Shiraz University

Faculty of Veterinary Medicine

Ph.D. Thesis in Bacteriology

EVALUATION OF POLYMORPHISM OF COAGULASE GENE IN *STAPHYLOCOCCUS AUREUS* ISOLATES FROM DIFFERENT SOURCES USING PCR-PFLP AND SEQUENCING

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September 2012

In The Name of God

DECLARATION LETTER

I, Zahra Esmailnezhad Shirazi, 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.

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Thesis

Submitted to the school of graduate students in partial fulfillment of the requirements for the degree of PhD

In

Bacteriology

Shiraz University

Shiraz, Iran

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Dedication

I wish to dedicate this manuscript to my family who loved and faithfully supported me during my education and through this work and more importantly during the life journey. I sincerely thank them for all their unsparing support, patience, assistance and encouragement and providing ideal condition for my life. I greatly appreciate them for everything.

Acknowledgement

I profusely thank my supervisor, Dr. Masoud Haghkhah, for providing good conditions for my work and for all his patience, enthusiasm and encouragement through the work and all of his support and advice not only during this work but also throughout my education.

I wish to thank Dr. Roya Firouzi for all of her support, patience and advice throughout my education and during this work.

I thank Dr. Mohammad Tabatabaei for all his support during my education and giving useful comments for the manuscript.

I greatly appreciate Dr. Arsalan Hosseini for giving so much valuable scientific information, for giving critical comments and for all his patience and unsparing assistance throughout the work.

I thank Mr. Sorbi and Mr. Shahed for their kindly assistance during this work.

I wish to thank my dear friend, Dr. Narjes Ghaffari whose friendship I greatly value, with whom I shared many enjoyable hours throughout my education.

Thanks to my family for all their support, patient and help throughout my education. I do appreciate them for everything.

Abstract

Evaluation of Polymorphism of Coagulase Gene in *Staphylococcus aureus* Isolates from Different Sources Using PCR-PFLP and Sequencing

By
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Considerable genetic heterogeneity has been shown in natural populations of *S. aureus* isolates. *S. aureus* coagulase gene is a significant virulence factor whose typing is used to identify *S. aureus* genotypes. The present study was conducted to characterize coagulase gene in bovine mastitic, human and food *S. aureus* isolates. Collected samples were identified by routine biochemical tests and confirmed by species specific PCR. PCR amplification of coagulase gene yielded single bands with the molecular size ranging from 500 to 900 bp. The detection of single bands by coagulase PCR in bovine mastitic isolates showed that the isolates were of bovine origin suggesting that there was no human contamination of bovine mammary glands or milk. Detection of single bands by coagulase PCR in human *S. aureus* isolates suggests that these isolates may be of bovine origin and reveals the possibility of transmission of the bacteria from cattle to human. Detection of single bands by coagulase PCR in food *S. aureus* isolates suggests that these isolates may be of bovine origin not human one, and contamination of food samples may initiate from the animal source not the food-handlers. Digestion of coagulase gene PCR products with restriction endonuclease enzymes *Alu*I and *Hin*61 yielded different RFLP profiles that indicated the presence of heterogeneity in the coagulase gene of the isolates. The numbers of RFLP patterns gained following *Alu*I and *Hin*61 digestion of the isolates did not differ considerably, no significant discriminatory power was found between *Alu*I and *Hin*61. Analysis of coagulase gene sequences of selected isolates indicated high similarity between the coagulase gene sequences and other reports. The differences between the sequences of coagulase gene in the selected isolates with other reports were highly acceptable and were due to the point mutation.

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Chapter One

Introduction

1-1 General Characteristics of Staphylococci

1-1-1Taxonomy

The taxonomy of the phylum *Firmicutes*, to which the staphylococci belong, has been completely revised recently. 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 (Gyles *et al.* 2010).

1-1-2 Morphology

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 cultures 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).

1-1-3 Natural Habitat

Staphylococci occur worldwide as commensals in mammals. They colonise the human nasal cavity, skin and mucous membranes and can be transient in the intestinal tract. Many infections are endogenous but prolonged survival of staphylococci in the environment permits indirect transmission. Staphylococci are relatively stable in the environment. Staphylococcal strains display a selective affinity for particular animal

species. Transfer of *S. aureus* strains between animal species and between animals and man is limited (Queen *et al.* 1994).

1-1-4 *Staphylococcal* species

The genus *Staphylococcus* has at least 40 species (Brooks *et al.* 2010). The species most commonly associated with human disease are *S. aureus* (the most virulent and best-known member of genus), *S. epidermidis*, *S. saprophyticus*, *S. capitis* and *S. haemolyticus* (Murray *et al.* 2009). *S. aureus* is coagulase positive, which differentiates it from other species. *S. aureus* is a major pathogen for humans. Almost every person will have some type of *S. aureus* infection during a lifetime. The coagulase negative staphylococci are normal human flora and sometimes cause infection often associated with implanted devices. Approximately 75% of these infections caused by coagulase negative staphylococci are due to *S. epidermidis* (Brooks *et al.* 2010).

Most species are facultative anaerobes, except for *S. aureus* subsp. *anaerobius* and *S. saccharolyticus.* These two species grow anaerobically and, unlike the facultative species, are often catalase-negative (Winn *et al.* 2006).

1-1-5 Growth characteristic

The staphylococci are catalase-positive, oxidase-negative and non-motile (Queen *et al.* 1994). They 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). 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 hemolysins 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).

