

Original Article

Comparison of Relation between Resistance Pattern to Erythromycin and Tetracycline and the Prevalence of Superantigens Coding Enterotoxins A and B in *Staphylococcus aureus* Isolated from Broiler Poultry in Ilam, Iran

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ABSTRACT

Staphylococcus aureus is a gram-positive coccus that, in specific conditions, is able to generate various diseases. By secreting different enterotoxins, this bacterium prepares the settings to attack the host; among these, enterotoxins A and B play the most important roles in food poisoning. This study was performed to trace the genes coding enterotoxins A and B in *Staphylococcus aureus* isolated from the clinic and poultry slaughterhouse. In addition, the present study analyzed the relation between the prevalence of these genes and resistance to erythromycin and tetracycline. This study was performed from October 2015 to December 2016. A total of 200 samples of noses and cloaca from broiler poultry farms in Ilam, Iran, were collected, including 150 samples from the slaughterhouse and 50 samples from the clinic isolated for separating *Staphylococcus aureus*. After bacterial culture and confirmation of biochemical tests, the samples were evaluated for the identification of *Staphylococcus aureus* and the resistance pattern to antibiotics regarding the presence of femA, tets, ermB, sea, and seb genes using the disk diffusion method and polymerase chain reaction test. Out of 200 tested samples, 112 strains of *Staphylococcus aureus* (56%) were identified from which 91 and 21 strains were associated with the poultry slaughterhouse and clinic, respectively, and all the samples were identified using biochemical tests. After the detection of femA gene as an exclusive gene for the identification of *Staphylococcus aureus* strain, 100 strains (50%) were confirmed to be contaminated with this bacterium. Out of 100 strains, 46%, 14%, and 5% possessed the genes coding enterotoxin A, the genes coding enterotoxin B, and both genes, respectively. The results of antibiotic tests showed that 85% and 86% of the examined strains were resistant to erythromycin and tetracycline, respectively. In the present study, the analysis performed using QuickCalcs software showed that the strains resistant to these two antibiotics possessing the sea gene were more prevalent than those possessing seb genes in the samples isolated from the poultry slaughterhouse. This comparison revealed that during the short period of broiler poultry farms growth, resistant strains were able to proliferate sea gene among the herd, and its prevalence increased until reaching into the slaughterhouse. This study showed that the relation between the genes resistant to erythromycin and tetracycline and the sea gene was close and significant.

Keywords: *Staphylococcus aureus*; Poultry; Enterotoxins A & B

Comparaison de la Relation entre le Profil de Résistance à l'Erythromycine et à la Tétracycline et la Prévalence des Super-antigènes Codant pour les Entérotoxines A et B chez *Staphylococcus aureus* Isolé à Partir des Volailles de Chair à Ilam (Iran)

Résumé: *Staphylococcus aureus* est un coccus à Gram positif qui, dans certaines conditions, est capable de causer diverses maladies. En sécrétant différentes entérotoxines, cette bactérie met en place les conditions pour attaquer l'hôte. Parmi celles-ci, les entérotoxines A et B jouent le rôle le plus important dans l'intoxication alimentaire.

