Vol. 20, No. 1, Spring, 2024, 97-108
DOI: 10.22055/IVJ.2024.415896.2634
Journal Homepage: http://www.ivj.ir/

Original Article

Prevalence of selected virulence factors of *Staphylococcus aureus* isolated in bovine mastitis in Chaharmahal and Bakhtiari province- Iran

Behnam Rozbahan[1], Naser Shams Esfandabadi[2*], Ali Kadivar[3], Azam Mokhtari[4] and Najmeh Davoodian[5]

[1] DVSc Student of Theriogenology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
[2] Professor, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
[3] Associate Professor, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
[4] Associate Professor, Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord , Iran
[5] Associate Professor, Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran

Received: 11.09.2023 Accepted: 08.01.2024

Abstract

It has been determined that there is a direct relationship between the severity of mastitis and the virulence factors produced by bacterial agents. Identifying bacterial virulence factors is neccecary for designing suitable vaccines against mastitis. The aim of the present study was molecular diagnosis of selected virulence factors of endemic isolates of *S. aureus* involved in bovine mastitis. A total of 180 milk samples were collected from cows with clinical (37 samples, 20.6%) and subclinical (143 samples, 79.4%) mastitis from 8 semi-industrial dairy farms of Chaharmahal and Bakhtiari province, Iran. After culture and purification, coagulase, catalase and oxidase tests were performed. DNA was extracted from *S. aureus* suspected colonies. Final confirmation was performed using PCR test on the specific 23S rRNA gene of the bacteria. Thirty one (17.22%) out of the 180 collected samples were found to be positive for *S. aureus* by PCR, of which 2 cases were related to clinical mastitis and 29 cases were related to subclinical mastitis. The highest frequency of virulence genes was related to the Coa gene (90.32%), followed by ClfB (87.09%), LukD, and fnbB (80.64%), LukE (77.41%), fnbA (74.19%), Hla (48.38%). The lowest frequency was related to the Hlb gene (45.16%). Based on the obtained results, the diagnosis of the virulence factors of *S. aureus* has the potential of being used in the development of vaccines for the prevention of mastitis.

Key word: Mastitis, *Staphylococcus aureus*, Virulence factors

Introduction

Pathogenicity level of a pathogen is called virulence, which is affected by microbial virulence factors, the number of contaminating microbes, the penetrating route, as well as specific and non-specific defense mechanisms of the host (Peterson,1996). Microbial virulence is brought about by a wide range of secreted products such as toxins, enzymes and exopolysaccharides, cell surface molecules such as capsules and lipopolysaccharides, as well as modification in intracellular regulatory signaling networks. In the recent years, advances in molecular biology,

* **Corresponding Author**: Naser Shams Esfandabadi, Professor, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
E-mail: drn_shams@yahoo.com

genomics, and bioinformatics have contributed to the molecular identification and functional analyses of a wide range of microbial virulence factors (Leitão, 2020). Identifying virulence factors can potentially lead to using the genetic characterization of bacteria for designing suitable vaccines for prevention of mastitis (Parker, 2018). In addition, anti-virulence compounds may be used as an adjuvant treatment of antibiotics to improve the therapeutic and safety effects; therefore, it is important to know, study and investigate the virulence factors (Sommerhäuser et al, 2003; Wu, 2019). Today, the drug resistance of different strains of *S. aureus* as the most important cause of clinical and subclinical mastitis has been reported to different antibiotics, and alternative strategies include anti-virulence treatment, photodynamic therapy, bacteriophage therapy, vaccines, and more. It is believed that there is a direct relationship between the severity of mastitis and virulence factors produced by *S. aureus* (Momtaz et al, 2010). *S. aureus* uses the Agr quorum sensing system to regulate the secretion of virulence factors in response to the non-inducing peptide signal (AIP). In addition, the Agr quorum sensing system regulates biofilm formation and heterogeneous resistance to escape death by the host immune system and antibiotics (Tan et al, 2018). Members of the family of enterotoxins and exotoxins, which act as superantigens and induce pro-inflammatory cytokine responses, are unique to *S. aureus*; they have not been identified in isolated from *S. epidermidis* or other coagulase-negative staphylococci (Gill, 2005). To invade and survive in the host, *S. aureus* needs to produce different pathogenic factors, some of which are as follows:

Hemolysin-Alpha (Hla or α-toxin), a 33 kDa polypeptide secreted by 95% of clinical strains of *S. aureus*, has been confirmed to play an essential role in the pathogenesis (Divyakolu 2019). It has the ability to bind the heptamer structure on the host cell and oligomerize it which makes it dangerous although it is not toxic by itself. Binding to its target, Hla oligomerizes a pre-pore structure and invades the cell membrane by extrusion of the β-barrel through the lipid bilayer and forms a hydrophilic transmembrane channel (Lee, 2010). Indeed, cell signaling pathways undergoes alterations including cell proliferation, inflammatory responses, cytokine secretion, and cell-cell interactions (Seilie and Wardenburg, 2017).

Hemolysin-Beta (Hlb) or beta toxin is a virulence factor that plays a role in *Staphylococcus aureus* immune escape and survival. Beta-toxin is a single-domain protein composed of four layers: two β-sheets in the center and two outer layers containing α-helices and β-strands (Tam and Torres, 2016). The beta toxin is also known as hot-cold hemolysin. Beta toxin is a biofilm ligase and increases biofilm formation by catalyzing the formation of nucleoprotein matrix in biofilms. (Salgado-Pabón et al, 2014). Moreovere, the red blood cells of sheep, cattle, and goats are very sensitive to this toxin. Beta toxin shows species-dependent hemolytic activity related to the amount of sphingomyelin in red blood cells (Bohach, 2006).

Leukotoxins E and D were initially reported more than a decade ago and are composed of the νSaβ gene cluster. Lecotoxins E and D have shown lytic activity in rabbit and human red blood cells and neutrophils. LukED genes have been isolated in 88–99% of MRSA strains worldwide, suggesting a potential role for this toxin in *S. aureus* pathogenesis (Oliveira et al, 2018).

