

Surveillance of Antibiotic Resistant *Staphylococcus aureus* in Agricultural Production Chain of Mongolia

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Abstract: Monitoring of food borne pathogens in food is the primary tool for the implementation of food safety systems. It is necessary to monitor the prevalence of food borne pathogens for effective food safety planning and targeted interventions. *Staphylococcus aureus* is considered as the third largest cause of food related illness in worldwide. The present study aimed at surveillance of *S. aureus* contamination of meat on meat supply chain stages, which is a common benchmark of meat market in Mongolia, and characterization of isolated and collected strains from other agricultural sources. The cultural and polymerase chain reaction (PCR) methods were used for isolation, identification and characterization of *S. aureus*. In 216 cultures of *S. aureus* among 634 *Staphylococci* isolates obtained from different sources throughout the agricultural production chain in this study, common gene for *S. aureus* (98.74%), and *nuc* (97.47%), *mecA* (44.12%), *msrA* (9.66%), *gyrA* (32.77%) and *ermC* (29.41%) genes were identified. As seen in the surveillance result, the prevalence of methicillin-resistance *S. aureus* (MRSA) is 44% among *S. aureus* isolates from agricultural production chain. Confirmed cases of food-borne infections and intoxications caused by *S. aureus* should be considered as one of mean criteria of food safety issues in Mongolia, and special attentions should be paid on antibiotic resistant bacteria, such as *S. aureus*.

Key words: Meat supply chain, *mecA* positive *Staphylococcus aureus*, polymerase chain reaction.

1. Introduction

Staphylococcus aureus is a bacterium, which causes food-borne and fomite-borne infections and intoxications [1]. Among healthy humans, 30% carry *S. aureus*, which causes various infections, intoxications, postsurgical infections, pustule and sepsis [2]. Virulence factors of *S. aureus* include: (1) surface proteins that promote colonization of host tissues; (2) invasins (leukocidin, kinases, hyaluronidase); (3) surface factors (capsule, protein A); (4) biochemical properties (carotenoids, catalase production); (5) immunological disguises (protein A, coagulase); (6) membrane-damaging toxins (hemolysins, leukotoxin, leukocidin); (7) exotoxins (SEA-G, TSST, ET); (8) inherent and acquired resistance to antimicrobial agents [3].

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S. aureus is considered as the third largest cause of food related illness in worldwide [4]. Monitoring the presence of food borne pathogens in food is the primary tool for the implementation of food safety systems. It is necessary to monitor the prevalence of food borne pathogens for effective food safety planning and targeted interventions [5]. Methicillin-resistant *S. aureus* (MRSA) that is resistant to virtually all β -lactam antibiotics is mediated by the chromosomally located *mecA* gene [6].

Livestock constitutes a potential reservoir of MRSA isolates belonging to a recently derived lineage within clonal complex 398 (MRSA CC398-IIa). Since its discovery in the early 2000s, this lineage has become a major cause of human disease in Europe, posing a serious public health challenge in countries with intensive livestock production. Various studies suggest that environmental contamination of air and

contacted surfaces may also contribute to MRSA CC398 transmission [7-10]. Moreover, MRSA CC398 is a relatively common contaminant of retail meat in Europe, and food-borne transmission has been hypothesized as a possible source of infections in people with no livestock contact. However, epidemiological data suggest that food-borne transmission is rare [11].

Results of the studies in last years demonstrate that the use of antibiotics is now out of control and antibiotics resistance of bacteria is broadening in Mongolia [12]. Among cases of food-borne infections and intoxications in Mongolia, it caused by *S. aureus* is not rare. For instance, intoxications occurrence increased two times during last two years according to the Bacteriological Laboratory of National Center for Communicable Disease (NCCD), and a total of 216 coagulase-positive *Staphylococci* cultures were determined, as all cultures were sensitive to cefazolin and 79% to ciprofloxacin, but 60% were resistant to penicillin and ampicillin in 2012. Also an outbreak among soldiers in the Army Unit 167 in Umnugovi aimag was caused by *S. aureus* and *S. aureus* were detected in textbooks of school children in Orkhon aimag [13].

With development of molecular techniques, polymerase chain reaction (PCR) has become recently an important tool for detecting pathogenic microorganisms in food products by replacing the time-consuming culture-based classical techniques [14]. It is rapid, easy to handle, sensitive and specific, and constitutes very valuable tools for microbiological applications.

Therefore, it has been essentially important to detect bacteria resistance to antibiotics, conduct surveillance of them, make risk assessments, improve diagnostic capacity and take control on veterinary drug use. Thus, the present study aimed to conduct surveillance of *S. aureus*, detect its virulence and antibiotic resistance and improve their diagnostic technology and proficiency testing.

2. Materials and Methods

2.1 Sampling and Strains Collection

Isolates, cultures and strains from six sources were used for studying antibiotic resistance of *S. aureus* as shown in Table 1. The strains were collected from laboratories, including NCCD Bacteriological Laboratory, State Central Veterinary Laboratory (SCVL), Veterinary Laboratory of Implementing Agency of the City Mayor (VLUC), National Reference Laboratory of Food Safety of General Agency for Specialized Inspection (NRLFS-GASI), Food Safety and Hygiene Laboratory of Institute of Veterinary Medicine (FSHL-IVM) and Laboratory of Infection Diseases and Immunology (LIDI-IVM) Mongolia.