Cette étude a été réalisée pour tracer les gènes codant les entérotoxines A et B chez *Staphylococcus aureus* isolé en clinique et en abattoir de volailles. En outre, la présente étude a analysé la relation entre la prévalence de ces gènes et la résistance à l'érythromycine et à la tétracycline. Cette étude a été réalisée d'octobre 2015 à décembre 2016. Un total de 200 échantillons de nez et de cloaque provenant d'élevages de volailles à Ilam, en Iran, ont été recueillis, dont 150 échantillons provenaient de l'abattoir et 50 échantillons ont été recueillis en clinique. Après culture bactérienne et l'identification de *Staphylococcus aureus* par tests biochimiques, les échantillons ont été évalués pour leur profil de résistance aux antibiotiques et la présence de gènes femA, tets, ermB, sea et seb en utilisant la méthode de diffusion sur disque et le test de réaction en chaîne par polymérase. Sur les 200 échantillons testés, 112 souches de *Staphylococcus aureus* (56%) ont été identifiées à l'aide de tests biochimiques, parmi lesquelles 91 et 21 souches étaient respectivement associées à l'abattoir et à la clinique. Après la détection du gène femA en tant que gène exclusif pour l'identification de *Staphylococcus aureus*, il a été confirmé que 100 échantillons (50%) étaient contaminées par cette bactérie. Sur les 100 isolats, 46%, 14% et 5% possédaient respectivement les gènes codant pour l'entérotoxine A, pour l'entérotoxine B et pour les deux entérotoxines simultanément. Les résultats des antibiogrammes ont montré que 85% et 86% des souches examinées étaient respectivement résistantes à l'érythromycine et à la tétracycline. Dans cette étude, l'analyse effectuée à l'aide du logiciel QuickCalcs a montré que les souches résistantes à ces deux antibiotiques possédant le gène de la mer étaient plus prévalentes que celles possédant les gènes seb dans les échantillons isolés de l'abattoir de volailles. Cette comparaison a révélé que pendant la courte période de croissance des poulets de chair, les souches résistantes étaient capables de proliférer le gène de la mer au sein du troupeau et que sa prévalence augmentait jusqu'à atteindre l'abattoir. Cette étude a montré que la relation entre les gènes de résistance à l'érythromycine et à la tétracycline et le gène de la mer était étroite et significative.

Mots-clés: *Staphylococcus aureus*; Volaille; Entérotoxines A & B

INTRODUCTION

One of the important infectious agents spreading food poisoning is *Staphylococcus* that contaminates food products, including poultry products, (Shekarforoush et al., 2013). Previous studies showed that enterotoxins A and B, produced by *Staphylococcus aureus*, are known as the main *Staphylococcus* food poisoning agent in the world (Saadati et al., 2008). Common methods for the detection of *Staphylococcus* enterotoxins genes and toxic shock syndrome include the enzyme-linked immunosorbent assay, latex agglutination, immunodiffusion, latex immunoassay, radioimmunoassay, and polymerase chain reaction (PCR). In these methods, the conditions for the desired antigen should be provided; however, in PCR and combined PCR, there is no need for antigens, and the genes are detectable with little or no toxins (Wang et al., 2002).

MATERIAL AND METHODS

Sampling. The samples were obtained from the nose and the cloaca of 15 herds of broiler poultry farms in Ilam, Iran, including Manesht Eyvan industrial poultry slaughterhouse, from October 2015 to December 2016. Moreover, 5 broiler poultry herds of different ages were referred to Day veterinary laboratory of Ilam. Then, 10 samples (5 from the nose and 5 from the cloaca) were isolated from each herd, and the herds were labeled from A to T. In addition, the site of sampling that is (N) for the nose and (C) for cloaca, was also labeled.

DNA extraction and molecular tests. After transferring the samples to Microbiology Laboratory of Ilam University, biochemical diagnostic tests were performed, including Gram staining, mannitol salt agar, hemolysis, catalysis, coagulase, DNase, and disk diffusion antimicrobial susceptibility. After isolating the strains suspicious of *Staphylococcus aureus* by biochemical tests, molecular PCR for the detection of femA gene was performed on the samples to confirm

the diagnosis. The *Staphylococcus aureus* strains identified by PCR were analyzed for detecting the presence of enterotoxin A and B genes, as well as genes resistant to erythromycin (*ermB*) and tetracycline (*tets*). The primers and materials used for molecular tests were obtained from Gene Fanavaran Company. The boiling method was used for DNA extraction (Reischl et al., 2000). Oligonucleotide sequences of the primers were used for the molecular tests (Table 1), and the heating schedule of the thermal cycler machine was utilized for gene replication in five separate reactions (Table 2). The products of PCR were analyzed by electrophoresis in a 1.2 % agarose gel and then

photographed under ultraviolet light.

Statistical analysis. The results of the tests were analyzed using QuickCals software in order to compare the samples isolated from the clinic and slaughterhouse and compare the relationship of resistance pattern to erythromycin and tetracycline with the prevalence of *sea* and *seb* genes.

