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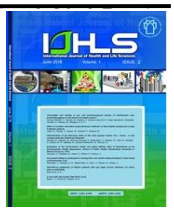

Biotyping of *Staphylococcus aureus* strains isolated from humans and bovine raw milk samples in Hamedan province

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ABSTRACT

Staphylococcus aureus causes a wide range of diseases in humans and animals. Rapid and precise typing of *S. aureus* is a prerequisite for epidemiological surveillance and controlling of infection caused by this bacterium. In this case, biotyping is a simple, cheap, and effective method for epidemiological investigations. A total of 143 *S. aureus* strains isolated from human (40 patient strains, 20 carrier strains) and bovine raw milk samples (83 strains from 7 herds) were genotypically confirmed by polymerase chain reaction (PCR), and phenotypically assessed using a biotyping method to determine the possible sources of contamination. Of 143 examined strains by the biotyping method, 14 strains belonged to human ecovars, while 11, 25, and 12 strains were classified as bovine, sheep, and poultry ecovars, respectively. Meanwhile, 61 strains were found to be non-host specific (NHS) biotypes, and 20 strains were not typable by this method. The results of the present study showed that in Hamedan province, humans and bovine raw milk samples were frequently contaminated by strains belonging to the K-B-CV:C biotype. However, among host specific (HS) biotypes, sheep ecovar was the most common biotype. The results indicated possibility of transmission of different ecovars among various species.

Introduction

S. aureus which is found on the nasal membrane, the skin of warm-blooded animals, and food of animal origins, can potentially cause a wide range of infections in humans and animals. In humans, *S. aureus* is an important food-borne pathogen, and it is major causative agent of hospital and community-associated infection [1, 2]. In cattle, it can produce acute and chronic mastitises, which are too difficult to be cured [3, 4]. Therefore, it is considered a major pathogen, and the identification and typing of its isolates have diagnostic value. This bacterium could be characterized using molecular methods such as PCR. For this, a species-specific PCR has been introduced using primers which detect a gene called, *femA*. This highly conserved gene is universally present in all *S. aureus* isolates [5-7]. Knowing the types and ecovars of *S. aureus* isolates is a crucial step for epidemiological surveillance and the controlling of infections caused by this bacterium. Numerous biochemical and molecular techniques and methods can be used for the characterization of *S. aureus* strains [8]. In the past few years, *S. aureus* isolates have been typed by phenotypic methods such as antibiotic resistance typing, biotyping, and phage typing [9, 10]. Among these, biotyping is a rapid and inexpensive method for the typing of *S. aureus* strains. This can differentiate strains isolated from various sources into host specific (NH) ecovars, including human, bovine, sheep, and poultry biotypes and non-host specific (NHS) ecovars [9].

Moreover, although several researchers tried to type *S. aureus* isolates based on the molecular assays, few studies have been conducted to phenotypically type staphylococcal strains into various ecovars [11-13]. The determination of origins of strains can help in delineating their circulation among different species. This is absolutely important, as there is an increasing concern about the potential transmission of *S. aureus* strains among humans and animal species through the consumption and handling of foods of animal origin. Thus, the present study was carried out to compare the sources of contamination and distribution of the strains that were isolated from different human and bovine raw milk samples using the biotyping method.

Materials and Methods

Bacterial isolates

A total of 143 *S. aureus* strains, which had been previously isolated from bovine raw milk samples (83 strains from 7 herds) and human samples (60 isolates: 40 and 20 from patients and healthy carriers, respectively), were examined to determine their biotypes using Devriese's method.

DNA extraction and molecular identification of isolates

DNA extraction was carried out from 10 ml overnight cultures of strains in tryptic soy broth (TSB) by a phenol-chloroform method [14]. The identity of 143 *S. aureus* isolates was confirmed using amplification of the *femA* gene in a species-specific PCR. The sequence of previously described oligonucleotide primers are given in Table 1 [15]. The PCR reaction (25 µl) contained 3 µl of template DNA, 2.5 µl of 10× PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.25 µl of 5 U/µl of Taq DNA polymerase, and 25 pmol of each of the primers. The PCR amplification was performed under the following conditions: initial denaturation at 94°C for 6 min followed by denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, extension at 72°C for 1 min (35 cycles), and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide (0.5 µg/mL).

