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fbl Gene as a Species-Specific Target for *Staphylococcus lugdunensis* Identification

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Staphylococcus lugdunensis is an unusually virulent coagulase-negative species, associated with severe infections. The present report describes the development of a single-step, species-specific PCR protocol for *S. lugdunensis* identification. *fbl* gene, encoding a fibrinogen-binding adhesin, was exploited and assessed as a suitable nucleic acid target. The gene was detected in all 17 *S. lugdunensis* isolates examined, while no amplification product was obtained from 98 isolates representing 11 staphylococcal and 17 nonstaphylococcal species. Forty-seven percent of the *S. lugdunensis* strains produced a positive slide coagulase reaction, which is consistent with varying levels of Fbl protein expression within the species. J. Clin. Lab. Anal. 24:119–122, 2010. © 2010 Wiley-Liss, Inc.

Key words: *fbl* gene; identification; PCR; *Staphylococcus lugdunensis*; fbl protein; expression

INTRODUCTION

Since its original description in 1988, *Staphylococcus lugdunensis* role as an important, though uncommon, human pathogen is increasingly being appreciated. *S. lugdunensis* has become particularly notorious as a causative agent of infective endocarditis associated with serious complications, poor response to conventional antimicrobial therapy, and increased morbidity of the affected patients without surgical intervention (1). The enhanced virulence of *S. lugdunensis* is further supported by its implication in clinically relevant bacteremia, abscesses, wounds, bone and joint infections (2).

Definite phenotypic identification of a gram-positive, catalase-positive coccus as *S. lugdunensis* implies a negative tube coagulase test and positive pyrrolidonyl-arylamidase and ornithine decarboxylase activities (3). However, complete hemolysis, yellow pigmentation, and detection of a fibrinogen affinity factor, although not consistently expressed by *S. lugdunensis*, may lead to its misidentification as *S. aureus* (2). Identification may be also problematic by certain commercial systems commonly used for routine testing (4).

Only very recently the first PCR protocol for detection of the species has been published targeting the *tanA* gene (5). Another suitable species-specific marker for *S. lugdunensis* identification could be the *fbl* gene, which codes for a surface-located fibrinogen-binding adhesin, referred to as Fbl protein (6,7). It mediates binding to the fibrinogen γ-chain and has close sequence and organizational similarity to clumping factor A (ClfA) of *S. aureus*, although the latter interacts with fibrinogen with a tenfold higher affinity. Both Fbl and ClfA possess a ligand-binding A region divided into three domains N1, N2, and N3. As N2 and N3 constitute the minimal fibrinogen-binding region of both proteins their residues are highly conserved resulting in a 60% identity. The function of the N1 region is not clear and it is more divergent (19% identity) between the two species with regard to its amino acid sequence, constituting a preferable target for the design of *S. lugdunensis* species-specific primers.

The objective of this study was to develop a single-step PCR assay for *S. lugdunensis* identification, using part of the *fbl* gene as a species-specific target.