RESULTS

Out of 200 samples isolated from noses and cloaca obtained from the clinic and slaughterhouse, 112 strains (56%) showed the existence of *Staphylococcus aureus* (Figure 1). Out of the 112 strains isolated from the

Table 1. Oligonucleotide sequences of used primers

Primers	Oligonucleotide sequences (3'-5')	Product size base pair (bp)
<i>sea</i> (Reverse)	5'ATTAACCGAAGGTTCTGT3'	270
<i>seb</i> (Reverse)	5'ATAGTGACGAGTTAGGTA3'	165
<i>Seu*</i> (Forward)	5'TGTATGTATGGAGGTGTA3'	-
<i>femA</i> (Forward)	5'CGATCCATATTTACCATATCA3'	450
<i>femA</i> (Reverse)	5'ATCACGCTCTTCGTTAGTT3'	
<i>tets</i> (Forward)	5'ATCAAGATATTAAGGAC3'	590
<i>tets</i> (Reverse)	5'TTCTCTATGTGGTAATC3'	
<i>ermB</i> (Forward)	5'AGACCTCGTCTAACCTTCGCTC3'	640
<i>ermB</i> (Reverse)	5'TCCATGTACTACCATGCCACAGG3'	

Seu*: Universal primer

Table 2. Polymerase chain reaction cycles

Duration (sec)	Stage	Temperature (°C)	Number of cycles
300	Initial denaturation	94	1
60	Denaturation	94	
		<i>femA</i> 58	
		<i>sea</i> 58	
30	Annealing	<i>seb</i> 60	35
		<i>tets</i> 55	
		<i>ermB</i> 50	
60	Extension	72	
300	Final extension	72	1



Figure 1. Number of *Staphylococcus aureus* identified by biochemical tests

clinic and slaughterhouse based on biochemical tests, *Staphylococcus aureus* strains were identified, and 100 strains contained *femA* gene (Figure 2).

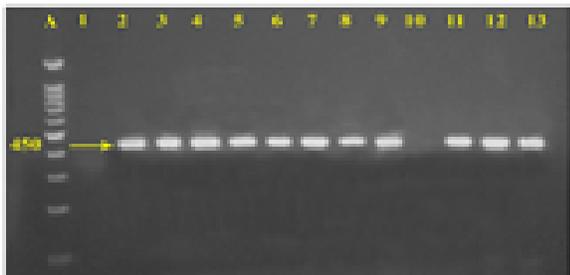


Figure 2. Electrophoresis products from polymerase chain reaction for identification of *femA* genes
 Column A: 100 base pair (bp) marker
 Column 1: Negative control (distilled water and no DNA)
 Column 2: Positive control
 Columns 3, 4, 5, 6, 7, 8, 9, 11, 12, 13: Polymerase chain reaction products of strains containing *femA* genes; length: 450 bp marker
 Column 10: Without *femA* gene

Out of 100 analyzed strains of *Staphylococcus aureus*, 46 (46%), 14 (14%), and 5 (5%) strains contained *sea* gene (Figure 4), *seb* gene (Figure 5), and both genes, respectively (Figure 3).

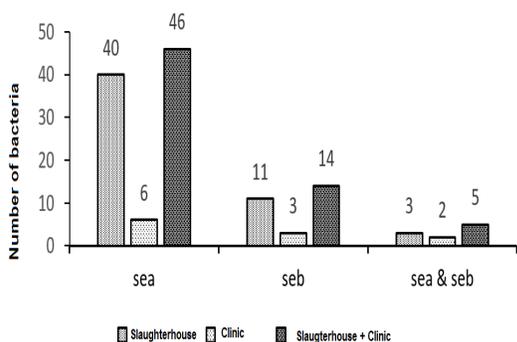


Figure 3. Results of polymerase chain reaction tests for tracing *sea* and *seb* genes

In the analyses performed to determine the sensitivity of isolated bacteria to erythromycin and tetracycline, the following results were obtained: The slaughterhouse samples were 90% resistant, 7% sensitive, and 3% semi-sensitive to erythromycin.

