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Multidrug resistance, extracellular enzymatic activity and biofilm formation of *Staphylococcus aureus* isolates from various animal foods in East China.

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Abstract

Background: *Staphylococcus aureus* is the most common pathogenic bacteria that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. The development of multiple drug resistance to this organism is posing serious threat to human health; these bacteria also have plenty of virulence factors which contribute to infection.

Results: Ampicillin, penicillin, and amoxicillin were the least effective. Norfloxacin, cephalexin, ciprofloxacin, rifampicin, clindamycin and vancomycin were the most effective antibiotics by in vitro sensitivity testing.

Conclusion: *S. aureus* isolates from foodstuff were producers of a variety of extracellular hydrolytic drug resistance.

Keywords: *Staphylococcus aureus*, Antibiotic susceptibility, Virulence factors, Biofilm, Animal foods.

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Introduction

Staphylococcus aureus is one of the most common pathogenic bacteria that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. Staphylococcal food poisoning (SFP) symptoms generally have a rapid onset, appearing around 3 hours after ingestion (range 1-6 hours). Common symptoms include nausea, vomiting, abdominal cramps and diarrhoea. SFP now is a major concern in food safety and public health and possesses a threat to the food production and human healthy all around the world. Foodstuff contamination may occur directly from infected animals or poor hygiene food preparation processes, or non-hygienic storage environment for food. As such, food products such as meat, milk, cheese and other related products have been considered as potential sources for the transmission of *S. aureus* to humans. Moreover, foods contaminated with drug resistant bacteria represent ideal reservoir for the transmission of drug resistant strains. Drug resistance is now a major global public health problem worldwide. In recent decades, the increasing prevalence of drug-resistant *S. aureus* is receiving widespread attention. In 1961, only 1 year after the introduction of methicillin into clinical practice, Methicillin-resistant *S. aureus* (MRSA) was first described in the United Kingdom as a hospital-acquired pathogen [1]. Now MRSA is prevalent in most of the countries. These strains are not only resistant to methicillin, but also resistant to all other β-lactams, such as cephalosporin [2,3]. Moreover, MRSA with reduced susceptibility to vancomycin was recognized [4]. MRSA cause nosocomial infections and are associated with increased rates of illness and death [5].

The pathogenicity of *S. aureus* is attributed to various virulence factors that allow it to adhere to surface, invade or avoid the immune system, and cause harmful toxic effects to the host. Over 40 different virulence factors have been identified in *S. aureus* [6]. The most associated virulence factors with this microorganism have been described in the literature including toxins such as hemolysins (α, β, γ), leukocidins, and heat-stable staphylococcal enterotoxins and extracellular enzymes such as proteases, nuclease, hyaluronidase, and lipases, the main function of these proteins may be to convert local host tissue into nutrients required for bacterial growth [7], which also involved in tissue invasion of the host cells [3,7]. As well as degrading host proteins directly, proteases also have the ability to dysregulate the kallikrein-kinin system, resulting in increased vascular permeability and

hence ensuring the supply of nutrients to the site of infection, in addition to having numerous modulatory effects on the host immune response [8]. Extracellular enzymes produced by *S. aureus* have been implicated in the pathogenesis of skin disorders such as atopic dermatitis [9]. Biofilms are commonly associated with many diseases of human and can form on virtually any surface [10]. It is considered to be one of the virulence factors in many bacterial pathogen. Moreover, they are linked to many persistent and chronic bacterial infections [11]. In *S. aureus*, biofilm help it adhere to its target tissues, mainly implants, and other foreign body materials, through adhesive mechanisms. Microcolonies encased in extracellular polysaccharide of biofilm are protected from antimicrobial agents [12].

The aim of this study was performed to determine the antimicrobial resistance of *S. aureus* isolates from different foods and to investigate their potency to secrete extracellular virulence factors, hemolysins, and the ability of biofilm formation was also evaluated. These results may provide insight into the spread of *S. aureus* isolates that are associated with outbreaks and may ultimately improve the control of SFP in East China.

Materials and Methods

Sample collection

Between June 2016 and September 2018, a total of 940 animal food samples were randomly collected from different street vendors, butchers, farms and supermarkets in Nanjing, Jiangsu Province and Lishui, Zhejiang Province, China (Table 1). All these meat and dairy products samples were collected in sterile bags, labeled and transferred in ice-boxes as soon as possible and investigated immediately after arrival at the laboratory.