Coagulase (Coa), von Willebrand factor-binding protein (vWbp), and staphylokinase (Sak) are cofactors produced by *S. aureus* that have no enzymatic activity by themselves, but can activate host zymogens. These three proteins hijack different aspects of the host's coagulation system, thereby manipulating the host's innate defenses to promote the survival of bacterial and dissemination. Coagulases must be present

simultaneously as the infecting strain to increase the severity of the disease. Coagulase is required to form a pseudo capsule immediately around the abscess; in fact, coagulases create a fibrin shield to protect *S. aureus* from infiltrating immune cells (McAdow et al, 2012).

Fibronectin-binding proteins (FnbA and FnbB) are effective in tissue invasion in various pathological conditions such as ocular keratitis and osteomyelitis (Soltani et al, 2019). Fibronectin A and B are also mediators of actin cytoskeleton rearrangement. Fibronectin-binding homologs A and B are two surface proteins that have been shown to be involved in host cell adhesion and invasion. Fibronectin proteins interact with host cell integrins through the fibronectin bridge to induce actin rearrangement leading to bacterial internalization. Some clinical strains of *S. aureus* have both fibronectins A and B genes. Fibronectin A or B alone is sufficient for invasion. It has been shown that fibronectin-binding proteins A and B play an important role in invading *S. aureus* into various non-phagocytic eukaryotic cells (Fowler et al, 2000; Niemann et al, 2004).

Clamping factor beta (ClfB) is a member of a family of proteins that are covalently attached to cell wall peptidoglycan. Staphylococci can express up to 22 proteins on their cell surface (Geoghegan and Foster, 2017). *Staphylococcus aureus* binds to squamous epithelial cells through cell wall binding proteins, including clamping factor-beta (Pollard and Pollard, 2018). In vitro, biochemical analysis and animal model studies have shown that the main target ligand for clamping factor beta is the squamous epithelial cell envelope protein loricin (herein Lor) (Niehues, 2017). This molecule consists of Gly-Ser-rich regions, and the highest affinity binding site for clamping factor-beta is located in the Lor 2 loop region. Clamping factor beta has recently been shown by Yazarlu and collogues to be a major adhesion molecule for the interaction of *S. aureus* with dermal keratinocytes (Yazarlu et al, 2021)

Identifying virulence factors has an important role in providing new treatment solutions, developing control and prevention programs, and laying the groundwork for eradicating diseases (Oaks et al, 1992). Therefore, the present study was carried out with the aim of molecular tracking of selected virulence factors of local *S. aureus* isolates involved in bovine mastitis.

Materials and Methods

Sampling of cows with clinical and subclinical mastitis was carried out from 8 semi-industrial dairy farms located in Chaharmahal and Bakhtiari provinces, Iran. Cows were milked there times a day. The patity of cows in these herds ranged between one and seven. The animals were kept in open shed barns with concrete floor. The cows were fed a total mixed ration on the basis of their milk yield production. The cows in this study were in mid lactation (DIM of 90-150). Clinical mastitis samples were taken from cows with at least one of the following symptoms: macroscopic abnormalities in milk (discoloration, clots, pus particles), physical abnormalities in udder (acute and diffuse swelling, warmth, pain, redness) or the general response of the body (varying degrees of anorexia, toxemia, dehydration, fever, increased heart rate, cessation of rumen movements, grounding). Subclinical mastitis samples were obtained from quarters that were positive in the California Mastitis Test (CMT). All samples were collected according to National Mastitis Council (NMC) instructions (Nickerson et al, 2004) and transferred to the microbiology laboratory, faculty of veterinary medicine, Shahrekord University, located in the middle part of Iran. Milk samples were collected aseptically immediately before milking. The teat end was disinfected with 70% ethanol and allowed to dry. The first three streams were discarded and milk samples

(about 10 ml) were collected in sterile tubes and tranfered to labarotory in cool bags. The milk samples were centrifuged at the speed of 6000 rpm for 30 minutes in a refrigerated centrifuge. Then, the resulting sediment was used for culture and molecular tests. Milk samples were cultured on blood agar and mannitol salt agar (MSA) (Merck; Germany) and suspected *S. aureus* colonies were evaluated by routine microbial tests such as: Gram stain, coagulase, catalase and oxidase tests.

DNA extraction from milk sediment was performed using the DNP Kit (Sinaclon, Tehran, Iran) according to the manufacturer's instructions. One hundred µL of protease buffer was added to 100 µL of milk sediment. After adding 5 µL of protease and mixing the set, the mixture was incubated for 30 minutes at 55°C. The lysing buffer was placed at 37 °C for 10 minutes, then 400 µL of this buffer was added to 100 µL of milk precipitate. Vortex was performed for 15-20 seconds to make the sample uniform. 300 µL of sedimentation buffer was added to the collection, vortexed for 5 seconds, and centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded, then 1 mL of washing buffer was added and vortexed for 3-5 seconds, and then centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded, and the remaining pellet was placed at 65°C for 5 minutes to dry completely. The precipitate was dissolved in 50 µL of dissolving buffer with gentle shaking and placed at 65 °C for 5 minutes. Undissolved materials were precipitated by centrifugation at 12000 rpm for 30 seconds, and the supernatant containing DNA was used. The extracted DNA was stored in -20 °C until the PCR test was performed.

In order to definitively diagnose and confirm *S. aureus*, PCR was used on the specific 23S rRNA gene of the bacteria. The volume of each PCR reaction was considered to be 25µL, which included 1 µL of extracted DNA, 12.5 µL of Taq polymerase mastermix 2X solution (Ampliqon, Denmark), 1 µL of each of the primers with the concentration of 10 picomoles. and 9.5 µL of sterile deionized distilled water. In addition to extracted milk DNA samples, positive control (*S. aureus* strain, ATCC: 25923) and negative control (distilled water) were included in each PCR run. After completion of the reaction, the PCR products was electrophoresed next to the DNA ladder on 1.2% agarose gel which was mixed with green viewer solution (Pars Tous) (Holtzhauer, 2006). The list of used primers and PCR programs for used genes are given in table 1 and 2, respectively \\\\\\\\. In order to confirm the products obtained in the PCR reactions, 50 µL of two samples of the PCR products of each gene were sent for sequencing (Gene-Fanavaran).