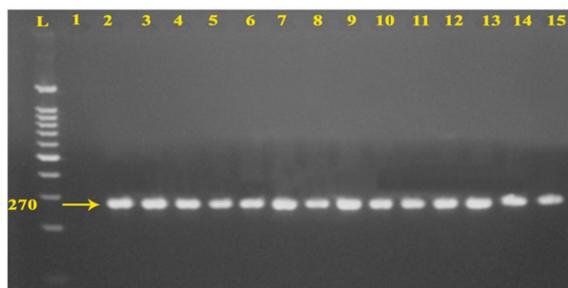


Figure 4. Electrophoresis of products from polymerase chain reaction for identification of *sea* gene
 Column L: 100 base pair (bp) marker
 Column 1: Negative control (distilled water and no DNA)
 Column 2: Positive control
 Columns 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13: Polymerase chain reaction products of strains containing *sea* genes; length: 270 bp marker

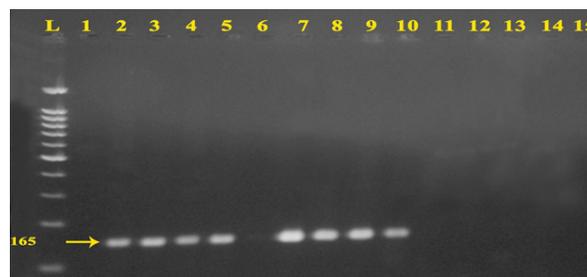


Figure 5. Electrophoresis of products from polymerase chain reaction for identification of *seb* gene
 Column L: 100 base pair (bp) marker
 Column 1: Negative control (distilled water and no DNA)
 Column 2: Positive control
 Columns 3, 4, 5, 7, 8, 9, 10, 11, 12, 13: Polymerase chain reaction products of strains containing *seb* genes; length: 165 bp marker

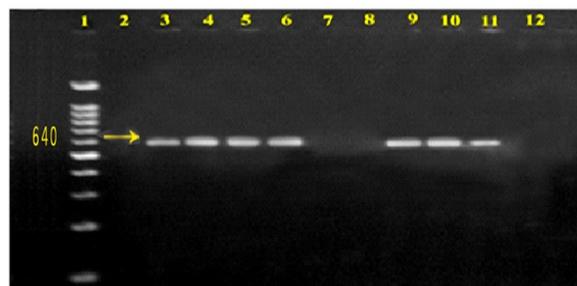


Figure 6. Electrophoresis of products from polymerase chain reaction for identification of erythromycin
 Column 1: 100 base pair (bp) marker
 Column 2: Negative control (distilled water and no DNA)
 Column 3: Positive control
 Columns 4, 5, 6, 9, 10, 11: Polymerase chain reaction products of strains containing *ermB* genes; length: 640 bp marker
 Column 7, 8, 12: Without *ermB* gene

Furthermore, the slaughterhouse samples were 88% resistant, 11% sensitive, and 1% semi-sensitive to tetracycline antibiotic. Moreover, the clinic samples were 81% resistant, 15% sensitive, and 4% semi-sensitive to erythromycin. In addition, the clinic samples were 84% resistant, 8% sensitive, and 8% semi-sensitive to tetracycline. In general, all the samples, including slaughterhouse and clinic samples, were 85% resistant, 11% sensitive, and 4% semi-sensitive to erythromycin (Figure 6). Moreover, all the samples were 86% resistant, 10% sensitive, and 4% semi-sensitive to tetracycline (Figure 7).

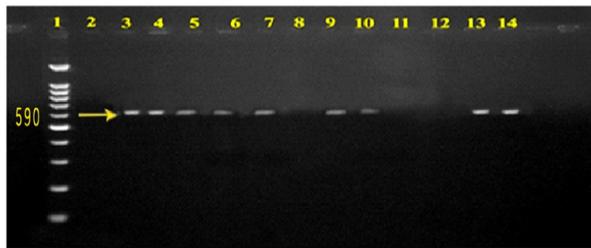


Figure 7. Electrophoresis of products from polymerase chain reaction for identification of tetracycline

Column 1: 100 base pair (bp) marker

Column 2: Negative control (distilled water and no DNA)

Column 3: Positive control

Columns 4, 5, 6, 7, 9, 10, 13, 14: Polymerase chain reaction products of strains containing tets genes; length: 590 bp marker

