Shiraz University

Faculty of Veterinary Medicine

Ph.D. Thesis in Bacteriology

PCR-RFLP ON THE *GROEL* AND *GAP* GENES FOR IDENTIFICATION OF STAPHYLOCOCCI SPECIES ISOLATED FROM HUMAN AND ANIMALS

By
Narjes Ghafari Sarvestani

Supervised by:
Dr. M. Tabatabaei

October 2012

In The Name of God

DECLARATION LETTER

I, Narjes Ghafari Sarvestani, Ph.D student in the field of Bacteriology the School of Veterinary Medicine of Shiraz University, declare that this thesis is the result of my research and I have written the exact references and full indication wherever I used others sources. I also declare that the research and the topic of my thesis are not reduplicative and guarantee that I will not disseminate its accomplishments and not make them accessible to others without the permission of the University. According to the regulations of the mental and spiritual ownership, all rights of this belong to the Shiraz University.

Name: Narjes Ghafari Sarvestani
Date: October 2012

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Evaluated and approved by the thesis committee as: Excellent

Dr. M. Tabatabaei, DVM, PhD, Assistant Professor of Microbiology, Shiraz University

Dr. M.Haghkhah, DVM, PhD, Associate Professor of Molecular Microbiology, Shiraz University

Dr. A. Hoseini, DVM, PhD, Associate Professor of Immuniology, Shiraz University

Dr. A. Mohammadi, DVM, PhD, Assistant Professor of Virology, Shiraz University

Dr. M. Motamedifar, DVM, PhD, Associate Professor of Microbiology, Shiraz Medical University

October 2012

This thesis is dedicated to my parents.

For their endless love, support and

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Abstract

PCR-RFLP on the *groEL* and *gap* genes for identification of staphylococci species isolated from human and animals

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Use of nucleic acid targets provides an alternative technique for accurate identification of *Staphylococcus* species. The *groEL* gene has well-conserved DNA sequences but with sufficient sequence variations that allow species-specific identification. The *gap* gene commonly has been considered a constitutive housekeeping gene and encodes a 42-kDa transferrin-binding protein located within the cell wall of the staphylococci. This study was aimed to compare phenotypic and genotypic identification methods used for *Staphylococcus* spp. isolates with bovine and human origins. PCR-RFLP of both the *groEL* and *gap* genes might be useful in reference laboratories for characterization of strains that could not be assigned to a species on the routine microbiology laboratories. This approach is confirmed by sequencing.

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Chapter One

Introduction

Staphylococci are spherical gram-positive bacteria that divide in several planes to form irregular clusters. They are present in the upper respiratory tract and on other epithelial surface of all warm-blooded animals (*Hirsh et al. 2004*).

To date, more than 50 *Staphylococcus* species and subspecies have been characterized. The genus is divided into coagulase-positive staphylococci and coagulase-negative staphylococci (CNS) based on their ability to coagulate plasma (*Pyorala and Taponen, 2009*). The staphylococci are the causative agents of many opportunistic human and animal infections and are considered among the most important pathogens isolated in the clinical microbiology laboratory (*Yugueros et al. 2000*).

1-1-Taxonomy

Recently, the taxonomy of the phylum *Firmicutes*, to which the staphylococci belong, has been completely revised (*Ludwig et al. 2009*). The genus *Staphylococcus* belongs to the class *Bacilli*, order *Bacillales*, family *Staphylococcaceae*, together with the genera *Macrococcus*, *Jeotgalicoccus*, and *Salinicoccus*. The minimal standards for assigning an organism to the genus *Staphylococcus* include genotypic as well as phenotypic criteria (*Freney et al. 1999; Gyles et al. 2010*).

1-2-Morphology and staining

The name staphylococcus is derived from the Greek term *Staphyle*, meaning "a bunch of grapes". This name refers to the fact that the cells of these gram-positive cocci grow in a pattern resembling a cluster of grapes (*Murray et al. 2009*).

Staphylococci are spherical cells about 1μm in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid clusters and clinical material. Young cocci stain strongly gram-positive. On aging, many cells become gram-negative. Staphylococci are non-motile and do not form spores (*Brooks et al. 2010*).