Table 1: Sequence of primers used in this study

Primer name	Primer sequence	Product length bp
23S rRNA F	AGCGAGTCTGAATAGGGCGTTT	894
23S rRNA R	CCCATCACAGCTCAGCCTTAAC	
nuc-F	GCGATTGATGGTGATACGGTT	270
nuc-R	AGCCAAGCCTTGACGAACTAAAGC	
HlbF	ACGCGCTGATTTAATCGGGCAA	393
HlbR	TCATGTCCAGCACCACAACGTGA	
HlaF	TCCTGTCGCTAATGCCGCAGA	431
HlaR	TTGCACCAATAAGGCCGCCA	
CoaF	TCAACCGACGACACCGAACCC	758
CoaR	GGCGAGCCCCGTATGACACT	
fnbAF	TGGTGTCGGTGGCGTTGGTG	950
fnbAR	GCGAAGCGGGTCACGTTGGA	
fnbBF	AGAGCCGCCAGTGGAGAAGCA	972
fnbBR	GGTGTCGGTGGCGTTGGTGG	
lukEF	TGCACCTTTAGCATCTCCGATTCAAG	614
lukER	CTCTTGCTGAACCTGTTGGACCATT	
lukDF	GCTCAACATATCACACCTGTAAGCGA	504
lukDR	CGCCTCAACACCCCAGCCAA	
ClfBF	CCGCCGGTTGACCCAGAACC	556
ClfBR	CGCTGTCTGAGTCCGAATCGC	

Table 2: PCR programs for used genes

	PCR steps				
Gene	Initial denaturation Temp°C (time)	Denaturation Temp°C (time)	Annealing Temp°C (time)	Extension Temp°C (time)	Final extension Temp°C (time)
23S rRNA	94 (5 min)	94 (30 s)	52 (35 s)	72 (40 s)	72 (10 min)
Nuc	94 (5 min)	94 (30 s)	59 (30s)	72 (30 s)	72 (5 min)
Hlb	94 (5 min)	94 (30 s)	53 (30 s)	72 (30 s)	72 (5 min)
Hla	94 (5 min)	94 (30 s)	53 (30 s)	72 (30 s)	72 (5 min)
Coa	94 (5 min)	94 (30 s)	55 (30 s)	72 (30 s)	72 (5 min)
fnbA	94 (5 min)	94 (30 s)	54 (40 s)	72 (45 s)	72 (10 min)
fnbB	94 (5 min)	94 (30 s)	57 (40 s)	72 (45 s)	72 (10 min)
lukD	94 (5 min)	94 (30 s)	55 (30 s)	72 (35 s)	72 (10 min)
lukE	94 (5 min)	94 (30 s)	55 (30 s)	72 (35 s)	72 (10 min)
ClfB	94 (5 min)	94 (30 s)	57 (35 s)	72 (35 s)	72 (10 min)

The number of cycles for Hlb and fnbB were 35 and for other genes were 30.

Results

The suspected cultured colonies were determined to be *S. aureus by* colony morphology, Gram staining, catalase, coagulase and oxidase tests. These isolates formed large, round, golden-yellow and often hemolytic colonies in the blood agar medium and produced cream-colored colonies surrounded by yellow areas in the mannitol-salt-agar medium. After gram staining, gram-positive cocci were seen in grape-like clusters. The selected isolates were positive for catalase and coagulase tests but had negative results in the oxidase test. Out of 180 milk samples, 29 samples were suspected to be *S. aureus* based on bacterial culture results.

Out of 180 milk samples, 31 samples were positive for the presence of *S. aureus* based on the PCR test (17.22%). Of these cases, 2 samples were related to clinical mastitis and 29 samples were related to sub-clinical mastitis (Table 3). In the electrophoresis of the PCR products, the band size of 894 base pairs for the 23S rRNA gene and 270 base pairs for the nuc gene were observed in positive samples for *S. aureus* (Figures 1 and 2).

Table 3: Frequency of *S. aureus* contamination in cases of mastitis using PCR

Mastitis type	Total Frequency	Frequency of positive cases	Positive frequency percentage
Clinical	37	2	5.4
Subclinical	143	29	20.27
Total	180	31	17.22

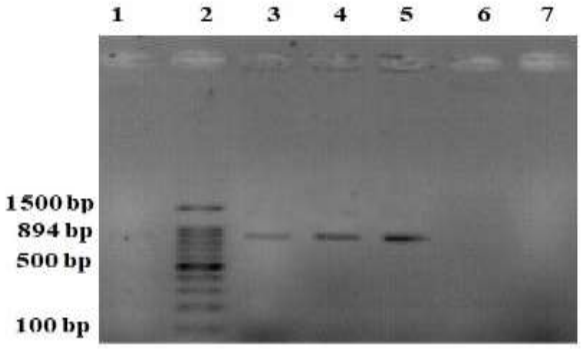

**Figure 1: Agarose gel electrophoresis of PCR product of 23s rRNA gene;
2: ladder (100 bp), 1, 3, 4: positive PCR products for *S. aureus* (894 bp), 5: positive control (894 bp), 6: negative control, 7: negative PCR product**

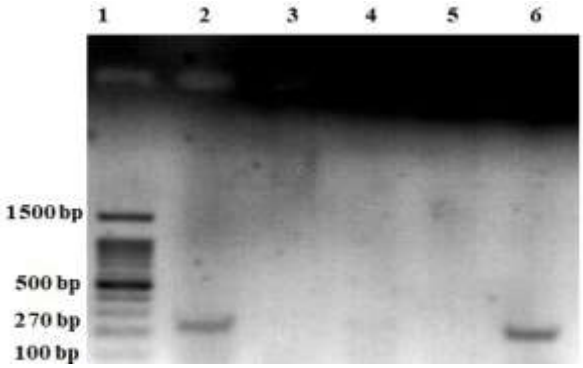

Figure 2. Agarose gel electrophoresis of PCR product of nuc gene; 1: Ladder (100 bp), 2: positive PCR product for *S. aureus* (270 bp), 3, 4: negative PCR product, 5: negative control, 6: positive control (270 bp)

PCR test was performed to identify the desired virulence genes on the DNA samples that were positive for *S. aureus*. Electrophorese of PCR products showed the band of 393 bp for Hlb gene, 431 bp for Hla gene, 758 bp for Coa gene, 950 bp for fnbA gene, 972 bp for fnbB gene, 504 bp for lukD gene 614 bp for lukE gene and 556 bp for ClfB gene for positive samples (Figures 3-10).

