

Species Diversity and Antimicrobial Susceptibility Properties of *Staphylococcus* Isolated from Broiler Feces in Selected Farms, Thailand

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ABSTRACT

In this study, 60 isolates of *Staphylococcus* species were isolated and characterized from 100 samples of broiler feces collected from 11 selected farms in northeast and central Thailand. The occurrence of antimicrobial susceptibility and resistant genes were investigated using phenotypic and molecular methods. The results revealed that coagulase-negative staphylococci were the main species of *Staphylococcus* consisting of *S. gallinarum*, *S. lentus*, *S. sciuri*, *S. saprophyticus*, *S. arlettae*, *S. cohnii*, *S. simulans*, *S. carnosus* and *S. kloosii*. Twelve antimicrobial drugs were administered to test antimicrobial susceptibility using an automated VITEK 2 (bioMérieux)

system, and most of the isolations showed resistance to oxacillin (87%), erythromycin (52%) and clindamycin (48%). Molecular analysis of the *mecA* gene showed some strains of coagulase-negative staphylococci harboring the methicillin-resistant gene. Our study indicated coagulase-negative staphylococci were the principal species showing high antimicrobial resistance and the presence of antibiotic resistant genes among the studied groups.

Keywords: *Staphylococcus* spp., coagulase-negative staphylococci, antimicrobial susceptibility, poultry feces, litter



Introduction

Staphylococci are one of the most common causes of infections in poultry. Coagulase-positive staphylococci typically cause illness, especially *S. aureus*, but coagulase-negative staphylococci (CoNS) have become an increasing concern in human and veterinary medicine¹⁻³. CoNS are opportunistic pathogens that can be isolated from the skin or mucous membrane flora among humans and animals, and can be associated with a variety of animal and human infections⁴⁻⁶. This has given rise to growing awareness throughout the world regarding the spread of pathogenic and opportunistic bacteria.

Antimicrobial resistance has become one of the most serious public health issues to be addressed today. Resistance to antimicrobial drugs is associated with difficult-to-treat infections and high levels of morbidity. Staphylococci were the primary bacteria to form resistance to antimicrobial drugs. Within one year of the introduction of penicillin, *S. aureus* became resistant to the drug⁷. This resistance spread fast and is now worldwide. The most broadly documented staphylococci resistance is that of methicillin resistance. The production of penicillin-binding protein (PBP2a), mediated by the *mecA* gene, was found to be the major cause of the methicillin resistance in *Staphylococcus* spp. In addition to beta-lactam antibiotics, *Staphylococcus* spp.,

isolated from chickens, have revealed resistant patterns with other groups of antimicrobial drugs⁸⁻¹⁰.

In 2015, a total of 418,330,613 domestic fowls were documented in the report published by the Information and Communication Technology Center, Department of Livestock Development (DLD), Thailand¹¹. The poultry house environment is generally rich in microorganisms, dust particles and chemical elements (toxic gases) that can be easily colonized, aerosolized and contaminated when the underlying poultry litter and feces are moved or disturbed. Microorganisms in poultry barns usually originate from feed, manure, litter, feathers, animal skin and the animal itself. Among aerobic bacteria isolated from poultry litter or feces, staphylococci were the most prevalent microbes^{12, 13}. The purpose of the current study was to determine the status of primary gram-positive bacteria often found in broiler farms in Thailand, using an automated system: firstly species identification among staphylococci, and secondly their phenotypic and genotypic resistance properties.

Materials and methods

Study area

The study was performed during 2015 within selected poultry farms. Purposive sampling was applied to select the broiler farms according to 1) logistical reason 2) cooperation

of farmer 3) implemented Good Agricultural Practices (GAP) and certified by the DLD and 4) number of chickens per flock $\geq 7,000$ to 10,000. A total of 11 farms were previously selected in this study. Six farms were located in four provinces of the northeast region (Buriram, Chaiyaphum, Khon Kaen and Nakhon Ratchasima), and five farms located in three provinces of central Thailand (Lopburi, Nakhon Nayok, and Saraburi). The northeast and central regions were selected due to the high poultry population in the area¹¹ and convenience in transferring samples to the laboratory. Written informed consent was obtained from all farmers before participating in the study.

Sample collection

Feces samples came from two sources: broiler cloaca and litter. Cloacal swab and litter (a mixture of poultry excreta/feces and bedding material) swabs were collected to identify the *Staphylococcus* spp. of poultry origin. The estimated number of feces specimens to be sampled from each farm was determined at three samples according to the formula¹⁴, so at least three samples from each source on a farm were collected. Samples were kept in tubes containing trypticase soy broth (TSB) with 10% NaCl and 1% sodium pyruvate and then sent to the laboratory for identification.

