALDI-TOF MS analysis step first considers the entirety of each amplicon, before a specialised software package (iSEQ, version 1.0 Sequenom) identifies the allele within the core region of each locus. Mass signal patterns, representing each allele of each isolate, were automatically collated and alleles identified using the iSEQ software. Sequence types were identified by querying the output data (allelic profiles) in batch-format at the *S. suis* MLST database. eBURST software, version 3.0, was used to resolve isolates into clonal complexes (CCs, groups of isolates sharing ≥6 alleles with one or more members of the same group) using default settings (Feil et al., 2004).

2.4. MLST by dideoxy sequencing

To assess the competency of MS-MLST, all alleles were concurrently identified using dideoxy sequencing, as described (King et al., 2002). Consensus sequences were generated and STs automatically interpreted using the MLST plug-in function in BioNumerics software, version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). To estimate the duration of analysis that could be expected when using non-specialist or open-source bioinformatics software, sequences from each locus of 10 randomly selected isolates were manually assembled and trimmed using the Assembler program available with BioNumerics software, version 6.1. Once processed, the sequences were manually queried and STs identified using the *S. suis* MLST database.

3. Results

3.1. Validation of MS-MLST

MS-MLST identified a total of 18 distinct alleles among the 45 isolates of *S. suis* type 2. The concordance between MS-MLST and conventional MLST was 100%. Both identified a single nucleotide polymorphism (C to T substitution)