Column 7, 8, 12: Without tets gene

DISCUSSION

Staphylococcus aureus can be isolated from different foods, including milk and dairy products, meat and its by-products, and especially the foods that require long-term processing, due to its easy growth in different conditions (Eshraghi et al., 2009). Feizi et al. (2012) reported the contamination of poultry meat by *Staphylococcus aureus* as 81.75%. In a study carried out by Khakpoor et al. (2013), it was announced that individuals who carry *Staphylococcus aureus* in the nose are considered a source of the infection spread. This finding indicates that despite hygienic measures in broiler poultry farms, nearly all bacterial contaminations under study are observed in the samples of broiler poultry products processed in industrial

slaughterhouses across the country. Considering the role of following hygienic guidelines exactly in the economic output of broiler poultry farms and the fact that the rate of applying hygienic regulations in industrial poultry slaughterhouses has a close connection with the percentage of their product contamination, it seems that most secondary contaminations occur during slaughter or after that. Therefore, it is necessary to pay special attention to this crucial issue in order to reduce contamination and microbial count (Shekarforoush et al., 2013). Targeting virulence factors of bacteria, such as enterotoxins, hemolysins, has become the focus of many food industry researchers as a replacement for developing new kinds of anti-microbial preservatives (Parsaeimehr et al., 2010). The results of the present study using PCR among the identified *Staphylococcus aureus* strains showed that 46%, 14%, and 5% of the strains had *sea* gene, *seb* gene, and both genes, respectively. In a study carried out by Barati et al. (2006), out of 98 isolates that were investigated, 89 isolates were confirmed by multiplex PCR as *Staphylococcus aureus*. Moreover, 6 (6.74%) *Staphylococcus aureus* isolates were observed to be positive for *sea* gene (Barati et al., 2006). A result of a study reported that the prevalence of enterotoxin in *Staphylococcus aureus* from the blood and nose samples was 73%. In a study conducted by Lim et al. (2004), it was determined that 22.28% of studied *Staphylococcus aureus* carried *sea* and *seb* genes, especially with a higher prevalence of *sea* gene. In a study performed by Chiang et al. (2008) regarding food poisoning agents in Taiwan, *sea* gene was reported to be the most effective agent. Bergdoll (1983) announced that 95% of *Staphylococcus aureus* enterotoxin poisoning was due to *sea*, *seb*, *sec*, *sed*, and *see* genotypes, and the remaining 5% were reported as other types of this bacterium. In this study, by analyzing the data using QuickCalcs software, it was observed that there was a significant difference between the prevalence of *sea* and *seb* genes in the samples isolated from the poultry slaughterhouse. Such

a difference was considerable in the prevalence of *sea* and *seb* genes. Using the primers for enterotoxin genes A to C, 4 of the 11 isolated *Staphylococcus aureus* strains showed a positive result in PCR. Three of the isolates represented *sea* gene, and the remaining one demonstrated *sec* gene (Bystron et al., 2005). This result is in line with the findings of Lim et al. (2004), Chiang et al. (2008), and others (the researches were briefly mentioned earlier). It can be said that the prevalence of enterotoxin-producing strains is different in samples taken from various infections. However, it cannot be shown why a bacterial strain is capable of producing more than one enterotoxin. Furthermore, the production of several enterotoxins has been reported by other researchers. In a study conducted by Ifesan and Voravuthikunchai (2009), it was shown that some *Staphylococcus aureus* strains produce more than one enterotoxin. This result is also in line with the findings of the present study indicating that 5% of strains are capable of coding both enterotoxin A and B. In addition, the above-mentioned difference was not significant based on the comparison between the samples taken from the clinic and slaughterhouse regarding the prevalence of enterotoxin genes using QuickCalcs software. In other words, the prevalence of these genes was not different in the samples obtained from the clinic and slaughterhouse. Although there have been many studies regarding the identification of enterotoxin genes in *Staphylococcus aureus* in food products, there are few studies for the identification of this agent in clinical cases (Salari-Sharif et al., 2012). However, there are some studies performed on clinical cases. For example in a study carried out by Imani-Fooladi et al. (2009), 32% of nonclinical samples had *sea* and *seb* genes, which is in line the findings of the present study. Nonetheless, in a study performed by Casman et al. (1967), the variations in the enterotoxin production strength of *Staphylococcus aureus* strains isolated from clinical sources, was reported as 47% and about 31% for nonclinical strains, which is not in line with the findings of the present study. Considering the fact that, the presence of enterotoxin itself was

