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Original Article

Prophage Typing of *Staphylococcus aureus* Strains Isolated from Broiler Poultry

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Abstract

Staphylococcus aureus is a well-known commensal and pathogen agent of many wild and domestic animals. A wide variety of infections can be caused by *S. aureus*, from suppurative skin infections to life-threatening septicemia. This study was conducted to determine the prophage typing and the pattern of antibiotic resistance of *S. aureus* isolated from broiler poultry before they have been slaughtered. In this study, 200 nasal and cloacal swab samples from 20 different flocks were collected for bacterial isolation. Staphylococci were identified using biochemical and molecular methods before being examined for *mecA* gene detections in all samples resistant to oxacillin and cefotaxime. The highest value of antibiotic resistance was observed against ciprofloxacin (94%), and the maximum value of susceptibility was to gentamicin (85%). Twenty-eight (27%) samples were resistant to oxacillin. In methicillin-resistance *Staphylococcus aureus* (MRSA) isolates, 5 prophage types were observed, where the *SGB* prophage with a frequency of 75% was identified as a dominant prophage; in isolates of *S. aureus* susceptible to methicillin, 8 prophage types were observed, where *SGFa* prophage with a frequency about 82% was the dominant prophage. The high prevalence of MRSA isolates can indicate the risk of transmission of these bacteria to the food cycle. Furthermore, existence of various prophages in these isolates can be considered a threat to public health in producing pathogenicity factors in this bacterium while also empowering other bacterial pathogenicity, even other bacterial genera.

Keywords: *S. aureus*, methicillin resistance, prophage typing, broiler

Typage par Prophage des Souches de *Staphylococcus aureus* Isolées de Volailles de Chair

Résumé: *Staphylococcus aureus* est un agent commensal et pathogène bien connu de nombreux animaux sauvages et domestiques. Une grande variété d'infections peut être causée par *S. aureus*, des infections cutanées purulentes à la septicémie potentiellement mortelle. Cette étude a été menée pour déterminer le typage du prophage et le profil de résistance aux antibiotiques de *S. aureus* isolés de volailles de chair avant leur abattage. Dans cette étude, 200 échantillons d'écouvillonnage nasal et cloacal de 20 troupeaux différents ont été collectés pour l'isolement bactérien. Les staphylocoques ont été identifiés à l'aide de méthodes biochimiques et moléculaires avant d'être examinés pour la détection du gène *mecA* dans tous les échantillons résistants à l'oxacilline et au céfotaxime. La valeur la plus élevée de résistance aux antibiotiques a été observée contre la ciprofloxacine (94%) et la valeur maximale de sensibilité était la gentamicine (85%). Vingt-huit (27%) échantillons étaient résistants à l'oxacilline. Dans les isolats de *Staphylococcus aureus* résistants à la méthicilline (SARM), 5 types de prophages ont été observés, où le prophage SGB avec une fréquence de 75% a été identifié comme un prophage dominant; dans les isolats de *S. aureus* sensibles à la méthicilline, 8 types de prophages ont été observés, où le prophage *SGFa* avec une fréquence d'environ 82% était le prophage dominant. La forte prévalence des isolats de SARM peut indiquer le risque de transmission de ces bactéries au cycle alimentaire. De plus, l'existence de divers prophages dans ces isolats peut être considérée comme une menace pour la santé

publique en produisant des facteurs de pathogénicité dans cette bactérie tout en renforçant la pathogénicité d'autres bactéries, voire d'autres genres bactériens.
Mots-clés: *S. aureus*, résistance à la méthicilline, typage des prophages, poulets de chair

1. Introduction

Staphylococcus aureus is one of the most important pathogens in the expansion of bacterial infections in developing countries, and it is the third causative agent of food poisoning in the world. This bacterium generates a wide range of diseases in humans such as folliculitis, superficial and deep skin abscesses, wound infections, osteomyelitis, septicemia, endocarditis, toxic shock syndrome, pneumonia, and food poisoning (1, 2).

S. aureus has different virulence factors, such as immune-modulating elements, surface protein A, enterotoxins, exfoliative toxin, nucleases, and hemolysins, which play an important role in bacterial binding to host cells, survival in the body, resistance to the immune system, and penetration into host tissue (3). Virulence factors are transmitted through plasmid, transposon, pathogenicity islands, chromosome sets, insertion sequences, and bacteriophages (4).