Isolation and preliminary identification

Preliminary isolation of *Staphylococcus*

spp. of poultry origin was initiated by incubating collected samples for 48 h at 37°C. After the growth period, a loopful of inoculum broth was subcultured onto blood agar and then incubated at 37°C for 24 h. The distinct colonies on the blood agar were subjected to gram-staining, catalase test, coagulase test and triple sugar iron agar (TSI). Species identification was performed with the VITEK 2 (bioMérieux) system using GP ID cards and *S. aureus* ATCC 29213 was used as the quality control (QC) strain.

Analysis of susceptibility testing

A VITEK 2 (bioMérieux) was used for the antimicrobial susceptibility study. AST-GP73 cards were used for the gram-positive *Staphylococcus* isolates. Colonies from an overnight agar plate culture of each isolate were suspended in 3 mL of 0.45% saline and adjusted to a turbidity of 0.5 McFarland standards with VITEK Densicheck (bioMérieux). Quality control for the system was performed according to the manufacturer instructions. The VITEK 2 interpretive software used the species identification to determine the appropriate breakpoint. The MICs determined by VITEK 2 identified the category of microbial susceptibility as susceptible, intermediate, or resistant, according to the Clinical and Laboratory Standards Institute (CLSI) interpretative criteria^{15, 16} (Table 1).



Table 1 List of Antimicrobial Agents and CLSI Breakpoint Interpretative Criteria for Susceptibility Testing on Coagulase-negative Staphylococci.

Antimicrobial agent	CLSI breakpoint (µg/mL)		
	Susceptible	Intermediate	Resistant
Ampicillin/Sulbactam	8	16	32
Benzylpenicillin	0.5	1	2
Chloramphenicol	8	16	32
Clindamycin	0.5	1-2	4
Enrofloxacin	0.5	1-2	4
Erythromycin	0.5	1-4	8
Gentamicin	4	8	16
Nitrofurantoin	32	64	128
Oxacillin	0.25		0.5
Rifampin	1	2	4
Tetracycline	4	8	16
Trimethoprim/Sulfamethoxazole	8		16
Vancomycin	4	8-16	32

PCR detection of antibiotic resistant genes

PCR was used to detect *mecA* from *Staphylococcus* spp. Both resistant and susceptible phenotype strains were included in the PCR test. DNA was extracted from each bacteria isolate using a spin column-based nucleic acid purification commercial kit. PCR amplification, with primers M1 (TGGCTATCG-TGTCACAATCG-3') and M2 (5'-CTGGAAGTT-GTTGAGCAGAG-3') were used to amplify the *mecA* region¹⁷. The PCR mixture contained 5 µl 10x buffer, 1.5 mM MgCl₂, 200 µM dNTP mix, 0.1 µM of each primer, 1U Taq polymerase (Invitrogen), and 5 µl of DNA template. After

amplification, electrophoresis was performed with 3 µl of PCR samples on 1.5% agarose gel.

Results

A total of 100 swab test tube samples were included in the study. From 100 tube samples, 60 isolates (34 isolates from cloacal origin and 26 isolates from litter origin) of *Staphylococcus* spp. were identified and the main species associated with CoNS. From 60 isolates, nine species of *Staphylococcus* were detected in this study: *S. gallinarum* 15 (25%), *S. lentus* 13 (22%), *S. sciuri* 10 (17%),

S. saprophyticus 6 (10%), *S. arlettae* 4 (7%), *S. cohnii* 4 (7%), *S. simulans* 4 (7%), *S. carnosus* 2 (3%) and *S. kloosii* 2 (3%). *Staphylococcus gallinarum* was not only the main species found in bird feces but also the main species found in the investigated farms.

The results of antimicrobial susceptibility testing are shown in Table 2. All 60 isolates of the coagulase-negative staphylococci isolates were susceptible to ampicillin/sulbactam, gentamicin, nitrofurantoin and vancomycin.

However, resistance was highest to oxacillin at 87%, while erythromycin and clindamycin were at 52 and 48% respectively. Eighty percent of the erythromycin resistant staphylococci were also resistant to clindamycin. Fifteen resistance patterns from 60 isolates were found in this study (Table 3). Some isolates of *S. gallinarum* and *S. sciuri* revealed resistance to six antimicrobial drugs: enrofloxacin, erythromycin, clindamycin, oxacillin, tetracycline and trimethoprim/sulfamethoxazole.

Table 2 Antimicrobial Susceptibility Profile of 60 *Staphylococcus* Isolates.