Isolation and Identification of S. aureus

Standard microbiological methods for the identification of microorganisms were applied. All specimens were inoculated onto mannitol salt agar and incubated at 37°C. Preliminary identification was performed based on bacterial morphology, Gram staining, catalase tests, and the coagulase test with rabbit plasma. Then, Slidex Staph Plus latex agglutination was performed for rapid detection; agglutinating bacteria that could be visually observed within 30 s were determined to be *S. aureus*. Questionable bacteria were further identified using the Vitek ATB Expression System, version 2.7.8 (BioMe´rieux Deutschland GmbH, Nu¨rtingen, Germany), which uses 32 biochemical reactions. Bacterial isolates were stored as suspensions in a 15 % (w/v) sterilized glycerol solution at -70ºC until tests were performed.

Antimicrobial susceptibility test

The antimicrobial susceptibility testing of all *S. aureus* isolates were done according to the criteria of CLSI 2017 by Kirby-Bauer disk diffusion method (2017). Colonies confirmed to be *S. aureus* were suspended in LB broth until matching with a standard turbidity (0.5 McFarland). The suspension was used to inoculate Mueller – Hinton agar. The inoculated plates were left at room temperature to dry for 3-5 min and a set of 20 antibiotic discs (Oxoid ltd, Basingstoke, Hants, Chicago) with the following concentrations were then evenly distributed on the surface of a Muller Hinton plate:

Table 1. *Numbers and sources of meat and dairy product samples used in this study.*

Products	No. of samples	Source	No. of S. aureus isolates
I — Meat products			
1 — Beef products			39
a — Frozen meat	100	Street vendors, supermarkets	
b — Fresh meat	50	Butchers	
2 — Chicken products			31
a — Breast	100	Street vendors, supermarkets	
b — Legs	100	Street vendors, supermarkets	
3 — Pork products			55
a — Frozen meat	190	Street vendors, supermarkets	
b — Fresh meat	50	Street vendors, supermarkets	
II — Dairy products			
1 — Raw milk			68
a — fresh milk	300	Private farms, animal owners, street vendors, supermarkets	
2 — Cheese			0
a —cheese	50	supermarkets	
Overall total	940		193

Amoxicillin (AML) (25 µg), Ampicillin (AMP) (10 µg), Penicillin G (P) (10 mu g), Erythromycin (E) (15 µg), Gentamicin (CN) (10 µg), Oxacillin (Ox) (1 µg), Clindamycin (DA) (2 µg), Tetracycline (TE) (30 µg), Co-trimoxazole (SXT) (25 µg), Vancomycin (VA) (30 µg), Norfloxacin (NOR) (10 µg), Ceftriaxone (CRO) (30 µg), Cephalexin (CL) (30 µg), Tobramycin (TOB) (10 µg), Neomycin (N) (30 µg), Nalidixic acid (NA) (30 µg), Ciprofloxacin (CIP) (5 µg), Streptomycin 10 (S) (10 µg), Kanamycin (K) (30 µg) and Rifampicin (RD) (30 µg). The criteria used to select the antimicrobial agents to be tested were based on their availability and frequent prescriptions in the hospital. The susceptibility results of the drugs were interpreted according to CLSI.

β-lactamase production

Pure cultures of *S. aureus* isolates were inoculated on LB agar (Luria-Bertani broth, 0.5% yeast extract, 1.0% tryptone, 0.5% NaCl, 1.5% agar, pH 7.4). After overnight incubation at 37ºC with 120 rpm agitation in a shaking incubator, 10 ml LB agar

containing 0.2% starch, 200,000 IU Penicillin and 1.0% agar was poured onto the *S. aureus* grown culture plate. After solidified, 0.5 ml of iodine solution was added into the plate and spread over the growth and later the excess solution was removed by inverting the plate. Reading was taken after 30 min at room temperature. The isolate producing β-lactamase would produce characteristic decolorization around the β-lactamase producing colonies [13].

Extracellular enzymatic activities

The activities of various extracellular enzymes were determined after inoculation of cultures onto LB to which supplemented with different substrates at 37ºC for 24 hours. All strains were tested in duplicate, and when results were different, a third experiment was carried out to resolve the discrepancies. The following substrates had been added: 2% (w/v) skim milk for proteases, the production of protease was recognized as a clear zone or a broad zone of precipitation around the colonies; 3% (w/v) gelatin for gelatinase, colonies that had opaque zones around them were considered positive; 1% Tween 80 for lipase, which makes easy the detection of the enzymatic activity, visualized by a halo close around the colonies; 5% (v/v) egg yolk for phospholipase, the egg yolk digested by phospholipase produces precipitation around colonies [14-15]. Elastase activity was evaluated by using 1% (w/v) soluble elastin (Fluka Chemical, France) in Columbia agar base as described by Henderson et al. [16]. Extracellular nucleases (DNases) were determined on DNase agar plates (Difco) with 0.005% methyl green. The culture was streaked onto the plates and incubated at 37ºC for 24-36 hours, a pink halo around the growth indicated nuclease activity [17,18].