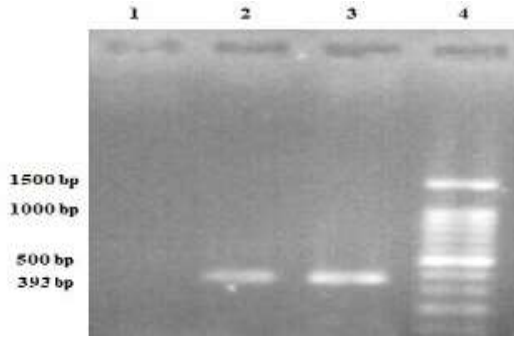

**Figure 3. Agarose gel electrophoresis of PCR product of Hlb gene;
1: negative control, 2, 3: positive PCR product for Hlb gene (393 bp), 4: ladder (100 bp)**

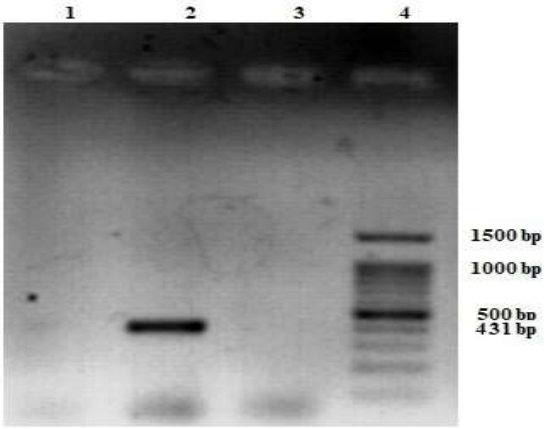

Figure 4. Agarose gel electrophoresis of PCR product of Hla gene; 1: negative control, 2: positive PCR product for Hla gene (431 bp), 3: negative PCR product, 4: ladder (100 bp)

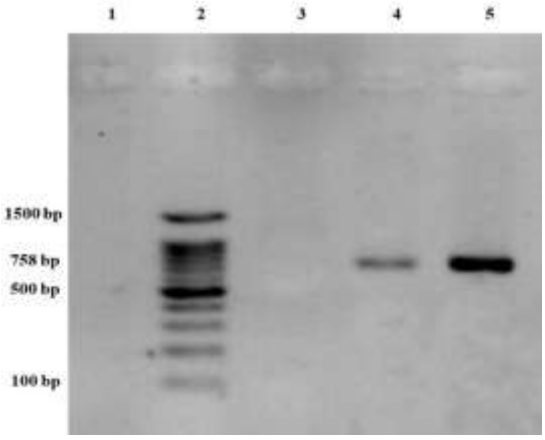

**Figure 5. Agarose gel electrophoresis of PCR product of Coa gene;
1: negative control, 2: leader (100 bp), 4, 5: positive PCR products for Coa gene (758 bp), 3: negative PCR product**

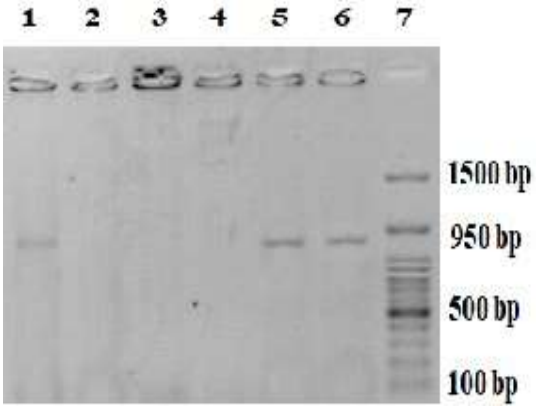

Figure 6. Agarose gel electrophoresis of PCR product of fnbA gene;
1, 5, 6: positive PCR product for fnbA gene (950 bp), 2: negative control, 3, 4 negative PCR product, 7: ladder (100 bp)

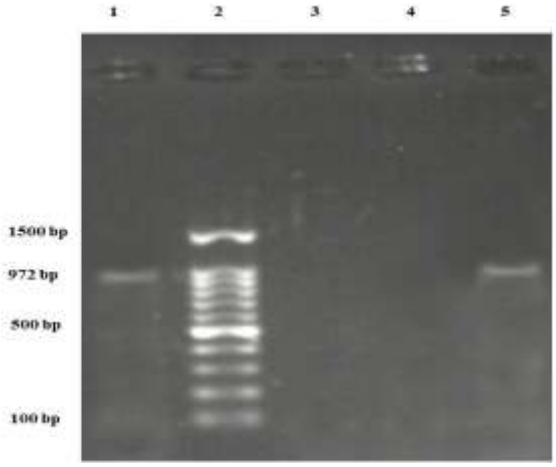

Figure 7. Agarose gel electrophoresis of PCR product of fnbB gene;
1, 5: positive PCR product for fnbB gene (972 bp), 2: ladder (100 bp), 3: negative control, 4: negative PCR product

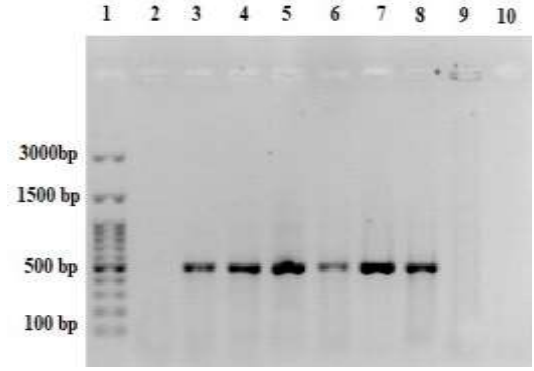

Figure 8. Agarose gel electrophoresis of PCR product of lukD gene;
1: ladder (100 bp), 3-8: positive PCR product for lukD gene (504 bp), 9, 10: negative PCR product, 2: negative control