Biotyping:

Biotyping of staphylococcal strains was performed according to the method described by Devriese and Rajamohan [5, 16], based on the evaluation of β-haemolysis on the sheep blood agar, colonial types on tryptic soy agar (TSA) containing crystal violet, the coagulating of bovine plasma in 6 h, and the ability of casein hydrolysis in skim milk agar (Merck).

Evaluation of β-hemolysis

B-hemolysis activity was evaluated using a culture of staphylococci strains on blood agar. Moreover, to get better results and remove any possible anti-hemolysin compounds in the blood, the red blood cells were washed three times with sterile saline and finally resuspended in saline to the original volume.

Coagulating of Bovine plasma

Bovine plasma was diluted three times with normal saline, and 0.5 ml of this suspension was added to 0.5 ml of an overnight broth culture of strains inside a tube. In the following, the tubes were incubated at 37ºC, and the results were observed after 6 h.

Colonial appearance on crystal violet TSA

The colors of grown strains on TSA contained 6 and 8 mg of crystal violet per liter, and they were recorded after 24 h of incubation at 37ºC. The blue/purple, yellow, and white colonies were considered as type C, type A, and type E, respectively.

Casein hydrolysis test

Ability to casein hydrolysis was examined on the skim milk agar medium contained 10 ml human serum per liter. The results were observed after 24 h of incubation at 37ºC. The emergence of clear halo around the colonies was considered as a positive result.

Results

According to the biochemical profile (catalase, DNase, mannitol salt agar, and slide coagulase tests) and amplification of the *femA* gene, all of the 143 isolates confirmed as *S. aureus* strains (Figure 1). The results obtained from grown colonies on

blood agar and skim milk agar media showed that 66 and 21 isolates had the ability to produce β-hemolysis and hydrolyze casein, respectively. In addition, 32 strains indicated a positive reaction in a tube coagulase test after 6 h of incubation. The colors of grown isolates on crystal violet TSA were recorded after 24 and 48 h of incubation. The results revealed that 85, 38, and 20 strains produced purple, yellow, and white colonies, respectively. However, the results were similar for TSA media, as they contained two different concentrations of crystal violet.

In the present study, 123 *S. aureus* strains (83.4% of all isolates) were typed based on the previously described method by Devriese. Accordingly, 61 isolates belonged to non-host specific biotypes, while 62 isolates were classified in the host specific group, including human, bovine, sheep, and poultry ecovars. The most frequent biotype in *S. aureus* strains isolated from both human and bovine milk samples were of sheep ecovar, whereas the less identified biotypes were of bovine ecovars. Detailed statistics are given separately for *S. aureus* strains isolated from human and bovine milk samples in Tables 2 and 3.

Table 1. Biotypes of *S. aureus* isolated from human sample

Sample	Group	Isolates(n)	Host specific (HS) biotypes				Non-host specific (NHS) biotypes					Non types
			Human	Bovine	Sheep	Poultry	K-B-CV:C	K-B+CV:C	K+B+CV:A	K+B-CV:A	K-B+CV:A	
Human isolates	Patient	40	5	1	5	3	5	10	4	0	0	7
	carrier	20	1	2	6	1	5	1	0	0	1	3
Total N(%)		60	6(10)	3(5)	11(18.3)	4(6.6)	10(16.6)	11(18.3)	4(6.6)	0	1(1.6)	10(16.6)

Table 2. Biotypes of *S. aureus* isolated from bovine raw milk

sample	Herd	Isolates(n)	Host specific (HS) biotypes				Non-host specific (NHS) biotypes					Non types
			Human	Bovine	Sheep	Poultry	K-B-CV:C	K-B+CV:C	K+B+CV:A	K+B-CV:A	K-B+CV:A	
Bovine raw milk	I	8	1	0	1	1	0	0	0	0	0	5
	II	3	0	0	0	0	1	0	0	1	0	1
	III	5	0	0	3	1	0	0	0	0	0	1
	IV	3	0	1	0	1	0	0	0	0	1	0
	V	6	3	0	0	0	2	1	0	0	0	0
	VI	27	0	4	4	1	16	0	0	1	0	1
	VII	31	4	3	6	4	4	1	6	1	0	2
Total N(%)		83	8(9.6%)	8(9.6%)	14(16.9%)	8(9.6%)	23(27.7%)	2(2.4%)	6(7.2%)	3(3.6%)	1(1.2%)	10(12%)