1-1-6 Antibiotic resistance

Staphylococci are variably sensitive to many antimicrobial drugs. Resistance falls into several classes:

1- β-lactamase production is common, under plasmid control, and makes the organism resistant to many penicillins (penicillin G, ampicillin, ticarcillin, piperacillin, and similar drugs). The plasmids are transmitted by transduction and perhaps also by conjugation.

2- Resistance to nafcillin (and to methicillin and oxacillin) is independent of β-lactamase production. Resistance to nafcillin is encoded and regulated by a sequence of genes found in a region of the chromosome called the staphylococcal cassette chromosome *mec* (*SCCmec*). Specifically, the *mec A* gene on this locus encodes a low-affinity penicillin binding protein (PBP2a) that is responsible for the resistance. There are several different *SCCmec* types. Types I, II, III are associated with hospital-acquired infections and may contain genes that that encode resistance to other antimicrobials as well. *SCCmec* type IV has principally been found in community-acquired methicillin resistant *S. aureus* (CA-MRSA) strains that tend to be less resistant, more transmissible, and responsible for outbreaks over the last decade in the United States and some countries in Europe.

3- In the United States, *S. aureus* and *S. lugdunensis* are considered to be susceptible to vancomycin if the minimum inhibitory concentration (MIC) is <= 2 μg/mL; of intermediate susceptibility if the MIC is 4-8 μg/mL; and resistant if the MIC is >= 16 μg/mL. Strains of *S. aureus* with intermediate susceptibility to vancomycin have been isolated in Japan, the United States, and several other countries. These are often known as vancomycin-intermediate *S. aureus*, or VISA. They generally

have been isolated from patients with complex infections who have received prolonged vancomycin therapy. Often there has been vancomycin treatment failure. The mechanism of resistance is associated with increased cell wall synthesis and alterations in the cell wall and is not due to *van* genes found in enterococci. *S. aureus* strains of intermediate susceptibility to vancomycin usually are nafcillin-resistant but generally are susceptible to oxazolidinones and to quinupristin/dalfopristin.

4- Since 2002, several isolates of vancomycin-resistant *S. aureus* (VRSA) strains were isolated from patients in the United States. The isolates contained the vancomycin resistance gene *vanA* from enterococci and the nafcillin resistance gene *mecA*. Both of the initial VRSA strains were susceptible to other antibiotics. Vancomycin resistance in *S. aureus* is of major concern worldwide.

5- Plasmid-mediated resistance to tetracyclines, erythromycins, aminoglycosides, and other drugs is frequent in staphylococci.

6- Tolerance implies that staphylococci are inhibited by a drug but not killed by, there is great difference between minimal inhibitory and minimal lethal concentrations of an antimicrobial drug. Patients with endocarditis caused by a tolerant *S. aureus* may have a prolonged clinical course compared with patients who have endocarditis caused by a fully susceptible *S. aureus*. Tolerance can at times be attributed to lack of activation of autolytic enzymes in the cell wall (Brooks *et al.* 2010).

1-1-7 Laboratory Diagnosis

1-1-7-1 Specimens

Surface swab pus, blood, tracheal aspirate, or spinal fluid for culture, depending on the localization of the process, are all appropriate specimens for testing (Brooks *et al.* 2010).

1-1-7-2 Direct microscopy

On direct Gram-stained smears from clinical specimens, staphylococci appear as gram-positive or gram-variable cocci ranging in diameter from 0.5 to about 1.5 μm. The organisms may appear singly, in pairs, in short chains, or in clusters, both within and outside of polymorphonuclear cells.

Variations in cell size and Gram reaction are probably due to the action of the inflammatory cells and their hydrolytic enzymes on the bacterial cells. On direct smears, pairs and short chains of organisms cannot be differentiated from other Gram positive cooci (Brooks *et al.* 2010).

1-1-7-3 Isolation

Clinical specimens should be inoculated onto sheep blood agar and other bacteriological media. For isolation of organisms from heavily contaminated specimens, specimens can also be inoculated onto Columbia colistin-nalidixic acid (CNA) 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).

1-1-7-4 Identification

1-1-7-4-1 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. Some strains may be pigmented yellow or yellow-orange, while other strains may produce off-white or gray colonies.

A- 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)

B- 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 blood 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 both of these animal species are susceptible to the alpha-haemolysins and beta-haemolysins that are produced commonly by staphylococcal isolates from animals. Both *S. aureus* and *S. intermedius* are usually haemolytic and often produce both the alpha-lysin and beta-lysin and so exhibit double-haemolysis. The alpha-lysin is responsible for the narrow zone of clear haemolysis immediately around the colony and the beta-haemolysin for the broader outer zone of incomplete (partial) haemolysis (Quinn *et al.* 1994).

1-1-7-4-2 Tests for pathogenicity of staphylococcal isolates

A- Coagulase test:

S. aureus produces coagulase, an enzyme-like protein that clots oxalated or citrated plasma. Coagulase binds to prothrombin; together they become enzymatically active and initiate fibrin polymerization. Coagulase may deposite fibrin on the surface of staphylocooci, perhaps altering their ingestion by phagocytic cells or their destruction within such cells. Coagulase production is considered synonymous with invasive pathogenic potential (Brooks *et al.* 2010).

B- Clumping factor:

Clumping factor is responsible for adherence of organisms to fibrinogen and fibrin. When mixed with plasma, *S. aureus* forms clumps. Clumping