Researchers have suggested that bacteriophages play a fundamental role in intensive horizontal gene transfer and can transform non-pathogenic isolates into pathogenic isolates by transforming lysogenic bacteriophage. Bacteriophages that have been replaced by prophage in *S. aureus* genomes play an important role in identifying this species. In general, prophage causes a morphological change in *S. aureus* isolates as a lysogenic transformation associated with virulence factors such as P, K, G, E, A enterotoxins, staphylokinase, beta-hemolysin, and Panton-Valentine Leucocidin (PVL) lipase (4, 5).

Bacteriophages have a high variety in MRSA strains. The high resistance of MRSA strains can indicate the important role of bacteriophages in the expansion and evolution of virulence factors in *S. aureus* strains. Most staphylococci have bacteriophages which can increase the pathogenicity power of this bacterium. Furthermore, when transferred to other bacteria, the bacteriophages cause genetic changes in them, thereby immunizing them against interference. Based on morphology and serology, *S. aureus* has six types of bacteriophages: *SGA*, *SGB*, *SGFa*, *SGFb*, *SGL*, and *SGD* (6).

Due to misuse of antibiotics, *S. aureus* can quickly resist many antibiotics, such as methicillin, semi-synthetic penicillins, and cephalosporins. The resistance of this bacterium, in addition to problems developing in the treatment, causes the establishment and dissemination of bacteria in the environment. The dissemination of bacteria from livestock to livestock and from livestock to humans is also an issue (7).

Since MRSA withstands all antibiotics of the β-lactam group as well as the heat of pasteurization and many proteolytic enzymes, it survives in food for a long time (8). Hence, evaluating the existence of MRSA is very important in foods, particularly poultry meat and its products.

The *mecA* gene is under the control of a chromosome, and it encodes a 78-kDa protein binding to methicillin. While this protein has a lower affinity to methicillin, *S. aureus* is resistant to methicillin. The gene is transported by a large *SCCmec* mobile gene cassette (9).

The *mecA* gene is well preserved in *Staphylococcus* species and is accepted as an indicator molecule. It is detected by PCR, which has been proposed as a standard method (3, 10). On the other hand, genotypic investigation of *Staphylococcus* species, because of the importance of distinguishing isolates, can be used extensively in tracking the source of infection and controlling its resulting contamination (11).

It is noteworthy that at the last stage of poultry life, i.e. at the time of slaughter, the bacteria are more likely

to transfer to the human consumption cycle. Accordingly, the current study investigated the antibiotic resistance pattern and prophage typing of *S. aureus* strains isolated from broiler poultry turned over to the slaughterhouse.

2. Material and Methods

2.1. Sample Collection

During the years 2015-2016, a total of 200 samples from 20 different flocks were collected prior to slaughter in Ilam Province. Ten birds from each flock were randomly sampled from the nose and the cloaca, and the samples were transferred to the microbiological laboratory in BHI media for bacteriological examination. In order to identify and validate isolates, standard biochemical methods for *S. aureus* detection, including gram staining, catalase test, oxidase, coagulase, DNase, and mannitol sugar fermentation, were used in mannitol salt agar medium in accordance with the method demonstrated by Kateete, Kimani (12).

2.2. Isolation of MRSA

Antibiogram testing was done in this study using Muller Hinton Agar (MHA) medium containing 4% salt, an antibiotic disk of oxacillin (1µg), and a cefotaxime antibiotic disk (30 µg) for the initial isolation of MRSA isolates.

2.3. DNA Extraction and PCR

For DNA extraction, the simple boiling method of Peng, Yu (13) was used with some modifications. In brief, 2 ml of the 48-hour culture of each isolate was poured into 2 ml microtubes and centrifuged at 6,000 g for two minutes. Next, 200 microliters of lysis buffer were added to each of the microtubes which were then placed in a thermomixer at 99 °C for 12 minutes. Finally, the samples were centrifuged at 10,000 g for five minutes, and their supernatant was used as the DNA template.

