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Short communication

Staphylococcus aureus subsp. *anaerobius* isolates from different countries are clonal in nature

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ABSTRACT

Staphylococcus aureus subsp. *anaerobius*, a microaerophilic, catalase-negative bacteria, is the etiological agent of abscess disease, a specific chronic condition of sheep and goats, characterized by the formation of necrotic lesions that are typically located in superficial lymph nodes. In this study, molecular analysis including pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and accessory gene regulator (agr) typing was carried out on 94 *S. aureus* subsp. *anaerobius* strains isolated in different countries (79 were isolated from 35 outbreaks of the disease in Spain from 1981 to 2009, 9 were isolated in Italy, 3 in Denmark and 3 in Sudan). All of the 94 *S. aureus* subsp. *anaerobius* isolates examined belonged to one PFGE type, within which four minority subtypes were identified. Representative isolates of all PFGE subtypes as well of all countries belonged to the same sequence type (ST), ST1464, which was a singleton, and to the agr type II. Our results support the view that abscess disease is caused by a single bacterial clone worldwide. This bacterium has existed for at least a century and, thus, has undergone long-term small ruminant host restriction.

1. Introduction

Staphylococcus aureus subsp. *anaerobius* is the etiological agent of abscess disease, a specific lymphadenitis of sheep and goats which affects mainly young animals up to 6 months of age. The disease is characterized by the presence of abscesses in superficial lymph nodes. Abscesses are the only obvious manifestation of the condition, and they are most frequently located in the lymph nodes of the mandibular region (mandibular, parotid and lateral retropharyngeal), followed by superficial cervical, subiliac and popliteal nodes, in that order of frequency (De la Fuente and Suarez, 1985). Abscess disease causes economic losses because of the reduced growth rate of affected animals and condemnations in abattoirs.

In Europe, abscess disease has been mainly diagnosed in France, where the condition was first described in 1911, and Spain. The disease has also been detected in Italy, Poland and Croatia, and a unique outbreak of the disease has been described in both Denmark and Hungary in animals imported from France. In Africa, abscess disease has been reported in Tunisia, Kenya, Nigeria, Egypt, Sudan and Somalia. The condition has also been diagnosed in occidental Asia (Saudi Arabia and Iran).

S. aureus subsp. *anaerobius* is closely related to *S. aureus* and both bacteria share the ability to produce extracellular toxins and enzymes that have been linked with staphylococcal pathogenicity (De la Fuente et al., 1985, 1987). *S. aureus* has been extensively investigated with regard to typing methods, and many different typing methods have been developed for epidemiological studies or for the analysis of genetic characteristics and relationships, including DNA fingerprinting techniques such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence

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typing (MLST). PFGE is one of the most discriminative typing methods for *S. aureus* and it is, therefore, considered the gold standard to investigate methicillin-resistant *S. aureus* (MRSA). MLST has been shown to be an effective method for studying the molecular evolution of *S. aureus* (Deurenberg and Stobberingh, 2008). This later method is based on the sequence analysis of fragments (~500 bp in length) of seven *S. aureus* housekeeping genes, i.e., *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*.

In this study, molecular analysis using a variety of methods, including PFGE, MLST and accessory gene regulator (*agr*) typing, was carried out on 94 *S. aureus* subsp. *anaerobius* strains isolated in different countries (Spain, Italy, Denmark and Sudan).

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 94 *S. aureus* subsp. *anaerobius* isolates were examined. Of these isolates, 79 were isolated from 35 outbreaks of the disease in Spain. These Spanish isolates were collected between 1981 and 2009 and represented diverse geographical origins since they were isolated from outbreaks of the disease in many provinces of several Spanish regions (Castilla y León, Castilla-La Mancha, Aragon, Andalucia and Extremadura). Of the other isolates, nine were isolated in Italy, three in Denmark and three in Sudan. The isolates were grown in blood agar at 37 °C, microaerophilically (candle jar system).