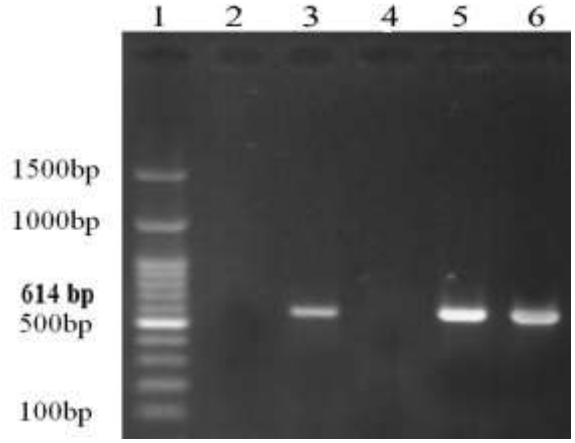

Figure 9. Agarose gel electrophoresis of PCR product of lukE gene;
1: ladder (100 bp), 2: negative control, 3,5,6: positive PCR products for lukE gene (614 bp), 4: negative PCR product

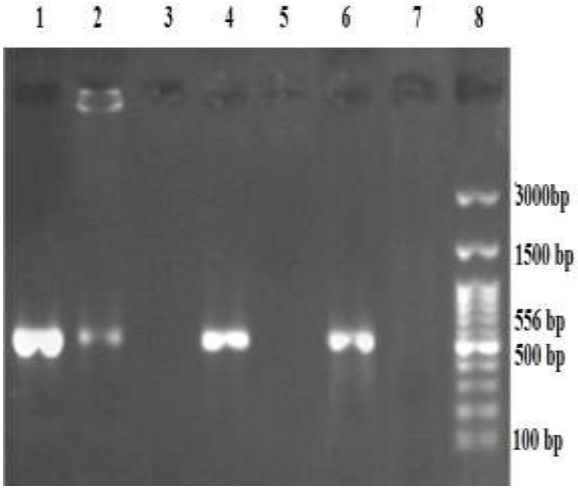

Figure 10. Agarose gel electrophoresis of PCR product of ClfB gene;
1, 2, 4, 6: positive PCR product for ClfB gene (556 bp), 3, 5: negative PCR product, 7: negative control, 8: ladder (100 bp)

Positive samples for *S. aureus* in PCR test were examined for the presence of virulence factors. The highest frequency was related to the Coa gene (90.32%), followed by ClfB (87.09%), LukD and fnbB (80.64%), LukE (77.41%), fnbA (74.19%), Hla (48.38%) and the lowest frequency was related to Hlb gene (45.16%) (Table 4).

Table 4: Frequency of virulence genes of *S. aureus* isolates involved in mastitis cases

Violent gene	Frequency of positive cases	Positive frequency percentage
Hla	15	48.38
Hlb	14	45.16
Coa	28	90.32
fnbB	25	80.64
fnbA	23	74.19
LukD	25	80.64
LukE	24	77.41
ClfB	27	87.09

Discussion

The aim of the current study was to trace the molecular virulence factors of selected local isolates of *Staphylococcus aureus* involved in bovine mastitis in Chaharmahal and Bakhtiari province. It has been proven that the virulence genes of isolated *S. aureus* are different in different geographical regions. It is also believed that there is a significant relationship between the severity of mastitis and virulence factors produced by *S. aureus* (Momtaz et al, 2010; Tegegne et al, 2021).

In this study, the prevalence rate of mastitis caused by *S. aureus* in the studied population was equal to 17.2% (31 positive out of 180 samples). Different percentages of mastitis caused by *S. aureus* have been reported in Iran and other countries. The difference in frequency can be due to differences in the type of management, herd size, use of bedding materials, the last lactation of mastitis cows, breed, age, fertility, stage of lactation, milk production, the health of the udder and feet, condition of the udder and morphology of the end of the udder and Season (Zadoks et al, 2001). Among the 31 positive samples, 29 (93.5%) and 2 (6.4%) were related to subclinical and clinical mastitis, respectively. In most published reports, *S. aureus* has been isolated from subclinical mastitis; in some cases, this infection in clinical mastitis has also been reported. In the study of Momtaz et al., the percentage of samples with *S. aureus* infection was stated as 23.88% (Momtaz et al, 2010).

In the current study, 28 (90.32%) of 31 positive samples had the Coa virulence factor gene. The observation of the 758 bp band for the Coa gene indicated positive results for the presence of this gene. According to the studies of Seyedi-Marghaki et al, (2019) and Pacha et al, (2020), coagulase is secreted by all strains of *S. aureus* and is commonly used to identify these bacteria in human infections. Dubin et al, (2013) have stated that the biological activity of coagulase is made by the biding domain of prothrombin. It nonproteolytically converts prothrombin to active thrombin which converts fibrinogen into fibrin filaments, stimulates plasma or blood coagulation and prevents digestion, and prevents their destruction by phagocytic cells or within such cells. In addition to the explored base pairs, according to Effendi et al. (2019), the presence of Coa genotypes varies by geographic location, the genotypes that predominate in each location, can be explained by pathogen-host coevolution and differences in management, nutrition, locations, and reservoir bacteria and the environment explained. It can also be concluded that the Coa gene is easily an epidemiological tool to detect the variant strain of *S. aureus* (Effendi et al, 2019). Upon the results of Mohammadi and Faghri (2019) 45 samples of *S. aureus* out of 150 studied samples, were confirmed by biochemical methods. Thirty-six isolates (80%) carried the Coa gene from the previous positive samples. Two different genotypes of the Coa gene were obtained, including the bp680 fragment in 20 samples and the bp750 fragment in 16 samples (Mohammadi and Faghri, 2019).