2.2 Isolation and Identification by Cultural Methods

Specimen from animal products and fomites were planted on both nutrient and nutrient agar, a total of 225 mL of tryptic soya broth (TSB; Oxoid, Basingstoke, Hampshire, UK) containing 10% NaCl was added to 25 g of aseptically ground sample in a stomacher bag. Bags were stomached using a Stomacher 400 circulator (Seward, Inc., London, UK) at 230 rpm for 2 min, then incubated at 35 °C for 24 h [15]. Then, smear was prepared and stained by Gram's method and Gram positive clustered cocci were selected. Colonies were selected based on whether the cocci cause beta hemolysis on blood agar and form black colonies on Baird Parker selective agar. In order to differentiate staphylococci from other cocci, catalase test was used, while coagulase test was used to identify *S. aureus* from other *Staphylococci* [15].

2.3 Biochemical Test

For identification of *Staphylococci* by biochemical characteristics, API Staph test kit (BioMerieux) was used as described in the manufacturers instruction [16]. Briefly, the following steps and procedures were used. The first step in this procedure is to make a saline suspension of the organism from an isolated colony. A

Table 1 Sampling and collection strains.

Collection	Sampling from food chains																	Total
	From slaughtering house				From food market			From beef production				From broiler				From horse	From patients	
Sources	Animals	Meat samples	Swabs	Carriers	Meat samples	Swabs	Carriers	Feed	Slaughter animals	Processing plant	Retail	Broilers	Slaughter	Processing plant	Retail	Nasa swabs	Nasa swabs	
No. of samples	52	52	52	52	104	102	87	-	-	-	-	-	-	-	-	360	-	861
No. of strains	2	3	5	2	15	31	3	7	8	6	9	23	22	18	13	13	36	216

Cultures taken from laboratories, such as NCCD Bacteriological Laboratory, SCVL, VLUC, NRLFS, FSHL-IVM and LIDI-IVM, and samples collected for last 3-4 years in these laboratories from the above mentioned sources.

-: unknown numbers of samples.

Table 2 Primers used for the study.

No.	Name of gene	Target sequences	PCR primer' sequences (5' to 3')	Product size (bp)	Reference
1	Common	<i>S. aureus</i>	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	108	[17]
2	<i>ermC</i>	Erythromycin resistance of <i>S. aureus</i>	CTT GTT GAT CAC GAT AAT TTC CC ATC TTT TAG CAA ACC CGT ATT C	190	[18]
3	<i>msrA</i>	Macrolide resistance efflux of <i>S. aureus</i>	TCC AAT CAT TGC ACA AAA TC AAT TCC CTC TAT TTG GTG GT	163	[19]
4	<i>mecA</i>	Methicillin resistance of <i>S. aureus</i>	AAC AGG TGA ATT ATT AGC ACT TGT AAG AAT TCC CTC TAT TTG GTG GT	174	[20]
5	<i>nuc</i>	Thermostable nuclease of <i>S. aureus</i>	GCGATTGATGGTGATACGGTT CAAGCCTTGACGAACATAAGC	276	[21]
6	<i>VSMec</i>	Penicillin binding protein of <i>S. aureus</i>	TGG CTA TCG TGT CAC AAT CG CTG GAA CTT GTT GAG CAG AG	310	[22]

staph strip is then placed in a tray that has a small amount of water added to it to provide humidity during incubation. Next, a sterile pipette is used to dispense 2-3 drops of the bacterial suspension to each micro cupule. The inoculated tray is covered and incubated aerobically for 18-24 h at 35-37 °C. Finally, a seven-digit profile number is obtained and used to identify the bacteria.

2.4 Antibiotic Resistance Test

Antibiotic resistance and susceptibility of 216 *S. aureus* cultures, isolated and collected during the study, were checked by use of disc diffusion test [23]. DNA of antibiotic resistant strains was extracted and the gene for antibiotics resistance was amplified by PCR using primer shown in Table 2.

2.4.1 Disk Diffusion Test (Kirby-Bauer Method)

The test is performed by applying a bacterial inoculation of approximately 1 CFU/mL to the surface diameter Mueller-Hinton agar plate. Commercially-prepared by Biolab, Zrt and HiMedia fixed concentration, antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16-24 h at 35 °C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium [24].

2.4.2 DNA Extraction and Purification

To extract bacterial DNA, 400 µL isolate was placed into microtube and centrifuged at 1,000 rpm for 30 min. Then, 200 µL of pellet was pipetted into new microtube and centrifuged at 8,000 rpm for 15 min. Supernatant was removed and 200 µL distilled water was added into the precipitate, followed by mixing in vortex. Then, the mixture was placed in boiling water for 15 min and template was prepared by centrifuging at 1,000 rpm for 10 min.