PCR testing was performed using primer and the specific programs in Table 1 for detecting *femA* gene for verification of the final diagnosis of all isolates identified as *S. aureus* and investigation of all samples for the existence of prophage encodings genes SGA, SGB, SGFa, SGFb, SGL, and SGD. Detection of *mecA* gene in all samples resistant to oxacillin and cefotaxime was also done.

Table 1. Primers and specific programs used

Primer	Primer sequence 5′→3′	PCR Production Length	
femA	F: 5′ CGATCCATATTTACCATATCA 3′ R: 5′ ATCACGCTCTTGCTTTAGTT 3′	450bp	58 °C
mecA	F: 5′ AAAATCGATGGTAAAGGTTGG 3′ R: 5′ AGTTCTGCACTACCGGATTTGC 3′	533bp	64 °C
SGA	F: 5′ TATCAGGCGAGAATTAAGGG 3′ R: 5′ CTTTGACATGACATCCGCTTGAC 3′	744bp	58 °C
SGB	F: 5′ ACTTATCCAGGTGGYGTTATTG 3′ R: 5′ TGTATTTAATTTCGCCGTTAGTG 3′	405bp	58 °C
SGFa	F: 5′ TACGGGAAAATATTCGGAAG 3′ R: 5′ ATAATCCGCACCTCATTCCT 3′	548bp	58 °C
SGFb	F: 5′ AGACACATTAAGTCGCACGATAG 3′ R: 5′ TCTTCTCTGGCACGGTCTCTT 3′	147bp	58 °C
SGL	F: 5′ GTCTAAAACAGTAACGGTGACAGTG 3′ R: 5′ TGCTACATCATCAAGAACACCTG 3′	648bp	58 °C
SGD	F: 5′ TGGGCTTCATTCTACGGTGA 3′ R: 5′ CTAATTTAATGAATCCACGAGAT 3′	331bp	58 °C

2.4. Determination of Antibiotic Resistance Pattern

Disc diffusion testing was done by antibiotic discs of MAST (Merseyside Company, United Kingdom) for penicillin (10 µg), oxacillin (1 µg), ciprofloxacin (30 µg), imipenem (10 µg), gentamycin (10 µg), doxycycline (30 µg), oxytetracycline (30 µg), cefotaxime (30 µg), clindamycin (2 µg), tetracycline (30 µg), erythromycin (15 µg), and ceftriaxone (30 µg). To determine MIC in methicillin-resistant isolates identified in the disc method, the antibiotic diffusion method (agar dilution test) was utilized with cefotaxime and oxacillin antibiotics and according to CLSI standards (14).

3. Results

Based on the phenotypic tests of all 200 collected samples, 112 samples were identified as *S. aureus* using standard biochemical methods. Thereafter, the *femA* gene was studied by PCR to confirm the genotypes of *S. aureus* isolates; among the 112 identified samples, 100 (89%) isolates of *S. aureus* were detected.

With the disk diffusion method using oxacillin antibiotics in this study, 28 isolates were found to be resistant, while 72 isolates were detected as susceptible to methicillin. In PCR testing to identify the *mecA* gene, 9 (32%) samples had this gene, while 19 (68 %) samples did not. The minimum growth inhibitory concentration (MIC) was 128 mg/ml (a very high concentration) in 13 isolates (46.43%), 32 mg/ml in 9 isolates (32.14%), and 4 mg/ml in 6 isolates (21.43%) (Table 2). Table 3 reports the values of susceptibility of different isolates to the used antibiotics. The highest values of resistance to antibiotics were associated with ciprofloxacin (94%), clindamycin (86%), erythromycin (86%), and oxytetracycline (83%), while the highest values of susceptibility were related to gentamicin (85%) and imipenem (78%) (Table 4).

In all of the *S. aureus* isolates in this study, prophage types *SGFa* (76%), *SGB*, (72%), and *SGA* (67%)) had the maximum frequency, while the *SGD* type prophage was not found in any of the samples. In total, 5 prophase resistance patterns were observed in MRSA strains; 8 patterns were found in methicillin-susceptible isolates (Table 5 and Table 6).