examined in this study, it is possible that the gene coding enterotoxins may exist in the bacteria; however, they are not expressed. One of the reasons for the difference in results may be in detecting the genes. The other factor that should be considered is the difference in the type of clinical samples. Molecular methods can only show the presence of enterotoxin gene in the microorganism, and cannot prove its expression and protein production (Rodriguez et al., 1996). In the present study, only the presence of enterotoxin genes was examined using the PCR genotype method, and the phenotype expression of enterotoxin genes was not evaluated. The present study analyzed the phenotypical and molecular resistance of *Staphylococcus aureus* strains to erythromycin and tetracycline and connection between these two antibiotics and the prevalence of *sea* and *seb* genes in the strains. The obtained results revealed that the sample resistance to antibiotics was considerable; on the average, the samples were 85% resistant, 11% sensitive, and 4% semi-sensitive to erythromycin. Moreover, the samples were 86% resistant, 10% sensitive, and 4% semi-sensitive to tetracycline. The results obtained by QuickCalcs software showed a significant difference between the frequency of *sea* and *seb* genes in both erythromycin and tetracycline in the slaughterhouse samples. However, this difference was not significant in the samples isolated from the clinic. In other words, the strains resistant to these antibiotics, including *sea* gene, had a higher difference in prevalence than *seb* gene in the samples isolated from the slaughterhouse. The above-mentioned comparison showed that during the growth period of broiler poultry farms in Ilam, resistant strains were able to carry *sea* gene, and its prevalence increased up to transfer to the slaughterhouse. The results of this study demonstrated a close connection between the genes resistant to erythromycin and tetracycline with *sea* gene that is in line with the findings of a study conducted by Mashouf et al. (2015). In the aforementioned study, it was reported that the most enterotoxin producing gene is *sea* gene (25.5%), and there was a significant relationship between

sensitivity pattern to erythromycin and tetracycline. In another study, the hens were reported to show 92.3% resistance to erythromycin and 88.5% sensitivity to gentamicin (Shapury et al., 2009). *Staphylococcus aureus* carries the genes of antibiotic resistance and virulence factors on genetic elements, such as plasmid, prophage, and pathogenicity islands, and can horizontally transfer them between strains (Alibayov et al., 2014). This is one of the reasons that justify the results of this study indicating that the resistance pattern to antibiotics was similar in the strains in each herd. In *Staphylococcus aureus* strains, 82.8% of them harbored one or more of enterotoxin genes (e.g., *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*), 39.8% of the strains demonstrated *se* genes, and 43% of the strains carried two to five *se* genes. However, 17.2 % of the strains possessed none of the examined genes. The most commonly detected toxin genes were *sea*, *seb*, *sec*, and *seg* genes (Alibayov et al., 2014). This can be a hazard for human health as a result of consuming broiler poultry products resistant to erythromycin and tetracycline with many medical uses. Therefore, elder individuals, patients with a deficient immune system, and children are more vulnerable to be contaminated with this bacterium and the associated genes (Cui et al., 2010). It can be concluded that the contamination of uncooked food products with *Staphylococcus aureus* with high prevalence, containing genes coding *sea* and *seb*, can be considered a serious threat to the consumers of broiler poultry products. Because the enterotoxins of this bacterium are resistant to heat and protease and are not eliminated by heating and cooking. Therefore, the high prevalence of the enterotoxin genes in this study isolated from the products of broiler poultry in Ilam revealed the potential role of this bacterium in food poisoning. In addition, elder individuals, patients with a deficient immune system, and children are more vulnerable to be contaminated with these bacteria and the associated genes (Cui et al., 2010). As mentioned earlier, there was a significant relationship between the prevalence of the *sea* gene and resistance to

erythromycin and tetracycline that have extensive functions in medicine.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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