DNA was extracted from blood and nasal swabs using the QIAamp DNA mini kit from Qiagen. The

QIAamp DNA mini kit was used for the protocol of commercial guideline. To isolate DNA from meat, phenol-chloroform extraction method was used. Yield and purity of isolated DNA were measured by spectrophotometer at 260 nm and 280 nm wavelength and the purity ranged between 1.72 to 1.94.

2.4.3 PCR Method

For the surveillance of genes of *S. aureus*, which is resistant to both β -lactam and non β -lactam antibiotics, including oxacillin, methicillin and erythromycin, the following primers shown in Table 2 and both of PCR and multiplex PCR methods for surveillance of antibiotics resistant genes were used [25].

2.4.3.1 Reaction Mixture

Total of 25 µL of mixture, containing 2.5 µL 10× PCR buffer, 2 µL dNTP (GeneAmp, UK) (each 2.5 mM), 2 µL template, 1.5 µL MgCl₂, 1 µL of each of primers 1 and 2, 0.175 µL of 5 unit/µM taq DNA polymerase (TaKaRa, Japan), and 14.815 µL ddH₂O/DW, was taken. In using multiplex PCR, subtractions from water equal to amounts of primer and MgCl₂ were estimated and each primer was taken in dependent on concentrations [25].

2.4.3.2 Amplification

In total of 35 cycles, there were such steps as initialization at 95 °C for 7 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min and final elongation at 72 °C for 7 min, which was 10 min for multiplex PCR [25].

2.4.3.3 Gel Electrophoresis

Mixture of 8 µL PCR product and 2 µL loading buffer was loaded in wells on the gel, and run in 1.5%-2% agarose gel depending on its DNA length. The gel was stained by ethidium bromide for 15 min and DNA fragments were visualized on transilluminator at 320 nm wavelength.

3. Results and Discussion

3.1 Prevalence and Identification of *S. aureus*

Of 861 samples taken from meat production and distribution chain, coagulase-positive *Staphylococcus*

samples represent 10% by microbiological method, whilst 12% were coagulase positive *Staphylococcus* by PCR method (Table 3). In these positive samples, *S. aureus* was detected in 3.8% of animal samples, 5.8% of meat samples, 9.6% of environmental swabs and 3.8% of patients (Table 3, Fig. 1).

For samples taken from food markets, *S. aureus* was detected in 14.42% of meat, 3.4% in carriers and 30.4% in fomite surface swabs (Fig. 2). Of 156 strains and cultures identified by laboratory examinations in the last three years, 83.7% were positive for *mecA S. aureus*. Furthermore, 3.6% of *S. aureus* identified from 360 horse samples (nasal swab and blood) of Selenge, Darkhan, Orkhon-Uul and Bulgan provinces

were positive for *mecA S. aureus* (Table 4).

3.2 Result of API Staph Test

Isolates from fomites and animal products accounted for 61.3% and 28.7%, respectively, in total isolates and serotyping of staphylococci by API test. When serotype of 634 cultures of *Staphylococcus* by their biochemistry and enzyme activity identified, there were *S. aureus* (35.3%), *S. xylosus* (29.4%), *S. hominis* (17.6%) and *S. saprophyticus* (8.8%) (Fig. 3). Results of the study demonstrated that portion of *S. aureus*, which is the cause of infection and intoxication, was greater than other types and *S. aureus* is seen to be indicator of fomite borne infection (Fig. 4).

Table 3 Results of isolation and identification of *S. aureus* from meat chain.

Collection	Kind	No. of isolation and identification of <i>S. aureus</i>		Sub-total	Total
		Slaughtering house	Food markets		
Sample	Animals	52	0	52	501
	Meat	52	104	156	
	Swabs	52	102	154	
	Carriers	52	87	139	
Isolates					
by CM	Animals	2	0	2	50
	Meat	2	12	14	
	Swabs	4	28	32	
	Carrier	1	1	2	
by PCR	Animals	2	0	2	61
	Meat	3	15	18	
	Swabs	5	31	36	
	Carrier	2	3	5	

CM: cultural method.

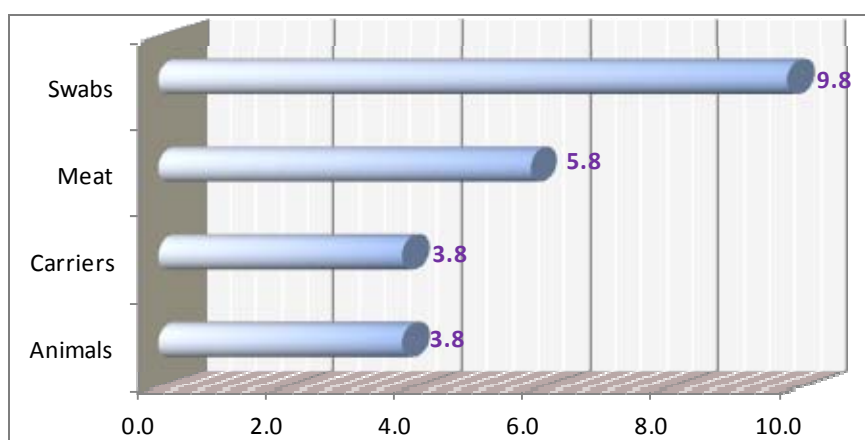


Fig. 1 Percentage of *S. aureus* of total samples on slaughter house.