Hemolytic activity

The hemolytic activity was evaluated by plating isolated strains on bacteriological agar supplemented with 5% sheep’s blood for hemolysin production [18]. The haemolytic activity was determined by observing a clear zone of haemolysis (beta-haemolysis), a partial and greening haemolysis zone (alpha-haemolysis).

Quantitative assay for biofilm formation

Tube test method was used to qualitative detection of biofilm as described by Christensen et al. [19]. A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hours. After incubation, tubes were decanted and washed with phosphate buffered saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with de-ionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The experiment was performed in triplicate and repeated three times.

Detection of virulence factors genes

Total genomic DNA template used for PCR analysis was extracted from the strains using a commercial bacterial Genome DNA Extraction Kit (Tiangen, China). The presence of protease, lipase, and α and β-hemolysin genes in *S. aureus* strains was detected by PCR using the primers listed in Table 2. Each singleplex PCR was performed using Ready-To-Use PCR Kit (Sangon Biotech, Shanghai, China) according to the manufacturer’s protocol. DNA amplification was carried out in a PCR mixture that contained 25 μL Taq Master Mix, 0.2 μM forward primer, 0.2 μM reverse primer, 100 ng of DNA template. Amplifications were performed according the following cycle conditions:

Table 2*. Primers used in this study.*

Primer	Sequence (5’-3’)	Products	Annealing TºC
sspA-F	GACAACAGCGACACTTGTGA	292	45
sspA-R	AGTATCTTTACCTACAACTACA		
sspB-F	TGAAGAAGATGGCAAAGTTAG	493	47
sspB-R	TTGAGATACACTTTGTGCAAG		
hla-F	CGCGGATCCAAAACACGTATAGTCAGC	960	47
hla-R	CGCGAGCTCATTTGTCATTTCTTCTTTTTC		
hlb-F	GGAGGATCCATGATGGTGAAAAAAACAA	1018	46
hlb-R	GGAGTCGACCGAGTTATTAGTTAGTTGAGC		
geh-F	GCACAAGCCTCGG	473	41
geh-R	GACGGGGGTGTAG		

sspA=serine protease; sspB=cysteine protease; hla =α-hemolysin; hlb =β-hemolysin; geh=lipase; F=forward; R=reverse.

For all genes an initial denaturation at 94ºC for 10 min was followed by 30 cycles of denaturation at 94ºC for 1 min, annealing for 1 min at temperature determined for each gene as described in Table 2 and elongation at 72ºC for 1 min, followed by 10 min of final extension at 72ºC. PCR products were then electrophoresed in a 1.5% (w/v) agarose gel.

Results and Discussion

Antimicrobial susceptibility of S. aureus isolates

A collection of 193 non-duplicate *S. aureus* isolates were obtained from different foods. *S. aureus* was identified by phenotypic markers and biochemically by means of the Vitek ATB Expression System, version 2.7.8 (BioMe´rieux Deutschland GmbH, Nu¨rtingen, Germany), which uses 32 biochemical reactions. The proportions of the strains varied depending on the sample origin.

Among the 193 *S. aureus* isolates, all isolates (100%) were resistant to Penicillin, Ampicillin, while, Amoxicillin showed resistance (95.9%), Nalidixic acid (87.6%), Ceftriaxone (74.6%), Streptomycin (53.9%), Erythromycin (32.6%), Tetracycline (20.7%), Kanamycin and Neomycin (25.4%), Oxacillin (55.4%), Gentamicin (21.8%), Co-trimoxazole (45.6%), and Tobramycin (29%), and there was low resistance found to Norfloxacin, Cephalexin, Ciprofloxacin, Rifampicin, Clindamycin and no resistance to Vancomycin (Table 3). Out of the 193 isolates165 strains of β-lactamase were positivethe positive rate was 85.5%; which probably accounted for the 100% resistance obtained for both Ampicillin and Penicillin.

Staphylococcus aureus is an important cause of food poisoning pathogen, which frequently harbours antibiotic resistance genes. In this study, we found that all animal foods isolates showed high susceptibility to Norfloxacin, Cephalexin, Ciprofloxacin, Rifampicin, Clindamycin and Vancomycin, though the majority of them were resistant to Ampicillin, Penicillin, Amoxicillin. The resistance to beta-lactamase Penicillin and ampicillin was highest of all antibiotics at 100%. The high prevalence of penicillin-resistant *S. aureus*, observed in our study, is in agreement with earlier findings [20-24]. We attributed this to the presence of β-lactamase producing *S. aureus* in animal feeding environment and ‘selection pressure’ due to the use of the β-lactam antibiotics for the diseases treatment, offering advantage for the selection colonization to more resistant β-lactamase strains. Vancomycin has been used to treat serious infections caused by MRSA in human bacterial infectious diseases, which are becoming increasingly common globally. In this study, the 193 isolates were sensitive to vancomycin, maybe this is the result of no vancomycin used in animal husbandry. But Bhattacharyya et al. [25] found seven isolates from bovine and caprine milk exhibited resistance to Vancomycin.