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MATERIALS AND METHODS

Bacterial Strains

A set of 16 *S. lugdunensis* clinical isolates recovered from blood ($n = 4$), pus ($n = 10$), urine ($n = 1$), and a catheter tip ($n = 1$) were used to verify ubiquity of *fbl* in the species. These were identified by means of the API Staph (BioMerieux) and partial sequencing of the 16S rRNA gene (8); the sequences were submitted to GenBank (Sequence Accession Numbers: EU130682-EU130685, EF546485-EF546487, DQ923425-DQ923433). Type strain *S. lugdunensis* ATCC 700328 was also included in the study. A battery of 70 staphylococcal strains comprising 17 *S. aureus*, 20 *S. epidermidis*, 10 *S. hominis*, 8 *S. cohnii*, 8 *S. haemolyticus*, 4 *S. saprophyticus*, 2 *S. sciuri*, and 1 *S. simulans* were tested by the same PCR protocol developed for *S. lugdunensis* to exclude amplification of nonspecific products. These isolates were speciated by PCR-Restriction fragment length polymorphism (RFLP) analysis targeting the *tuf* gene (9). For the same purpose a random collection of nonstaphylococcal clinical isolates was investigated. One isolate of each of the following species was included: *Enterococcus casseliflavus/gallinarum*, *E. faecium*, *E. faecalis*, *Streptococcus pyogenes*, *S. pneumoniae*, *S. agalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa*, *Acinetobacter baumanni*, *Proteus vulgaris*, *Stenotrophomonas maltophilia*, *Enterobacter cloacae*, *Citrobacter koseri*, *Morganella morganii*, and *Serratia marcescens*. They were all identified by Phoenix Automated Microbiology System (Becton Dickinson). Reference strains *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *S. epidermidis* ATCC 12228, *S. haemolyticus* ATCC 29970, *S. saprophyticus* ATCC 49453, *S. simulans* ATCC 27851, *S. capitis* subsp. capitisATCC 35661, *S. warneri* ATCC 49454, *S. xylosus* ATCC 29971, *S. sciuri* subsp. *sciuri* ATCC 29060 were also investigated.

Primer Design

The only available *fbl* gene sequence was retrieved from GenBank (accession number AF 404823). The highly variable part of the gene (nucleotides 1 to 1020) corresponding to the initial signal peptide and N1 and N2 regions of the ligand-binding A domain was targeted for the design of primers using Primer 3 software (http://frodo.wi.mit.edu/). Oligonucleotide primers Fbl1 (5′-CCGATGTATCACCTGCAACG-3′) and Fbl2 (5′-ACATCATCTGGGGCTTGG-3′) were selected to amplify a 390 bp fragment of the *fbl* gene from nucleotide position 433 to 823. These primers were checked against all available sequence data for nonspecific amplification using the Blast Software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA Extraction

DNA extraction from solid agar cultures was carried out as previously described (10) using the chaotropic agent guanidinium thiocyanate for bacterial cell lysis.

PCR Amplification

In order to standardize the optimal conditions of the protocol, two concentrations (20 pmol and 50 pmol) and five annealing temperatures (58.0, 56.4, 54.8, 51.5, and 50.0°C) for the oligonucleotide primers were tested. In detail, the 50 µl reaction mixture contained 5 µl of template DNA, 2.5 U Taq DNA polymerase (Qiagen), 10X PCR buffer (Qiagen), 25 mM of each deoxyribonucleoside triphosphate (Invitrogen), 2 mM $MgCl_2$ (Qiagen), and either primer concentration. The reaction was performed under the following conditions: 10 min at 94°C, then 35 cycles of 30 sec at 95°C, 30 sec at each annealing temperature, 30 sec at 72°C, and a final extension step of 10 min at 72°C. Amplification products were detected by 2% agarose gel electrophoresis in the presence of ethidium bromide and visualized under a UV transilluminator.

Slide Coagulase Assay

All 17 *S. lugdunensis* strains under investigation were tested for their ability to produce a clumping factor positive reaction using the BBL Staphyloslide Latex test (Becton Dickinson).

RESULTS

Primers Fbl1 and Fbl2 amplified the expected 390 bp fragment of the *fbl* gene from all *S. lugdunensis* isolates (16 clinical isolates and the reference strain *S. lugdunensis* ATCC 700328), lending support to the view that *fbl* may possibly be ubiquitous in the species. Amplification was successful under all annealing temperatures and for both concentrations of the primers. The same PCR conditions including annealing temperature of 50°C and primer concentration of 20 pmol were also applied for the rest of the strains. No amplification product was yielded for any of the other species under investigation. The lowest annealing temperature and primer concentration were chosen, since the specific genetic target is currently evaluated as part of a multiplex PCR protocol, being optimized for the detection of several staphylococcal species. As for their slide coagulase phenotype, only 8 out of the 17 strains (47%) tested positive in the

latex agglutination assay (all positive were clinical isolates).