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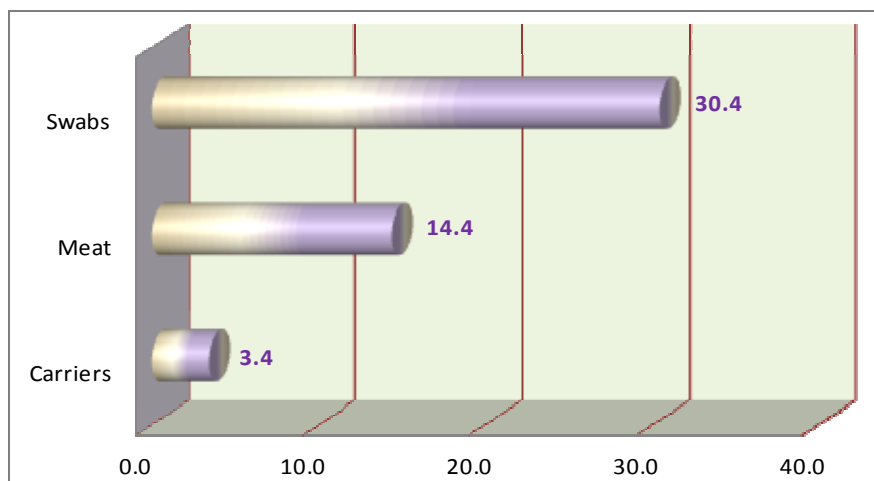


Fig. 2 Percentage of *S. aureus* of total samples on slaughter house.

Table 4 Strains collected from laboratories and *mecA* positive results.

No.	Host	Sources	Laboratories	Cultures and strains	<i>mecA</i> positive <i>S. aureus</i>
1	Cattle	Feed	SCVL	7	6
		Slaughtering animal	SCVL	8	5
		Processing plant	SCVL/FSHL	6	6
		Retail	SCVL/FSHL	9	8
2	Broiler	Broilers	VLCC & FSHL	23	19
		Slaughtering	VLCC & FSHL	22	16
		Processing	VLCC & FSHL	18	13
		Retail	VLCC & FSHL	13	9
3	Horse samples (360)		IVM-LIDI	13	13
4	Patients		NCCD	37	36
Total cultures and strains from labs				156	131

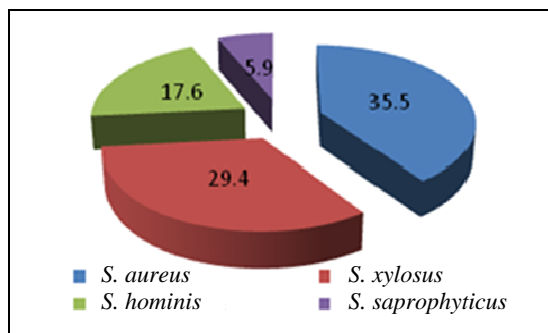


Fig. 3 Serotypes of *Staphylococci*.

3.3 Result of Disk Diffusion Test

Result of antibiotic disk diffusion test showed that of 216 isolates of aureus from meat and related environment, 72.7% are resistant to amoxycillin (Am10), 63.6% to penicillin (P10), 18.8% to cefazolin (CZ30), 9.1% to nitrofurantoin (F/M), 89.8% to ampicillin (A10), 89.9% to oxacillin (OX10), 36.4% to

doxycycline (D30), 9.1% to erythromycin (E15) and 45.5% to chloramphenicol (C30) and all were sensitive to gentamicin (GM 10) (Figs. 5 and 6).

3.4 Result of PCR Analysis on Virulence and Antibiotic Resistance Genes of *S. aureus*

Molecular epidemiological survey was performed by using all *S. aureus* from about 10 sources, such as meat animals, meat, by-products, feed, carriers, patients, and the PCR results were shown in Fig. 7.

The analysis of virulence and antibiotic resistance of *S. aureus* showed that 216 cultures of *S. aureus* among 634 *Staphylococci* isolates obtained from different sources throughout the agricultural production chain in this study, common gene for *S. aureus* (98.74%), and *nuc* (97.48%), *mecA* (44.12%), *msrA*

(9.66%), *gyrA* (32.77%) and *ermC* (29.41%) genes were identified.