Antimicrobial drug	Susceptibility profile (n = 60)		
	S (%)	I (%)	R (%)
Benzylpenicillin	52 (87)	0 (0)	8 (13)
Ampicillin/Sulbactam	60 (100)	0 (0)	0 (0)
Oxacillin	8 (13)	0 (0)	52 (87)
Gentamicin	60 (100)	0 (0)	0 (0)
Enrofloxacin	44 (73)	6 (10)	10 (17)
Erythromycin	21 (35)	8 (13)	31 (52)
Clindamycin	23 (38)	8 (13)	29 (48)
Vancomycin	60 (100)	0 (0)	0 (0)
Tetracycline	39 (65)	0 (0)	21 (35)
Nitrofurantoin	60 (100)	0 (0)	0 (0)
Chloramphenicol	56 (93)	2 (3)	2 (3)
Trimethoprim/sulfamethoxazole	52 (87)	0 (0)	8 (13)

S: susceptible, I: intermediate, R: resistant



Table 3 Antimicrobial Resistance Patterns Observed Among 60 *Staphylococcus* Isolates.

Number of antimicrobial resistance patterns	Phenotype ¹	Number of resistant isolates (%)
1	OXA	16 (27)
1	ERY	2 (3)
1	TET	2 (3)
2	OXA CLI	4 (7)
2	OXA CHL	2 (3)
2	PEN OXA	6 (10)
2	ERY TET	2 (3)
3	OXA ERY CLI	6 (10)
3	OXA ERY TET	2 (3)
4	OXA ERY CLI TET	4 (7)
4	ERY CLI TET TRI	2 (3)
4	OXA ERY CLI TRI	2 (3)
5	OXA ENR ERY CLI TET	4 (7)
5	PEN OXA ENR ERY CLI	2 (3)
6	OXA ENR ERY CLI TET TRI	4 (7)

¹ENR = enrofloxacin; ERY = erythromycin; CHL = chloramphenicol; CLI = clindamycin; PEN = benzylpenicillin; OXA = oxacillin; TET = tetracycline; TRI = trimethoprim/sulfamethoxazole

Twenty-two isolates from 60 identified isolates of CoNS were included in the PCR test for analyses of methicillin resistant staphylococci, and methicillin resistant staphylococcus genes. Therefore, PCR was performed to detect the methicillin-resistant strains from the identified CoNS isolates; the predicted size of the *mecA* amplicon was 320 bp. Finally, the presence of the *mecA* genes was detected among CoNS from poultry feces.

The resistant gene existed in 4 of 22 isolates (18%), namely, two strains showing oxacillin resistance and a positive-cefoxitin screen, and two strains showing oxacillin resistance and a negative result in the cefoxitin screen. Moreover, all four strains presenting oxacillin-susceptible and cefoxitin screen-negative phenotype from the VITEK 2 gave negative results on electrophoresis gel.

Discussion

According to restaurants and food shops in the Phitsanulok Municipality, boiled chicken and à la carte dishes were the most bacteria contaminated menu¹⁸. One of the most common and frequent pathogens in food poisoning and food-related infections is *S. aureus*¹⁹. However, this study found no coagulase-positive *S. aureus* in any of the samples. In other terms, only very low numbers of *S. aureus* were found in the feces and litter of healthy poultry. Furthermore, another study of clinical cases among broilers in Oregon, USA found *Staphylococcus* to be the most prevalent bacterial isolates and most of these were coagulase-negative staphylococci²⁰. Similarly, the primary pathogenic *S. aureus*, associated with gangrenous dermatitis, was not found in poultry house litter²¹. The predominant CoNS species of staphylococci found in this study was *S. gallinarum*, which belongs to the commonly reported chicken skin flora^{20, 22}. Moreover, *S. gallinarum* is one of etiological agents that causes bumblefoot disease in birds²³.

This study revealed antimicrobial susceptibility to nitrofurantoin and chloramphenicol. This susceptibility to antimicrobial drugs may have resulted from a Thai law that has prohibited the use of these drugs in animal feed for a long time. Nevertheless, *S. gallinarum* was regularly detected in poultry and in their

environment^{22, 24}. Despite the fact that *S. gallinarum* was the principal bacteria isolated from chickens, it appeared to have developed resistance to many antimicrobial drugs in this study. Consequently, this species should not be overlooked as it might be a source of resistance patterns within the environment.

Conventional antibiotics that were frequently used in the study area showed resistant patterns among nonclinical isolates. However, results in this study revealed susceptibility to ampicillin/sulbactam, and the MIC was $\leq 2 \mu\text{g/mL}$. Still, when we used the deduced drug option in the VITEK 2 software, antimicrobial susceptibility predicted resistance to amoxicillin (78%). Furthermore, phenotypic oxacillin resistance was highly detected, so resistance to beta-lactam antibiotics tended to increase among staphylococci in this study. Vancomycin was considered as a drug of last resort when all other drug options failed to treat infection²⁵. Luckily, all the staphylococcal isolates in this study were vancomycin susceptible, which may have produced an adequate response to the pathogens.