The results indicate that *SGB* with 75% was the most dominant prophage in MRSA samples, while the *SGD* and *SGL* prophage types were not observed in these samples. In *S. aureus* isolates susceptible to methicillin, the most dominant prophage was *SGFa* with 81.94%. Table 2 lists the PCR results of the search for the encoding genes of *SGA*, *SGB*, *SGFa*, *SGFb*, *SGL*, and *SGD* prophage types.

Table 2. MIC results at different concentrations of oxacillin in MRSA samples

MIC value	≥128	32	4	Total
Number of samples	13	9	6	28

Table 3. Susceptibility of *S. aureus* isolates to different antibiotics by disc diffusion method

Susceptibility level (%)	**Average susceptibility (%)**	**Resistance level (%)**	**Antibiotic type**
73	0	27	**Oxacillin**
37	15	57	**Cefotaxime**
40	10	50	**Ampicillin**
42	20	38	**Penicillin**
60	0	38	**Erythromycin**
78	0	22	**Imipenem**
6	0	94	**Ciprofloxacin**
14	0	86	**Clindamycin**
17	0	83	**Oxytetracycline**
17	0	83	**Doxycycline**
60	25	15	**Gentamicin**
55	0	45	**Tetracycline**
61	14	25	**Ceftriaxone**
73	0	27	

Table 4. Frequency distribution of prophage types in *S. aureus* isolates

	MRSA						
Prophage		*SGA*	*SGB*	*SGFa*	*SGFb*	*SGD*	*SGL*
Number	28	18	21	17	10	0	0
Percentage	28	64.29	75	60.71	35.71	0	0
	MSSA						
Number	72	49	51	59	53	0	43
Percentage	72	68.06	70.83	81.94	73.61	0	59.72

Table 5. Frequency of prophage patterns in MSSA isolates

Prophage pattern	Prophage type					Number	Frequency percentage
	SGA	*SGB*	*SGFa*	*SGFb*	*SGL*		
1	+	+	+	+	+	10	14.10
2	-	+	+	+	+	11	15.38
3	+		+	+	-	18	25.64
4	+	+	+	+	-	8	11.54
5	+	-	-	-	-	7	10.26
6	+	+	+	-	+	6	7.69
7	-	-	+	-	+	6	7.69
8	-	+	-	+	-	6	7.69
						72	100

Table 6. Frequency of prophage patterns in MRSA isolates

Prophage pattern	Prophage type					Number	Frequency percentage
	SGA	*SGB*	*SGFa*	*SGFb*	*SGL*		
1	+	-	+	+	-	7	25.00
2	+	+	+	-	-	7	25.00
3	+	+	-	-	-	4	14.29
4	-	+	+	+	-	3	10.71
5	-	+	-	-	-	7	25.00
						28	100

4. Discussion

Recently, the progressive emergence of MRSA isolates has become a serious public health concern. Despite the prohibition of antibiotic consumption in the poultry industry, antibiotics are used to control and prevent diseases and increase the rate of feed conversion to weight. Moreover, many poultry farms that do not conduct antibiogram testing use different antibiotics to treat poultry diseases.

Such problems increase the use of antibiotics in the poultry industry and subsequently contribute to the development of antibiotic resistance in existing bacteria in poultry (15). Meat can be an important reservoir for methicillin-resistant *S. aureus*, and the consumption of contaminated meat allows these bacteria to enter the digestive system of the consumer, thereby transferring resistant genes to natural flora bacteria (16).

Given that food poisoning caused by *S. aureus* is the third most common cause of food-related diseases and due to the increasing number of breeding poultry and the rise in poultry consumption, the evaluation of chicken meat is very important in terms of contamination with MRSA isolates (1). The current study investigated the isolates of *S. aureus* isolated from the nose and cloacal of poultry samples for antibiotic resistance and different types of prophages.

The study results indicated that 50% of the collected samples were contaminated with *S. aureus* bacterium, 28 isolates of *S. aureus* were detected as being methicillin-resistant, while 72 isolates of *S. aureus* were susceptible to methicillin. MRSA was examined by PCR to identify the *mecA* gene. The PCR results revealed that 28% of the samples had the *mecA* gene, indicating the risk of the existence of this bacterium in poultry.

The results of this study have both congruence and discrepancies with other studies. For example, Andrea T. , Kristina (17) studied turkey and chicken meat products and reported the level of MRSA contamination to be 37.2%, which is partly consistent with the results of this study.