As seen in the surveillance result, the prevalence of

MRSA is 44% among *S. aureus* isolates. The majority of MRSA isolates (74.2%) were from human patients in the hospitals. Coagulase positive *Staphylococci* account

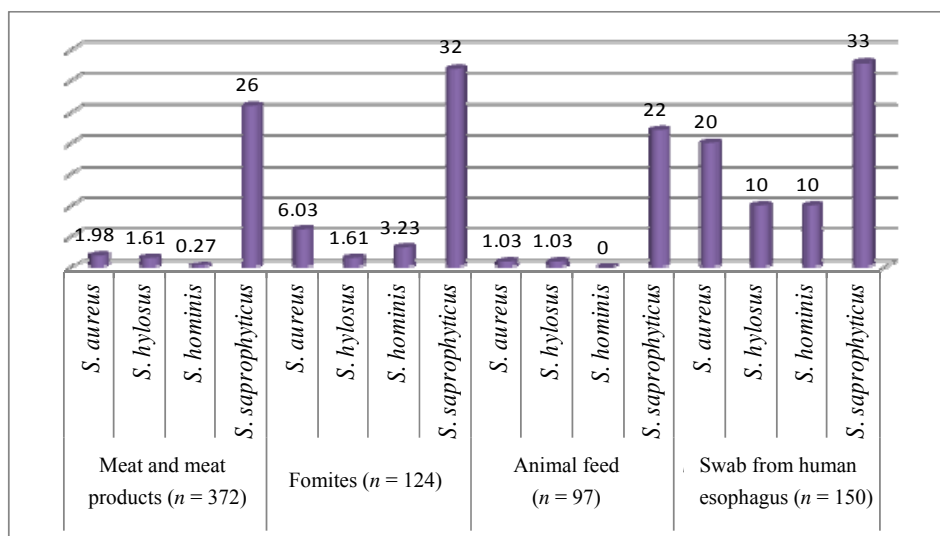


Fig. 4 Prevalence of *Staphylococci* serotypes.

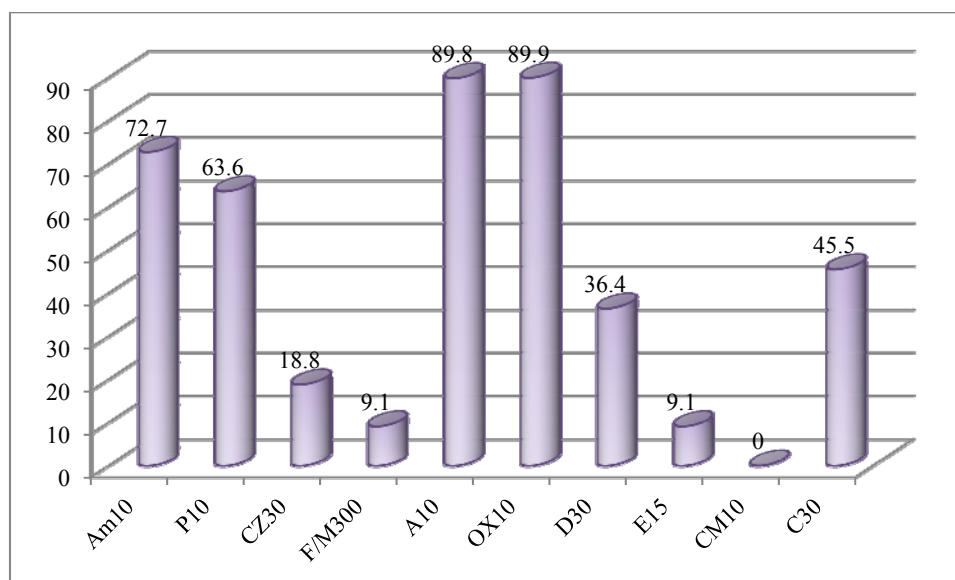


Fig. 5 Results of antibiotics testing by disk diffusion method.

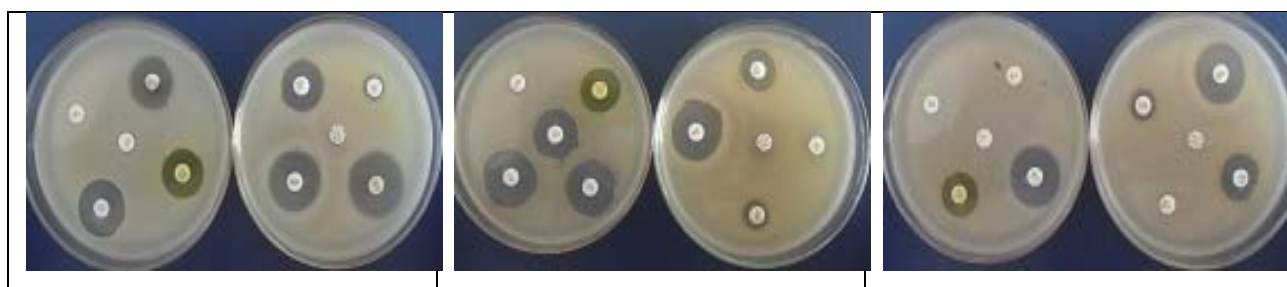


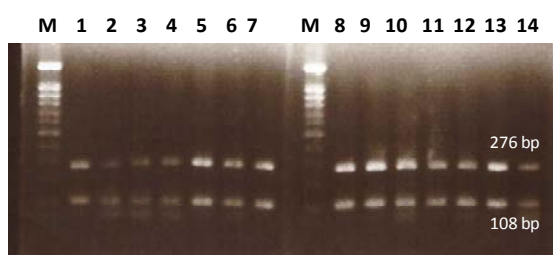
Fig. 6 Results of antibiotic resistance and susceptibility test by disk diffusion method.