Clinical microbiology laboratories now accept the use of commercial automated systems to rapidly identify and determine susceptibility of bacteria. Among commercial automated systems, VITEK 2 (bioMérieux) is a widely used commercial antimicrobial susceptibility testing system because of its



convenience, cost-effectiveness and labor extensive method. Several studies indicated that VITEK 2 showed good performance in identifying and testing antimicrobial susceptibility of the most frequently found and clinically relevant gram-positive cocci²⁶⁻³⁰. According to CLSI, oxacillin MIC interpretative criteria may exhibit overcall resistance for some CoNS, so testing for *mecA* or for PBP2a is recommended for some strains, MICs = 0.5-1 µg/mL, before reporting complete beta-lactam resistance. The phenotype of methicillin/oxacillin resistance in this study was confirmed by detecting the *megA* gene. A high presence of the gene in healthy animals might challenge the animals' impact on the environment, and staphylococci with *megA* positive isolates from animal feces were observed in another study¹.

Our results indicated that VITEK could exactly predict methicillin susceptibility in CoNS. The VITEK result will show oxacillin-susceptible and cefoxitin screen-negative in a sample when an isolate sample is methicillin susceptible and lacking *mecA*. On the other hand, when the VITEK oxacillin result was discordant from the cefoxitin screen, such as oxacillin resistance with a negative cefoxitin screen, detecting the resistant gene was necessarily encouraged. The reason the VITEK system gave inaccurate results for antimicrobial agents was because the VITEK reports

used only qualitative values for cefoxitin as either a positive or a negative result for methicillin resistance. Also, the VITEK cefoxitin determined susceptibility using only 6 µg/mL of the cefoxitin drug. A limitation of VITEK cefoxitin testing on *S. saprophyticus* was listed in the product insert.

Bacteria from animals and animal housing play important roles in the spreading of pathogens to other animals, communities or the environment. Movements of domestic fowls or their wastes may propagate the disease by air, other vectors or through contact. For instance, antibiotic resistant staphylococci were isolated from flies caught near poultry feeding operations⁸. Poultry farms and houses were described as sources of antimicrobial resistance, with the presence of dust, endotoxins and aerosolized bacteria that included *Staphylococcus*^{10, 13, 31}. However, the reason methicillin-resistant staphylococci are found among chickens, so far remains unclear. However, our study provided limited information regarding types and antimicrobial susceptibility by focusing on some specific areas, but the results underline that staphylococci isolated from broiler feces presented a resistant phenotype and could also harbor resistant genes. Further studies are required to expand the knowledge of *Staphylococcus* spp. towards regional or national surveillance and other aspects such as aerosols and vectors.

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ชนิดความหลากหลายและความไวของเชื้อ *Staphylococcus* ที่แยกได้จากมูลไก่เนื้อในบางฟาร์มประเทศไทย

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บทคัดย่อ

การศึกษาในครั้งนี้เป็นการศึกษาการแยกและจำแนกชนิดเชื้อ *Staphylococcus* จากมูลไก่เนื้อ 100 ตัวอย่าง ซึ่งเก็บจาก 11 ฟาร์ม ในภาคตะวันออกเฉียงเหนือและภาคกลาง จำนวน 60 ไอโซเลต และการทดสอบความไวของเชื้อแบคทีเรียต่อยาต้านจุลชีพ ในด้านการแสดงออกและยีนดื้อยา ผลการศึกษาการแยกและจำแนกชนิดพบว่า เป็นเชื้อกลุ่ม coagulase-negative staphylococci ได้แก่ *Staphylococcus gallinarum* *S. lentus* *S. sciuri* *S. saprophyticus* *S. arlettae* *S. cohnii* *S. simulans* *S. carnosus* และ *S. kloosi* ซึ่งเชื่อดังกล่าวได้ทำการทดสอบความไวของเชื้อแบคทีเรียต่อยาต้านจุลชีพ จำนวน

12 ชนิด ด้วยเครื่องอัตโนมัติ VITEK 2 (bioMérieux) ซึ่งพบผลดื้อยาส่วนใหญ่ คือ Oxacillin Erythromycin และ Clindamycin คิดเป็น 87% 52% และ 48% ตามลำดับ นอกจากนี้ยังพบยีนดื้อยา *mecA* ที่เป็นตัวแทนของการดื้อต่อยา Methicillin ในกลุ่มเชื้อดังกล่าว จากการศึกษาครั้งนี้แสดงให้เห็นว่า เชื้อที่พบหลักเป็นเชื้อกลุ่ม Coagulase-negative Staphylococci ทั้งยังมีการดื้อต่อยาต้านจุลชีพ และมียีนดื้อยาในระดับสูงในกลุ่มตัวอย่างที่ทำการศึกษา

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คำสำคัญ: *Staphylococcus* spp., coagulase-negative staphylococci, ความไวของเชื้อต่อยาต้านจุลชีพ, มุลสัตว์ปีก, สิ่งปฏิกูล