DISCUSSION

This study demonstrates that *fbl* gene may serve as a species-specific target for *S. lugdunensis* identification by means of a simple PCR protocol. In two previous reports the presence of this gene was verified in 20 *S. lugdunensis* strains by Southern blot analysis (6,7). In accordance with these results, the expected 390 bp *fbl* fragment was amplified from all 17 *S. lugdunensis* strains tested. Notably, the 16 clinical *S. lugdunensis* strains were single-patient isolates recovered during a 5-year period in the same hospital. All patients were epidemiologically unrelated, as also shown by PFGE analysis of 14 of these strains, which belonged to distinct pulsotypes. The specificity of the primer set designed for the development of the PCR protocol was verified with 11 staphylococcal and 17 nonstaphylococcal species (6 gram-positive and 11 gram-negative). It is noteworthy that no amplification products were obtained for *S. aureus*, whose *clfA* gene displays high similarity to *fbl* especially over the minimal binding domain.

Only a few virulence factors of *S. lugdunensis* and their corresponding genetic loci have already been identified. Biofilm formation is probably the predominant virulence mechanism employed by the species, whose genome harbors homologues of the *ica* locus. Others include a heat-stable δ-like hemolysin, a von Willebrand factor-binding protein and a membrane-bound peptidoglycan *O*-acetyltransferase conferring resistance to lysozyme, encoded by *slush*, *vwbl*, and *oatA* genes, respectively (2). To our knowledge, none of the aforementioned loci has been exploited so far for the development of a species-specific PCR assay for *S. lugdunensis* identification. *fbl* gene encoding the fibrinogen-binding adhesin Fbl, also assumed to be a major virulence factor of *S. lugdunensis*, is the first to be tested as a species-specific target for its identification.

To date, molecular species identification of *S. lugdunensis* using sequencing of genetic targets such as 16S rRNA, *hsp60*, sodA, tuf, and *rpoB* genes (11) is not available in all routine microbiology laboratories. The major drawbacks of this technology pertain to its high complexity, expensive instrumentation, and need for trained personnel. RFLP analysis on *hsp60* (12) and *tuf* (9) genes, as well as internal-trancribed spacer PCR followed by direct analysis of amplification products (13) are more simple alternatives, but the former method may be time-consuming and both may reveal intraspecific polymorphisms among *S. lugdunensis* strains. Automated ribotyping (14), real-time PCR (15), and microarray technology (16) are more sophisticated techniques recruited for coagulase-negative staphylococci speciation, including *S. lugdunensis*, that have not been extensively evaluated in the literature. This study is the second to report on the development of a species-specific single-step PCR protocol for *S. lugdunensis* identification.

Finally, a remarkable observation is the fact that a positive clumping reaction was observed only for 47% of the *S. lugdunensis* isolates under investigation by the latex agglutination assay. This is possibly due to the variable level of Fbl protein expression among *S. lugdunensis* strains, assuming that the associated gene is ubiquitous in the species. As previously shown, clumping factor positivity among *S. lugdunensis* strains varied significantly, from 6.7 to 60%, by using different commercial assays (17). Subsequently, the low rate of clumping factor detection in our study may reflect a low sensitivity of the used Staphyloslide Latex test (Becton Dickinson).

S. lugdunensis, previously characterized as a "wolf in sheep's clothing," (2) deserves particular attention among CNS, especially when isolated from sterile body sites. Its accurate and timely identification is required either by phenotypic or molecular methods. In this study we developed a simple PCR assay as a rapid and reliable diagnostic tool for recognition of this species. As the *fbl* gene appears to be conserved among *S. lugdunensis* isolates (6,7), the application of this assay in the clinical laboratory is expected to improve the accuracy of species identification. Adaption of the method for direct detection from clinical specimens was not attempted, as *S. lugdunensis* remains a rarely encountered staphylococcus. Still the protocol could possibly be combined in a multiplex format for concomitant identification of other pathogens.

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The GenBank (EMBL) accession numbers for the 16S rRNA gene sequences of the clinical *S. lugdunensis* strains that were studied are EU130682-EU130685, EF546485-EF546487, DQ923425-DQ923433.

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