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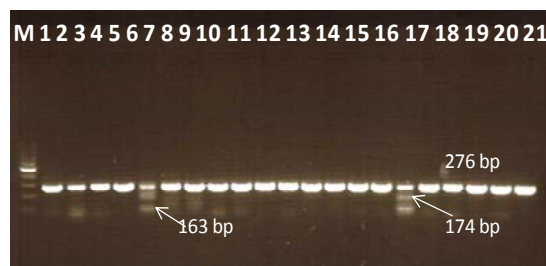
(a) PCR for common gene of *S. aureus*

Lanes M: 100 bp DNA ladder; L1: *S. aureus* ATCC 25923;
L2-L15: samples; L16: negative control.



(b) Multiplex PCR for common and *nuc* gene

Lanes M: 50 bp DNA ladder;
L1-L15: samples.



(c) Multiplex PCR for *msrA*, *mecA* and *nuc* gene

Lanes M: 100 bp DNA ladder;
L1-L21: samples of meat and swab of human throat swab;
L7 and L17 positive for *mecA* and *msrA*.



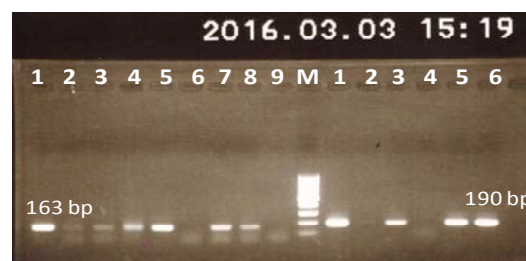
(d) Multiplex PCR for common and *mecA* gene

Lanes M: 100 bp DNA ladder; L1: *S. aureus* ATCC 2921;
L2: negative control; L3-L11: samples of meat and feed;
L12: ATCC 25923 *S. aureus* (University of Hokkaido).



(e) PCR for *ermC* gene

Lanes M: 100 bp DNA ladder;
L1-L14: samples of chicken, cattle, patients.



(f) PCR for *msrA* and *ermC* gene

Lanes M: 100 bp DNA ladder; Later L1-L6: samples of horse;
L1-L9: samples of cattle, horse, chicken, patient, carrier and beef.



(g) PCR for *gyrA* gene

Lanes M: 100 bp DNA ladder; Later LM: Hind III marker

L1: *S. aureus* ATCC 25923; L2-L4: positive with *gyrA* gene.

Fig. 7 Results of PCR analysis of *S. aureus*.

Table 5 Results of surveillance of antibiotics resistance genes of *S. aureus*.

No.	Sources	Isolates	<i>S. aureus</i>	<i>mecA</i>	<i>msrA</i>	<i>gyrA</i>	<i>ermC</i>	<i>nuc</i>
1	Animals	15	15	13	2	8	4	15
2	Feed	19	19	2	3	8	7	18
3	Meat	38	38	16	5	9	12	38
4	Swabs	55	54	7	2	10	3	53
5	Carrier	3	3	1		2		3
6	Patients	37	35	28	2	18	19	36
7	Sub products	15	15	11	7	6	8	14
8	Imported chicken	56	56	27	2	17	17	55
No.		238	235	105	23	78	70	232
Percentage (%)		100	98.74	44.12	9.66	32.77	29.41	97.48

for 10.8% of all samples, 15.9% of samples from meat production chain, and 3.6% of horse samples (Fig. 7, Table 5). So, MRSA is the most prevalent (Table 5, Figs. 8-11). Prevalence rate was also determined. Antibiotic resistance of *S. aureus* isolates, cultures and strains, which were cultured from products and environment contaminated by *S. aureus*, was variable as shown in Figs 8-11.

The majority of cases was identified from environmental swabs and imported chickens. The high percentage of occurrence of *S. aureus* highlights the need to improve the sanitation and hygiene procedures at all levels from production to the consumption of meat.

Although there were some differences in primers used for detection of antibiotics resistance gene and

antibiotics discs for some antibiotics in the study. Types of both β -lactam and non β -lactam antibiotics were consistent, and result of antibiotics disc test for oxacillin and erythromycin was also consistent with that for PCR. Results were summarized in Table 5. β -lactams are preferred antibiotics used to treat serious

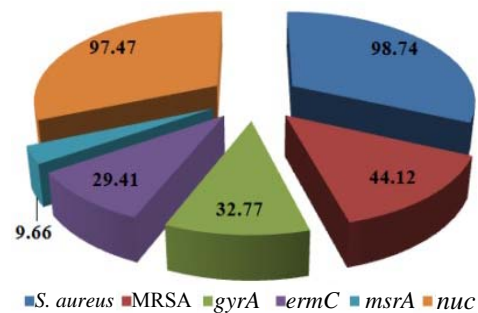


Fig. 8 Percent of virulence and antibiotic resistance genes.