Persoons, Van Hoorebeke (18) investigated poultry in Belgium and suggested that the level of MRSA contamination among them was 11%, which was less than the values obtained in the current study. Given the time and location of the study, this result may indicate an increase in the resistance level of this bacterium to methicillin. In the current study, a high prevalence of multiple antibiotic resistance was found in MRSA isolates from poultry samples.

Resistance level to ciprofloxacin, clindamycin, erythromycin, and oxytetracycline was found to be very high in the current study. High levels of resistance to erythromycin are attributable to the excessive use of spiramycin as a growth stimulus in poultry food on poultry farms in Ilam province. These results indicate the effect of antibiotic consumption in the food chain on human health.

The current study also investigated the relationship between the *mecA* gene and *SGL*, *SGD*, *SGFa*, *SGFb*, *SGB*, *SGA* prophage types in *S. aureus* isolates using the PCR method. In all *S. aureus* isolates, the dominant prophages were *SGFa* (86%), *SGFb* (82%), and *SGA* (70%), while in methicillin-resistant samples, the dominant prophage was *SGB* (67%), and in methicillin-susceptible samples, the dominant prophage was *SGA* (68%).

The studies performed by Rahimi et al. during the years 2012 to 2013 indicated the presence of *SGA*, *SGB*, *SGFa*, and *SGFb* prophage types in the clinical isolates of MRSA in Tehran province (19). In both studies, the *SGF* prophage type was introduced as the most dominant one. On the other hand, none of the studies on clinical samples and poultry in Iran identified the prophage types of *SGD* and *SGL*. It is noteworthy that in the current study, none of the isolates were positive for the *SGD* prophage, but the *SGL* prophage was observed in methicillin-susceptible samples.

The difference in the prophage pattern in studies can be due to geographical differences of the studies.

In a study conducted by Pantůček, Doškař (6) in the

Czech Republic on MRSA isolates in clinical samples, the five prophage types *SGF*, *SGFa*, *SGFb*, *SGB*, and *SGA* were identified, and the *SGFa* prophage type was introduced as the dominant type of prophage, which was consistent with this study.

In general, in *S. aureus* isolates, 8 prophage patterns were seen in MSSA and 5 patterns were found in MRSA. In both of them, the dominant prophage types were *SGFa*, *SGFb*, and *SGA*. Overall, the results obtained in this study suggest that there are differences among prophages in MRSA and MSSA. For example, the *SGP* prophage type was observed in MRSA samples with a frequency of 52%, while it was not detected at all in methicillin-resistant samples.

The results of this study also show that in Ilam Province, highly methicillin-resistant isolates exist on poultry farms. Moreover, the existence of different prophragmatic patterns among these isolates indicates that these isolates have the ability to express intensity of bacteriophage-dependent factors, such as Panton-Valentine leucocidin, exfoliative toxinA, TSST-1, various enterotoxins, staphylokinase, and beta-hemolysin, which can be transmitted through poultry meat to humans and cause resistance and increased food poisoning in them.

Poultry meat is one of the most highly-consumed foods in hospitals and is rarely controlled in such places. Unfortunately, methicillin-resistant *Staphylococcus* and the carriers of prophages reported in this study are prevalent in areas that are poorly sanitized and where antibiotics are used indiscriminately, as has been reported by Otalu, Junaidu (20)

Thus, effective and rapid actions to control the use of antibiotics and remove reservoirs of MRSA isolates in different centers and sources are needed. If the necessary attention is not given to eliminating or at least reducing these bacterial isolates, the prevalence of hospital infections and other potential pathogenic consequences of the disease will become serious for the society.

Authors' Contribution

Study concept and design: M. N.
Acquisition of data: Kh. R.
Analysis and interpretation of data: F. P.
Drafting of the manuscript: Kh. R.
Critical revision of the manuscript for important intellectual content: M. N.
Statistical analysis: F. P.
Administrative, technical, and material support: Ilam University

Ethics

All the procedures and animal handling were approved by the Ilam University, Iran under the project number of 2020-14125136-33.

Conflict of Interest

The authors declare that they have no conflict of interest.

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