In the present study, 27 positive samples (87.09%) had the virulence factor gene ClfB. The presence of 556 base pairs band for the Clfb gene was considered a positive result for this gene. Mulcahy et al, (2012) reported that the adhesion of *S. aureus* to surfaces during colonization is facilitated by the protein clamping factor B (ClfB)

through high-affinity interactions with the stratum corneum. The clfB gene is carried by most strains of *S. aureus* (Mulcahy et al, 2012). In the study of Lacey et al, (2019) it was mentioned that Clfb is involved in the formation of abscesses by *S. aureus*. Ri Jung et al, (2022) evaluated the identification of pathogens in enterotoxin-producing *S. aureus* from the milk tank. In this study, the prevalence of fnbA (92.5% vs. 52.8%), clfA (50.0% vs. 20.8%), and clfB (37.5% vs. 3.8%) genes were significantly different between enterotoxin-producing and non-enterotoxin-producing isolates.

In the present study, 25 positive samples (80.64%) and 24 positive samples (77.41%) had luk D and luk E virulence factor genes, respectively. The observed band was 504 bp for the luk D gene, and 614 bp for the luk D gene was considered to indicate positive results for the presence of these genes. *Staphylococcus aureus* produces Leukotoxin E and D, lyse host cells and increases bacterial virulence. Studies conducted in China used PCR to screen 180 *S. aureus* isolates and showed that 81.4 to 92% of the isolates contained leukotoxin E and D. In a study conducted in Germany, a similar result (82%) was observed in blood isolates (Bennett & Thomsen, 2020). In their study, Yamada et al, (2005) reported the isolation of 96% for leukotoxin D.

In the present study, 25 positive samples (80.64%) and 23 positive samples (74.19%) had virulence factor genes fnbB and fnbA, respectively. The observation of 972 base pairs band for the fnbB gene and 950 base pairs band for the fnbA gene indicating the positive results were considered for the presence of these genes. Fibronectin-binding proteins (FnbA and FnbB) are effective in tissue invasion in various pathological conditions and replacement levels. Some clinical strains of *S. aureus* have both fnbA and fnbB genes, but some have only one gene. It has been shown that either FnbA or FnbB alone is sufficient for invasion. Overexpression of fnb genes leads to increased invasion of bovine mammary epithelial cells (bMEC) (Soltani et al, 2019). Magro et al, (2017) showed that the most common virulence factors in high-prevalence isolates were fibrinogen-binding protein B (fnbB), aggregation factor B (ClfB), and serine aspartate repeat protein C (sdrC) genes. Abad et al, (2020) reported the distribution percentage of fnbA, fnbB, cna, and |Hla genes in *S. aureus* isolate as 81, 81, 73, and 30, respectively.

In the present study, 15 positive samples (48.34%) and 14 positive samples (45.16%) had Hla and Hlb virulence factor genes, respectively. The band size of 431 base pairs for the Hla gene and 393 base pairs for Hlb gene were considered positive for the presence of these genes. α-Hemolysin (Hla), a small β-barrel pore-forming toxin, is essential for *S. aureus* virulence in various animal models. The severity of *S. aureus's* clinical symptoms correlates with alpha toxin's expression. Most clinical isolates, including strains with antibiotic-resistant, express Hla. In addition, Hla is a primary virulence factor that is used by *S. aureus* to escape from the host's immune system or antibiotics. In the studies conducted, the Hla gene was expressed more than the Hlb gene (Wu et al, 2019). Hlb is a phospholipase with sphingomyelin properties for the production of ceramide and phosphocholine (Rohmer and Wolz, 2021). Jain et al, (2022) in their study, stated that among *S. aureus*, most isolates have the blaZ gene (92.73 percent), followed by coa (89.09 percent), Hlb (60 percent) and Hla (49.09 percent).

Reviewing the literature, it seems that genes of interest in the present study have been considered in numerous studies mentioning the severity determination, prevention, control, treatment and vaccine development worldwide. Moreover, the correlation between these gens and the virulence of *S. aureus* is well discussed. The characteristics are specific and different from other genotypes. The isolation percentage of the desired genes in this study

overlaps with other studies, and the differences could be due to genotype differences, farm management methods, sampling time, sampling method, geographic location, pathogen-host coevolution, nutrition, places, reservoir bacteria, and the environment. Indeed, molecular epidemiology studies have stated many less common strains to be found alongside the common strains of *S. aureus* in many herds.

Acknowledgments

The authors thank Shahrekord University (Shahrekord, Iran) for funding this research and providing facilities. The authors wish to give special thanks to the farmers that helped and supported us in sample collection.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was funded by Applied Research Centre, Vice Chancellor for Research of Shahrekord University, Shahrekord, Iran.

References

Abad, H. E. K., Sadeghi, J., Aghazadeh, M., Ahangarzadeh Rezaee, M., Samadi Kafil, H., & Ahangar Oskouee, M. (2020). Frequency of fnbA, fnbB, hla and cna genes in Staphylococcus aureus isolates obtained from blood cultures and their antimicrobial susceptibility pattern in Tabriz, Iran. *Archives of Pharmacy Practice*, *11*(S1), 137-43.

Bennett, M. R., & Thomsen, I. P. (2020). Epidemiological and clinical evidence for the role of toxins in S. aureus human disease. *Toxins*, *12*(6), 408.

Bohach, G. A. (2006). Staphylococcus aureus exotoxins. *Gram-Positive Pathogens*, 464-477.

Divyakolu, S., Chikkala, R., Ratnakar, K. S., & Sritharan, V. (2019). Hemolysins of Staphylococcus aureus—An update on their biology, role in pathogenesis and as targets for anti-virulence therapy. *Advances in Infectious Diseases*, *9*(2), 80-104.

Dubin, G., Koziel, J., Pyrc, K., Wladyka, B., & Potempa, J. (2013). Bacterial proteases in disease–role in intracellular survival, evasion of coagulation/fibrinolysis innate defenses, toxicoses and viral infections. *Current pharmaceutical design*, *19*(6), 1090-1113.

Effendi, M. H., Hisyam, M. A. M., Hastutiek, P., & Tyasningsih, W. (2019). Detection of coagulase gene in Staphylococcus aureus from several dairy farms in East Java, Indonesia, by polymerase chain reaction. *Veterinary World*, *12*(1), 68-71.

Fowler, T., Wann, E. R., Joh, D., Johansson, S., Foster, T. J., & Höök, M. (2000). Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell β1 integrins. *European journal of cell biology*, *79*(10), 672-679.