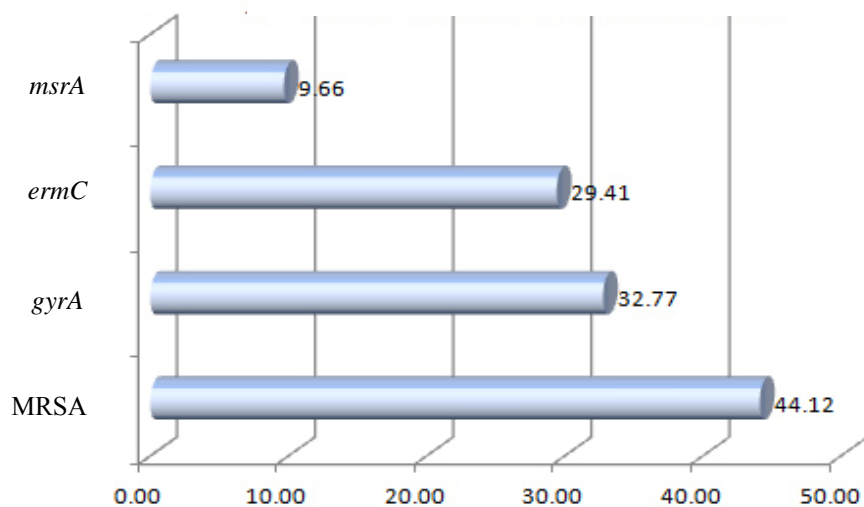


Fig. 9 Percent of antibiotic resistance *S. aureus*.

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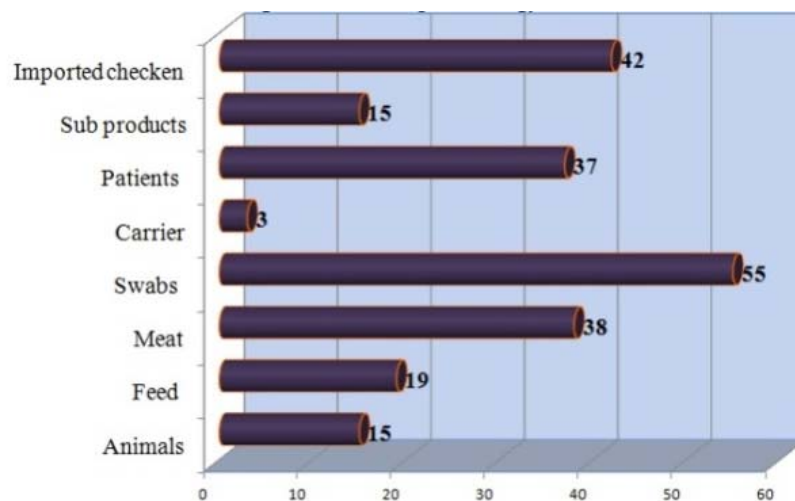
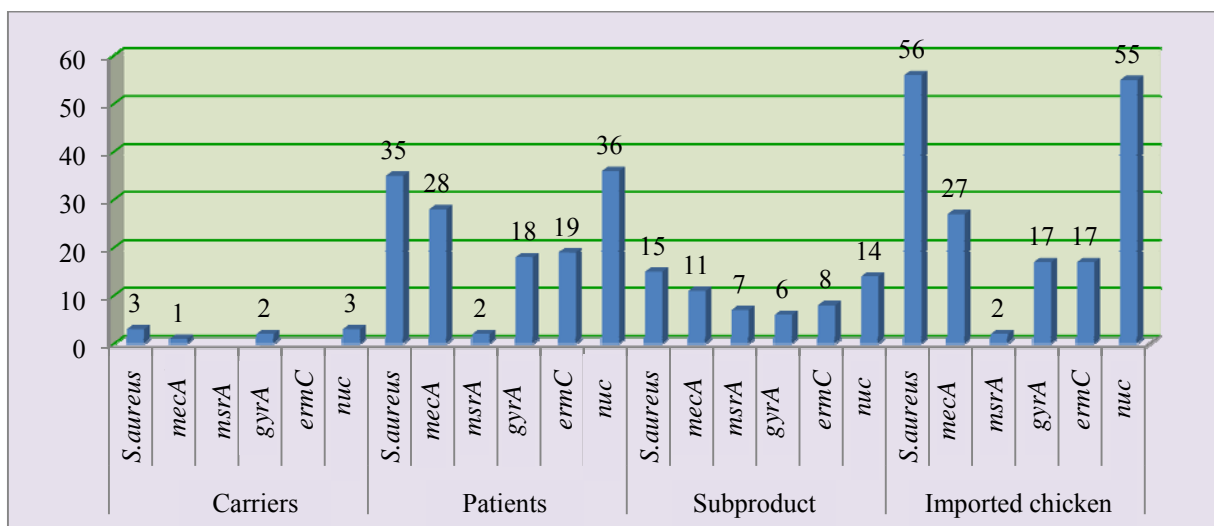
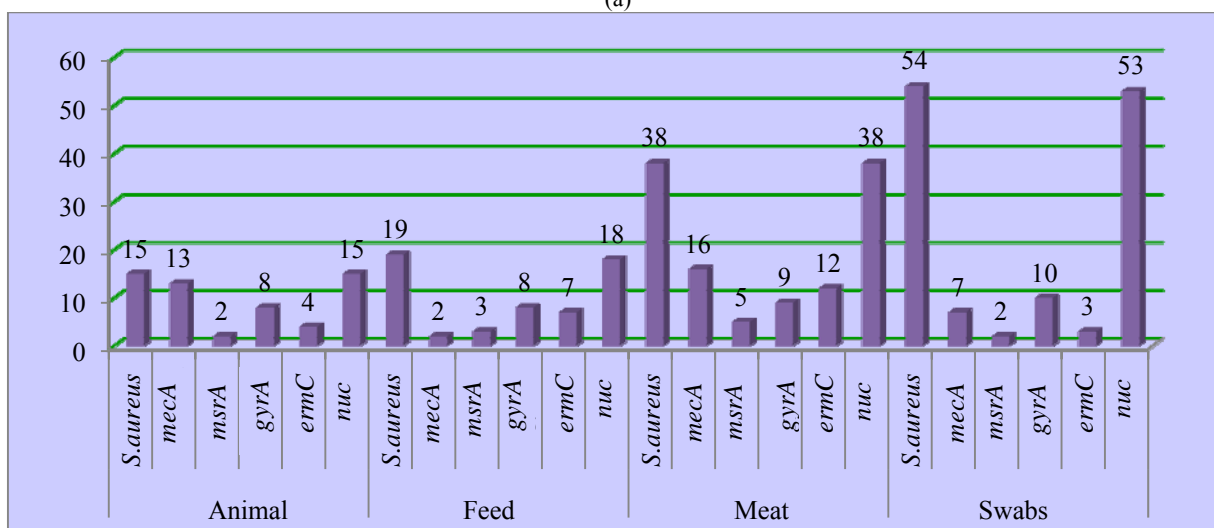