1-3-Structure and composition

The cell wall consists of proteins and polysaccharides. One protein ("clumping factor," "bound coagulase") is usually present in *S. aureus* and *S. intermedius*. Clumping factor interacts in vitro with fibrinogen to produce an agglutination-like reaction. Another, protein A, produces aggregation by combing with the Fc fragment of immunoglobulins. The predominant polysaccharide is teichoic acid linked to peptidoglycan. Its alcohol moiety is ribotol in *S. aureus*, and glycerol in *S. epidermidis* and *S. intermedius*. Carotenoid pigments in the cell membrane can impart a "golden" (latin: "aureus") colour to colonies of *S. aureus*. A capsule is sometimes produced by *S. aureus*, and often a "pseudocapsule" a loosely associated carbohydrate structure, is produced by strains causing bovine mastitis (*Hirsh et al. 2004*).

1-4-Growth characteristic

The staphylococci grow readily on most bacteriological media under aerobic or microaerophilic conditions. Most species are facultative anaerobes and grow in a medium containing 10% sodium chloride and at a temperature ranging from 18°C to 40°C *(Murray et al. 2009)*. The routine medium for inoculation of specimens is sheep or ox blood agar. Mannitol

salt agar and Baird-Parker medium are specifically selective for staphylococci but are used mainly in food microbiology (*Queen et al. 1994*).

They grow most rapidly at 37°C but form pigment best at room temperature (20-25°C). Colonies on solid media are round, smooth, raised and glistening. *S. aureus* usually forms gray to deep golden yellow colonies. *S. epidermidis* colonies usually are gray to white on primary isolation; many colonies may develop pigment only upon prolonged incubation. No pigment is produced anaerobically or in broth. Various degrees of haemolysis are produced by *S. aureus* and occasionally by other species. Staphylococci slowly ferment many carbohydrates, producing lactic acid but no gas. Proteolytic activity varies greatly from one strain to another (*Brooks et al. 2010*).

Relatively simple biochemical tests can be used to differentiate *S. aureus* and the other staphylococci. *S. aureus* has positive reactions for coagulase, heat-stable nuclease, alkaline phosphatase and mannitol fermentation. Differentiation of the coagulase-negative staphylococci is more complex, however, and is not routinely done in many clinical laboratories unless the isolates are demonstrated to be clinically significant (*Murray et al. 2009*).

1-5-Isolation

Bovine blood agar is best for the detection of β-toxin, which is diagnostic for coagulase positive staphylococci (*S. aureus*, *S intermedius*, *S. schleiferi* spp. *Coagulans*) (*Hirsh et al. 2004*). For isolation of organisms from heavily contaminated specimens, specimens can also be inoculated onto Columbia colistin-nalidixic acid (CNA) media or phenylethyl alcohol (PEA) agar, which inhibit the growth of gram-negative bacteria and allow the growth of gram-positive organisms. Mannitol salts agar is a good selective medium for assessing the presence of *S. aureus* in specimens such as nasal cultures. On sheep blood agar, most staphylococci produce good growth within 24 hours. Some species of staphylococci may also require more than 24 to 48 hours of incubation in order to discern whether a specimen contains a pure or a mixed culture.

Longer incubation may be necessary to ensure that identification and

susceptibility tests are being performed on a pure culture, especially if multiple colonies are being sampled to obtain a representative inoculum (*Winn et al. 2006*).

1-6-Identification and Colonial characteristics

Colonies of most staphylococcal species are 1 to 3 mm in diameter after 24 hours of incubation, although some may form smaller colonies during this time. Strains of some staphylococcal species will show considerable variation in the size of colonies on the same culture plate, giving the appearance of a mixed culture.

Staphylococcal colonies are usually round, smooth, butyrous, raised, glistening and have a low convex profile with an entire edge. Colonies of some *S. aureus* strains are usually large (4-6 mm in diameter) smooth, entire, and butyrous in consistency, although some strains may be wet looking or sticky.

1-6-1-Pigmentation

Staphylococci form pigment best at room temperature (20-25°C). Some strains of *S. aureus* may be pigmented yellow or yellow-orange, while other strains may produce off-white or gray colonies. Pigment enhancement in the staphylococci is said to be induced by the addition of milk, fat or glycerol monoacetate to the medium. *S. epidermidis* colonies usually are gray to white on primary isolation. Many colonies may develop pigment only upon prolonged incubation. No pigment is produced anaerobically or in broth (*Winn et al. 2006, Brooks et al. 2010*). The colonies of bovine and human strains of *S. aureus* are golden yellow. Colonies of some coagulase negative staphylococci are also pigmented (*Quinn et al. 1994*).

1-6-2-Haemolysis

the staphylococcal haemolysins (alpha, beta, delta and gamma) can be produced singly, in combination or not at all. The haemolysins differ antigenically, biochemically and in their effect on the red cells of various animal species. Blood agar prepared with either ovine or bovine erythrocytes is preferable in veterinary diagnostic work as the red cells from