Geoghegan, J. A., & Foster, T. J. (2017). Cell wall-anchored surface proteins of Staphylococcus aureus: many proteins, multiple functions. *Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis*, 95-120.

Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., DeBoy, R.T., Ravel, J., & et al. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. *Journal of bacteriology*, *187*(7), 2426-2438.

Holtzhauer, M. (2006). *Basic methods for the biochemical lab*. Berlin, Heidelberg: Springer Berlin Heidelberg.

Jain, V. K., Singh, M., Joshi, V. G., Chhabra, R., Singh, K., & Rana, Y. S. (2022). Virulence and antimicrobial resistance gene profiles of Staphylococcus aureus associated with clinical mastitis in cattle. *Plos one*, *17*(5), e0264762.

Jung, H. R., & Lee, Y. J. (2022). Characterization of Virulence Factors in Enterotoxin-Producing Staphylococcus aureus from Bulk Tank Milk. *Animals*, *12*(3), 301.

Lacey, K. A., Mulcahy, M. E., Towell, A. M., Geoghegan, J. A., & McLoughlin, R. M. (2019). Clumping factor B is an important virulence factor during Staphylococcus aureus skin infection and a promising vaccine target. *PLoS pathogens*, *15*(4), e1007713.

Lee, I. W., Kang, L., Hsu, H. P., Kuo, P. L., & Chang, C. M. (2010). Puerperal mastitis requiring hospitalization during a nine-year period. *American journal of obstetrics and gynecology*, *203*(4), 332-e1.

Leitão, J. H. (2020). Microbial virulence factors. *International journal of molecular sciences*, *21*(15), 5320.

Magro, G., Biffani, S., Minozzi, G., Ehricht, R., Monecke, S., Luini, M., & Piccinini, R. (2017). Virulence genes of S. aureus from dairy cow mastitis and contagiousness risk. *Toxins*, *9*(6), 195.

McAdow, M., Missiakas, D. M., & Schneewind, O. (2012). Staphylococcus aureus secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. *Journal of innate immunity*, *4*(2), 141-148.

Mohammadi, M., & Faghri, J. (2019). Genetic diversity of Staphylocoagulase genes (Coa) among methicillin-resistant Staphylococcus aureus isolates at clinical specimens of blood and urinary infections. *Tehran University Medical Journal*, *77*(4), 208-215.

Mulcahy, M. E., Geoghegan, J. A., Monk, I. R., O'Keeffe, K. M., Walsh, E. J., Foster, T. J., & McLoughlin, R. M. (2012). Nasal colonisation by Staphylococcus aureus depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS pathogens*, *8*(12), e1003092.

Momtaz, H., Rahimi, E., & Tajbakhsh, E. (2010). Detection of some virulence factors in Staphylococcus aureus isolated from clinical and subclinical bovine mastitis in Iran. *African Journal of Biotechnology*, *9*(25), 3753-3758.

Niehues, H. (2017). *From sequence to function: Understanding of genetic risk factors for psoriasis and atopic dermatitis* (Doctoral dissertation, [Sl: sn]).

Niemann, H. H., Schubert, W. D., & Heinz, D. W. (2004). Adhesins and invasins of pathogenic bacteria: a structural view. *Microbes and infection*, *6*(1), 101-112.

Nickerson, S. C., Saxon, A., Fox, L. K., Hemling, T., Hogan, J. S., Morelli, J., ... & Petersson, L. (2004). National Mastitis Council: Recommended protocols for evaluating efficacy of postmilking teat germicides. In *NMC Annual Meeting Proceedings* (pp. 379-399).

Oaks Jr, S. C., Shope, R. E., & Lederberg, J. (Eds.). (1992). Emerging infections: microbial threats to health in the United States. National Academies Press.

Oliveira, D., Borges, A., & Simões, M. (2018). Staphylococcus aureus toxins and their molecular activity in infectious diseases. *Toxins*, *10*(6), 252.

Pacha PA, Munoz MA, Paredes-Osses E, Latorre AA. Virulence profiles of Staphylococcus aureus isolated from bulk tank milk and adherences on milking equipment on Chilean dairy farms. Journal of dairy science. 2020 May 1;103(5):4732-4737.

Parker, D. (2018). A live vaccine to Staphylococcus aureus infection. *Virulence*, *9*(1), 700-702.

Peterson, J. W. (1996). Bacterial pathogenesis. *Medical Microbiology. 4th edition*.

Pollard, B. S., & Pollard, H. B. (2018). Induced pluripotent stem cells for treating cystic fibrosis: state of the science. *Pediatric Pulmonology*, *53*(S3), S12-S29.

Rohmer, C., & Wolz, C. (2021). The role of hlb-converting bacteriophages in Staphylococcus aureus host adaption. *Microbial Physiology*, *31*(2), 109-122.

Salgado-Pabón, W., Herrera, A., Vu, B. G., Stach, C. S., Merriman, J. A., Spaulding, A. R., & Schlievert, P. M. (2014). Staphylococcus aureus β-toxin production is common in strains with the β-toxin gene inactivated by bacteriophage. *The Journal of infectious diseases*, *210*(5), 784-792.

Seilie, E. S., & Wardenburg, J. B. (2017, December). Staphylococcus aureus pore-forming toxins: The interface of pathogen and host complexity. In *Seminars in cell & developmental biology* (Vol. 72, pp. 101-116). Academic Press.

Seyedi-Marghaki, F., Kalantar-Neyestanaki, D., Saffari, F., Hosseini-Nave, H., & Moradi, M. (2019). Distribution of aminoglycoside-modifying enzymes and molecular analysis of the coagulase gene in clinical isolates of methicillin-resistant and methicillin-susceptible Staphylococcus aureus. *Microbial Drug Resistance*, *25*(1), 47-53.

Soltani, E., Farrokhi, E., Zamanzad, B., Shahini Shams Abadi, M., Deris, F., Soltani, A., & Gholipour, A. (2019). Prevalence and distribution of adhesins and the expression of fibronectin-binding protein (FnbA and FnbB) among Staphylococcus aureus isolates from Shahrekord Hospitals. *BMC Research Notes*, *12*(1), 1-5.