Production of extracellular hydrolytic enzymes

In this study, all *S. aureus* tested strains were positive for proteases gelatinase and elastase. We noted that among the 193 *S. aureus* strains, 136 were phospholipase producers (70.5%), 125 (64.8%) isolates can produce lipase, DNase activity was found in 165 (85.5%) of 193 *S. aureus*. 47 out of 193 tested strains were beta-hemolytic (24.4%), 83 out of 193 tested strains were alpha-hemolytic (43%) and the other 62 strains can produce alpha-and beta-haemolysis (32.1%). Among the tested strains, 156 (80.8%) were positive for sspA and sspB. Our result revealed that 134 *S. aureus* isolated from animal foods harbor the geh gene (69.4%). The hla and hlb genes encoding the α-hemolysin and β-hemolysin were detected in 123 strains (63.7%) and 162 strains (83.9%).

S. aureus can secrete many proteins including various extracellular enzymes and pathogenic factors that favor the successful colonization and infection of host tissue. The production of *S. aureus* extracellular enzymes such as proteases, lipases, nucleases, gelatinase and elastase convert tissue components into nutrients, facilitating bacterial growth, and invasion [26]. In this study, the determination of hydrolytic enzymes production revealed that all strains produce proteases gelatinase and elastase production and we also found that all isolates have hemolysis (alpha or beta) characteristics. Wu et al. studies showed that *S. aureus* isolated from corneal ulcers produced caseinase (100%), gelatinase (80%), and elastase (70%) [27]. DNase activity is important to distinguish between

Table 3. *Antibiotic sensitivity pattern of the Staphylococcus aureus isolates recovered from different animal foods.*

Antibiotic	Resistant	(%)	Intermediate	(%)	Sensitive	(%)
Penicillin	193	100	0	0	0	0
Ampicillin	193	100	0	0	0	0
Amoxicillin	185	95.9	8	4.1	0	0
Norfloxacin	17	8.8	56	29	120	62.2
Ceftriaxone	144	74.6	49	25.4	0	0
Cephalexin	24	12.4	59	30.6	110	57
Tobramycin	56	29	20	10.4	117	60.6
Neomycin	49	25.4	119	61.6	25	13
Nalidixic acid	169	87.6	17	8.8	7	3.6
Erythromycin	63	32.6	22	11.4	108	56
Tetracycline	40	20.7	13	6.7	140	72.6
Ciprofloxacin	22	11.4	20	10.4	151	78.2
Gentamicine	42	21.8	17	8.8	134	69.4
Streptomycin	104	53.9	74	38.3	15	7.8
Kanamycin	49	25.4	59	30.6	85	44
Rifampicin	15	7.8	9	4.6	169	87.6
Clindamycin	16	8.3	81	42	96	49.7
Vancomycin	0	0	0	0	193	100
Oxacillin	107	55.4	0	0	86	44.6
Co-trimoxazole	88	45.6	31	16.1	74	38.3

pathogenic staphylococci and nonpathogenic resident flora. In our study, we found 91.1% isolates can produce DNase, this result was similar with Gundogan. In this study, we found that 75.6% strains can produce lipase. While Saising et al. suggest that 65.6% of the strains isolates from acne lesions were lipase positive, and Merghni et al. showed that 77% isolates from oral cavity can produce lipase [28-30]. Kot found that most of *S. aureus* strains from cows with mastitis showed haemolytic activity (93.9%), and above 48% produced proteases and esterase (42.4%). We found that the multi-drug resistant strains have more ability to produce extracellular enzymes.

S. aureus biofilm formation

The biofilm detection among *S. aureus* isolates has been done by tube methods. Out of 193 isolates of *S. aureus*, 116 (60.1%) isolates were found to be positive for biofilm production by standard tube (ST) method. Biofilm formation contributes to bacterial pathogenesis and resistance to antibiotics and harsh environment. *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced more frequently. In this study, we found that 61.5% of *S. aureus* isolates to be biofilm producers detected by tube method. Similar results have been reported by others. Indrawattana's study [30] showed that 72.8% of the isolates can form biofilm. Taj et al. found that 54.8% isolates showed biofilm formation by tube method [30-32].

Conclusion

The results of this study provide insight information on the drug resistant profile and phenotypic traits of *S. aureus* isolates from animal foods in East China which should be useful for future active surveillance that aimed to control a spread of existing antimicrobial resistant bacteria as well as early recognition of a newly emerged variant.

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