Fig. 10 The number of distribution of antibiotic resistance in *S. aureus* isolates from several sources.



(a)



(b)

Fig. 11 The number of occurrence of antibiotic resistance among *S. aureus*.

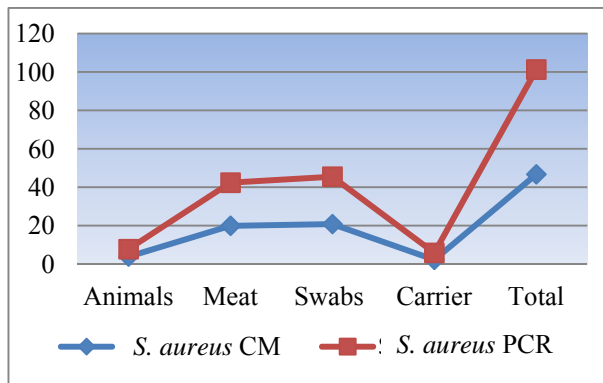


Fig. 12 Result of PCR sensitivity test.

S. aureus infections [26]. However, since 1961, when methicillin was introduced for clinical use, the occurrence of MRSA strains has steadily increased and MRSA infection have become a serious problem internationally [27, 28]. Identification of MRSA strains in food animals led to concerns regarding food-borne contamination, and MRSA has been identified in retail meat in Europe, Asia, and North America [29-31].

The prevalence of methicillin-resistance is known to more than 70% among *S. aureus* isolates from hospitals in Korea [32]. According to the present study, MRSA accounts for 44.7% of all *S. aureus* cultures in Mongolia. Many MRSA isolates exhibit multiple resistance to the commonly used antimicrobial agents amikacin, oxacillin, penicillin, erythromycin and tetracycline [33, 34].

3.5 PCR Sensitivity Test Result

The comparative study using McNemar's test showed that PCR has sensitivity of 96.2%, whereas culturing method was 78.4% sensitive. There was discrepancy of 16.4% between both methods (Fig. 12) and that means microbiological analysis for processed and stored samples will be more reliable and less time-consuming if it is done at DNA level.

In the present study, direct PCR was shown to be very effective in detection of the pathogens from meat sample homogenates, indicating that it is a robust method for rapid detection in comparison with culture technique which provides a significant contribution to

both regulatory agencies and meat. Especially, differences of testing results for carriers can depend on the presence of a number of issues, such as human nutrition, immunity and use of medicines, which affect bacterial viability. As well, it is observed that difference between both methods is probable to depend on the genera of bacteria.

4. Conclusions

Results of this surveillances of *mecA* positive *S. aureus* in the present study and confirmed cases of food-borne infections and intoxications caused by *S. aureus* should be considered as one of mean criteria of food safety issues in Mongolia, and special attentions should be paid on antibiotic resistant bacteria, such as *S. aureus*.

Due to the diversity of these resistance mechanisms and the constant appearance of new patterns, antibiotic utilization in developing countries should be under strict control and should be monitored to avoid the exhaustion of the antibiotic arsenal that is under intense use.

Acknowledgments

The authors want to acknowledge the support from the International Foundation for Science (IFS) for moral financial support.

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