2.2. PFGE

All isolates were genotyped by PFGE following SmaI digestion of chromosomal DNA, prepared using a modification of a previously described protocol (Cookson et al., 2007). The resulting agarose plugs were loaded onto 1% (w/v) agarose gels, and PFGE was performed in CHEF-DRII apparatus (Bio-Rad, Hemel Hempstead, UK) in 0.5× Tris–borate–EDTA buffer (1× TBE is 89 mM Tris, 89 mM boric acid and 1 mM EDTA), at 6 V/cm and 12–14 °C. The total run time was 23 h, comprising 5–15 s for 10 h and 15–60 s for 13 h. The gels were stained with ethidium bromide, visualized under UV illumination and photographed. Following analysis according to established criteria (Tenover et al., 1995), a dendrogram was constructed with Molecular Analyst Software (Bio-Rad) using the Dice correlation coefficient (Hunter, 1990) and the unweighted pair-group method with averages, with a tolerance position of 0.8%.

2.3. MLST

The sequence type (ST) of 7 selected isolates, including representatives from all of the PFGE subtypes as well of all countries, was determined according to a previously described method (Enright et al., 2000). Analysis of the results was performed using the database available at www.mlst.net. The possible assignment of the *S. aureus* subsp. *anaerobius* MLST to a clonal complex (CC) was analyzed using the eBURST (Based Upon Related Sequence Types) algorithm (Feil et al., 2004) (www.eburst.mlst.net). CCs were composed of STs that shared at least six out of the seven alleles in common and a predicted ancestral ST and its associated single locus variants (SLVs; variants that differ at one of the seven MLST alleles from the ancestor) and double locus variants (DLVs; variants that differ at two of the seven MLST alleles from the ancestor).

2.4. Determination of agr types

Determination of the four *agr* types was performed according to a previously described multiplex PCR method (Shopsin et al., 2003). This method utilized a universal forward primer (pan-*agr*, corresponding to conserved sequences from the *agrB* gene) and four reverse primers (*agr*I, in the *agrD* gene; *agr*II, in the *agrC* gene; *agr*III, in the *agrD* gene and *agr*IV, in the *agrC* gene) (Shopsin et al., 2003).

2.5. Determination of Panton–Valentine leukocidin (PVL) genes

The PVL genes (*lukS-PV* and *lukF-PV*) were detected by PCR as described previously (Lina et al., 1999). The PVL-positive *S. aureus* (PVL-SA) ATCC 49775 strain was used as a positive amplification control.

2.6. Phylogenetic analysis

The phylogenetic relationships between the *S. aureus* subsp. *anaerobius* ST and the most frequently described ovine, caprine, bovine and ruminant associated STs were determined using the MEGA 4 software package (Tamura et al., 2007). The concatenated sequences of all seven MLST alleles were used to construct neighbor-joining trees. Neighbor-joining trees were constructed using the absolute number of nucleotide differences between STs. Bootstrapping was performed with 500 replicates.

3. Results

All of the 94 *S. aureus* subsp. *anaerobius* isolates examined in this study belonged to the same PFGE type, and four minority subtypes were identified (Fig. 1). The majority of the isolates (81/93; 71 from Spain, 9 from Italy and 1 from Denmark) belonged to PFGE type 1, four isolates (two from Spain and two from Denmark) belonged to subtype 1b, two Spanish isolates belonged to subtype 1c and four other Spanish isolates belonged to subtype 1d, and the three isolates from Sudan belonged to subtype 1e (Fig. 1).

All of the isolates analyzed belonged to the same ST, ST1464, including isolates from all of the countries examined and from all of the PFGE subtypes detected. ST1464 was a singleton, that is, a ST with no associated SLVs in the database.

S. aureus subsp. *anaerobius* isolates were all of *agr* type II and all of the isolates studied were negative for the PVL genes.

The phylogenetic analysis of concatenated MLST allele sequences performed using MEGA 4 software showed that *S. aureus* subsp *anaerobius* ST1464 had grouped separately from the ovine, caprine, bovine and ruminant associated