Veterinary Microbiology 164 (2013) 370–377

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

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

Detection and discrimination of common bovine mastitis-causing streptococci

Alexandre Almeida [a,b,c], Pedro Albuquerque [b], Ricardo Araujo [a,c], Niza Ribeiro [d], Fernando Tavares [a,b,*]

[a] FCUP – Faculdade de Ciências, Departamento de Biologia, Edifício FC4, Via Panorâmica n° 36, Universidade do Porto, 4150-564 Porto, Portugal
[b] CIBIO – Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
[c] IPATIMUP – Instituto de Patologia e Imunologia da Universidade do Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal
[d] ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira n° 228, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 13 November 2012
Received in revised form 1 March 2013
Accepted 2 March 2013

Keywords:
Bacterial detection
Bovine mastitis
DNA markers
Dot blot hybridization
Infrasubspecific discrimination
Streptococccus

ABSTRACT

Detection and typing of bovine mastitis pathogens are currently limited by time-consuming and culture-based techniques. In this work, a novel genus-specific DNA marker for *Streptococcus* and species-specific DNA markers for the prevalent mastitis pathogens *Streptococcus agalactiae* and *Streptococcus uberis* were designed and assessed. In order to enable further discrimination of these mastitis-causing streptococci, metabolic and pathogenicity-related genes were used to infer additional functional markers. A total of 12 DNA markers were validated with a set of 50 reference strains and isolates, representative of the *Streptococcus* genus, of closely related species and of microorganisms with matching habitats.

The experimental validation, using dot blot hybridization under high stringency conditions, confirmed the specificity of the selected markers. The broad-spectrum taxonomic marker (ST1) was specific to the *Streptococcus* genus and the markers selected for *S. agalactiae* (A1 and A2) and *S. uberis* (U1 and U2) were shown to be species-specific. The functional markers revealed strain-specific patterns of *S. agalactiae* and *S. uberis*. Markers derived from the fructose operon (FO1 and FO3) were specific to bovine isolates of *S. agalactiae*, and the nisin operon markers (NU1 and NU3) were able to discriminate isolates belonging to *S. agalactiae* and *S. uberis*. The virulence-associated markers (V1, V2 and V3) allowed the detection of *S. uberis* and of closely related species.

This work suggests that the combined use of these novel taxa-specific markers coupled with discriminatory functional markers presents a promising approach for the rapid and cost-effective detection and discrimination of common bovine mastitis-causing pathogens, which will contribute to an improved treatment and control of this disease.

1. Introduction

Bovine mastitis infections can be caused by over 150 different microorganisms and several *Streptococcus* species have been identified as directly responsible for the disease (Kuang et al., 2009; Shome et al., 2011; Wyder et al., 2011).

* Corresponding author at: FCUP – Faculdade de Ciências, Departamento de Biologia, Edifício FC4, Via Panorâmica n° 36, Universidade do Porto, 4150-564 Porto, Portugal. Tel.: +351 220402727; fax: +351 220402799.
E-mail address: ftavares@fc.up.pt (F. Tavares).

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.03.011

Streptococcus agalactiae, although generally acknowledged as a well-controlled etiological agent, has been reemerging throughout Europe (Kalmus et al., 2011; Klimiene et al., 2011; Mweu et al., 2012; Radtke et al., 2012). Recent studies have identified distinct populations of human and bovine mastitis isolates of *S. agalactiae* (Richards et al., 2011; Zadoks et al., 2011) and among this seemingly host-related genomic patrimony for this species is the four-gene operon responsible for fructose utilization, present in the majority of bovine strains (Richards et al., 2011). In *Streptococcus uberis*, the distribution and frequency of a number of virulence-associated genes implicated in the pathogenesis of this species have been described, including the *hasC*, *gapC* and *oppF* genes as well as two loci within the nisin U operon (Reinoso et al., 2011; Richards et al., 2011; Wirawan et al., 2006).

Culture-based techniques and molecular approaches currently used for detection and genotyping of *Streptococcus* present limitations concerning their specificity and resolution (Ajitkumar et al., 2012; Ieven et al., 1995; Radtke et al., 2012; Rato et al., 2008; Shome et al., 2011). Despite these efforts, methods are still required to simultaneously detect and infer the infrasubspecific variability of important pathogenic streptococci.