Sommerhäuser, J., Kloppert, B., Wolter, W., Zschöck, M., Sobiraj, A., & Failing, K. (2003). The epidemiology of Staphylococcus aureus infections from subclinical mastitis in dairy cows during a control programme. Veterinary microbiology, 96(1), 91-102.

Tam, K., & Torres, V. J. (2016). Staphylococcus aureus Secreted Toxins & Extracellular Enzymes Kayan. *Physiology & Behavior*, *176*, 139-148.

Tan, L., Li, S. R., Jiang, B., Hu, X. M., & Li, S. (2018). Therapeutic targeting of the Staphylococcus aureus accessory gene regulator (agr) system. *Frontiers in microbiology*, *9*, 55.

Tegegne, D. T., Mamo, G., Waktole, H., & Messele, Y. E. (2021). Molecular characterization of virulence factors in Staphylococcus aureus isolated from bovine subclinical mastitis in central Ethiopia. *Annals of Microbiology*, *71*(1), 1-8.

Wu, S. C., Liu, F., Zhu, K., & Shen, J. Z. (2019). Natural products that target virulence factors in antibiotic-resistant Staphylococcus aureus. *Journal of agricultural and food chemistry*, *67*(48), 13195-13211.

Yamada, T., Tochimaru, N., Nakasuji, S., Hata, E., Kobayashi, H., Eguchi, M., Kaneko, J., Kamio, Y., Kaidoh, T.,& Takeuchi, S. (2005). Leukotoxin family genes in Staphylococcus aureus isolated from domestic animals and prevalence of lukM–lukF-PV genes by bacteriophages in bovine isolates. *Veterinary microbiology*, *110*(1-2), 97-103.

Yazarlu, O., Iranshahi, M., Kashani, H. R. K., Reshadat, S., Habtemariam, S., Iranshahy, M., & Hasanpour, M. (2021). Perspective on the application of medicinal plants and natural products in wound healing: A mechanistic review. *Pharmacological research*, *174*, 105841.

Zadoks, R. N., Allore, H. G., Barkema, H. W., Sampimon, O. C., Wellenberg, G. J., Gröhn, Y. T., & Schukken, Y. H. (2001). Cow-and quarter-level risk factors for Streptococcus uberis and Staphylococcus aureus mastitis. *Journal of Dairy Science*, *84*(12), 2649-2663.

Received: 11.09.2023
Accepted: 08.01.2024

DOI: 10.22055/IVJ.2024.415896.2634

میزان شیوع عوامل حدت منتخب جدایه‌های استافیلوکوکوس اورئوس دخیل در ورم پستان گاو در استان چهارمحال و بختیاری- ایران

بهنام روزبهان[۱]، ناصر شمس‌اسفندآبادی[۲*]، علی کدیور[۳]، اعظم مختاری[۴] و نجمه داودیان[۵]

[۱] دانشجوی دکترای تخصصی مامایی و بیماری‌های تولید مثل دام، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران
[۲] استاد گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران
[۳] دانشیار گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران
[۴] دانشیار گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران
[۵] دانشیار پژوهشکده فناوری جنین دام، دانشگاه شهرکرد، شهرکرد، ایران

تاریخ دریافت: ۱۴۰۲/۶/۲۰ تاریخ پذیرش: ۱۴۰۲/۱۰/۱۸

چکیده

مشخص شده است که بین شدت ورم پستان و فاکتورهای حدت تولید شده توسط باکتری رابطه مستقیم وجود دارد. شناسایی فاکتورهای حدت برای طراحی واکسن‌های مناسب جهت پیش‌گیری از ورم پستان لازم است. مطالعه حاضر با هدف شناسایی مولکولی عوامل حدت منتخب جدایه‌های بومی استافیلوکوکوس اورئوس دخیل در ورم پستان گاو در استان چهارمحال و بختیاری انجام گرفت. تعداد ۱۸۰ نمونه شیر از گاوهای مبتلا به ورم پستان بالینی (۳۷ نمونه، ۲۰/۶ درصد) و تحت بالینی (۱۴۳ نمونه، ۷۶/۴ درصد) از ۸ گاوداری شیری نیمه صنعتی استان چهارمحال و بختیاری- ایران جمع‌آوری شد. پس از کشت و خالص‌سازی، تست‌های کوگولاز، کاتالاز و اکسیداز انجام شد. استخراج DNA از کلنی‌های مشکوک به استافیلوکوکوس اورئوس انجام شد. تایید نهایی با استفاده از آزمون PCR بر روی ژن اختصاصی 23S rRNA باکتری انجام گرفت. از مجموع از ۱۸۰ نمونه اخذ شده، تعداد ۳۱ نمونه (۱۷/۲۲ درصد) با استفاده از آزمایش PCR از نظر استافیلوکوکوس اورئوس مثبت تشخیص داده شدند. از این تعداد ۲ مورد مربوط به ورم پستان‌های بالینی و ۲۹ مورد مربوط به ورم پستان‌های تحت بالینی بودند. بیش‌ترین فراوانی ژن‌های حدت مربوط به ژن *Coa* (۹۰/۳۲ درصد) بود و سپس به ترتیب *ClfB* (۸۷/۹ درصد)، *LukD* و *fnbB* (۸۰/۶۴ درصد)، *LukE* (۷۷/۴۱ درصد)، *fnbA* (۷۴/۱۹ درصد)، *Hla* (۴۸/۳۸ درصد). کم‌ترین فراوانی مربوط به ژن *Hlb* (۴۵/۱۶ درصد) بود. بر اساس نتایج به دست آمده در مطالعه حاضر، شناسایی فاکتورهای حدت استافیلوکوکوس اورئوس پتانسیل استفاده در برنامه‌های تولید واکسن جهت پیش‌گیری از ورم پستان را دارد.

کلمات کلیدی: ورم پستان، استافیلوکوکوس اورئوس، فاکتورهای حدت

* نویسنده مسئول: ناصر شمس‌اسفندآبادی، استاد گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران
E-mail: drn_shams@yahoo.com