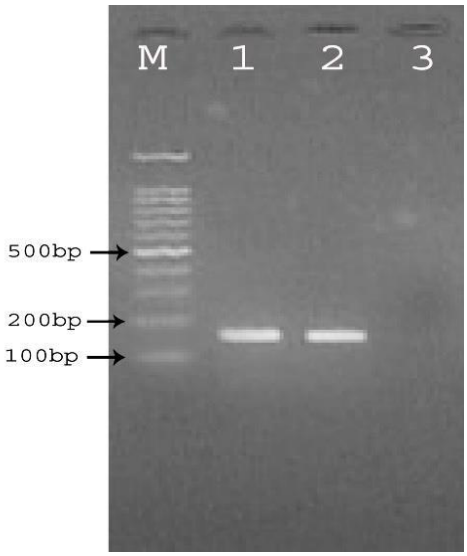

Figure 1. Electrophoresis of femA gene PCR products. Lane 1: femA positive control (ATCC 25923), Lanes 2: femA gene from S. aureus isolate. Lane 3: Negative control, Lane M: 100 bp DNA ladder.

Discussion

The typing of *S. aureus* is crucial to determine the possible sources of contamination and control infection caused by this bacterium, and different genotypic and phenotypic methods have been used for this purpose [17]. Although molecular methods are effective tools for *S. aureus* typing, they are often expensive, laborious, and technically demanding. Conversely, phenotypic methods are simple, cheap, and effective techniques for epidemiological investigations [8]. However, like other phenotypic methods, the discriminatory capability of biotyping is low. Meanwhile, due to the variable expression of bacterial genes under different conditions, the reproducibility of biotyping is lower than molecular methods [18]. Nevertheless, the biotyping method introduced by Devriese is the most appropriate method to determine ecovars of strains. The results of such a study can provide valuable information about the transmission of *S. aureus* isolates among humans and different animal species.

Rodríguez-Calleja et al. (2005), biotyped 26 S. aureus strains isolated from 51 different animals to assess their relationships. Their results showed that all of the isolates were typable by this method. However, they did not find any ovine, poultry, or bovine ecovars [19]. In another study, KITAI *et al*. (2004) tried to determine the relationship between different ecovars and enterotoxigenic strains. They reported that among 292 examined strains of *S. aureus* isolated from raw chicken meat, the majority of enterotoxigenic isolates belonged to human and poultry biotypes [20].

In the present study, a total of 143 S. aureus isolates from human and bovine samples were assessed by Devriese's biotyping method.

As results showed, different animal ecovars were characterized among human and bovine raw milk isolates. Rabello *et al*. (2005) also reported that different types of *S. aureus* could be found within a particular herd [21]. However, 42% of our strains were of NHS biotypes. This finding is in agreement with the report by Soltan Dallal *et al*. (2010) who showed that NHS biotypes were the predominant ecovars in food S. aureus strains [13]. Although the sheep ecovar was found to be the most prevalent HS biotype in the present study, depending on the tested samples, some studies reported different prevalent ecovars [13, 19]. Our finding also indicated that biotype K-B-CV:C had the highest frequency in collected samples from the Hamedan province. The predominance of a special biotype of *S. aureus* strains may result from its increased resistance to host immune responses with respect to other biotypes [22]. As recorded by Lee *et al*., the identification of the human ecovar among some of herds (I, V, III) may imply that *S. aureus* can be transmitted from human to cattle [23]. On the other hand, three human isolates were typed as bovine ecovar, suggesting that these strains were probably of human origin. In this case, Juhász-Kaszanyitzky *et al*. (2007) reported *S. aureus* can be transmitted from cows to humans who worked with these animals [24].

Conclusions

The results of the present study confirm the possibility of the transmission of *S. aureus* strains among several hosts, and this can be very important, especially when such strains carry antibiotic resistance genes. Therefore, it is recommended to take proper hygienic measures

on farms and food chains to reduce the risk of contamination of humans with these bacteria**.**

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