In this work, a hybridization platform comprising 12 genomic markers was developed for the simultaneous detection and discrimination of common bovine mastitis-causing streptococci. Insignia (Phillippy et al., 2007) was used to obtain DNA signatures for which five novel taxa-specific markers were designed. Seven additional functional markers were proposed to further investigate the overall diversity of the most relevant bovine mastitis pathogenic *Streptococcus*.

2. Materials and methods

2.1. Selection of DNA signatures and in silico analyses

Identification of taxa-specific DNA signatures on the bacterial chromosomes was carried out using the Insignia online application (Phillippy et al., 2007) with the available sequenced genomes as of October 2011. For each target taxon, the 10 largest outputted signatures were analyzed for their specificity using the BLAST (blastn) utility (Altschul et al., 1990), and the most promising regions were selected for further analyses. One broad range signature for the *Streptococcus* genus was selected (Ins1), taking into account the comparison of 63 sequenced genomes. In addition, specific regions for *S. agalactiae* (Ins2) and *S. uberis* (Ins3 and Ins4) were selected based on the available complete genome sequences for these pathogens: *S. agalactiae* 2603V/R, *S. agalactiae* H36B, *S. agalactiae* NEM316, *S. agalactiae* COH1, *S. agalactiae* 18RS21, *S. agalactiae* CJB111, *S. agalactiae* 515 and *S. agalactiae* A909 for Ins2 and *S. uberis* 0140J for Ins3 and Ins4.

The position of each genomic region was determined in *S. uberis* 0140J for signatures Ins1, Ins3 and Ins4 and in *S. agalactiae* 2603V/R for the Ins2 region, using Geneious Pro (Drummond et al., 2012). The Codon Adaptation Index (CAI), the expected CAI and GC content were calculated using the CAIcal server (Puigbo et al., 2008). Synteny analyses were performed using two applications from the CoGe platform of comparative genomics (Lyons et al., 2008), namely SynMap for obtaining whole genome syntenic dotplots and GEvo for high-resolution analysis of selected genomic regions.

2.2. DNA markers design

Primer pairs were designed for each of the four selected DNA regions, using the Vector NTI software (Invitrogen, Carlsbad, CA), in order to obtain taxa-specific DNA markers with a predicted amplicon size of 150–500 bp. Using the AlignX utility, primers specific to *Streptococcus* (ST1) and *S. agalactiae* (A1 and A2) were selected taking into account the alignment of several target sequences. The primers exclusive to *S. uberis* (U1 and U2) were chosen based solely on the sequence of *S. uberis* 0140J.

Concerning the functional markers, two primer pairs were designed for the amplification of genomic markers within two genes from the fructose operon of *S. agalactiae* FSL S3-026: one for a transcriptional regulator gene (FO1) and another for a phosphotransferase system (PTS) fructose-specific component gene (FO3). In addition, two primer pairs were designed for the nisin U operon of *S. uberis* strain 42: one for the gene responsible for the operon regulation (NU1), and another for the gene which confers immunity to nisin U (NU3).

Three additional primer pairs were selected based on virulence-associated genes described in *S. uberis* (Reinoso et al., 2011; Smith et al., 2002; Ward et al., 2001), specifically the hyaluronic acid operon gene *hasC* (V1), the glyceraldehyde 3-phosphate dehydrogenase gene *gapC* (V2) and the oligopeptide permease gene *oppF* (V3).

Overall, a total of 12 taxa-specific and functional markers were obtained for experimental validation (Table 1).

2.3. Bacterial strains, culture conditions and DNA extraction

In this work, 50 bacterial strains were used, corresponding to 15 reference strains and 35 isolates representative of the *Streptococcus* genus, of closely related species and of organisms with common habitats (Table S1 of Supplementary data). The bacterial isolates, obtained from different mastitic milk samples within the northern region of Portugal, were provided by SEGALAB (Laboratório de Sanidade Animal e Segurança Alimentar, S.A.). Species identification was done using the VITEK 2 system (bioMérieux, Durham, NC).

All strains were cultured in Brain Heart Infusion (BHI) medium (Oxoid, Hampshire, England) at 37 °C, with the exception of *Lactovum miscens* DSM 14925, which was cultured in MRS broth medium (Oxoid, Hampshire, England) pre-reduced with cysteine 0.05% and supplemented with N-acetylglucosamine 2 mM at 25 °C, in anaerobic conditions, using the CampyGen Atmosphere Generation System (Oxoid, Hampshire, England).

DNA was extracted from pure bacterial cultures using the EaZy Nucleic Acid (E.Z.N.A.) bacterial DNA purification kit (Omega Bio-Tek, Norcross, GA), following