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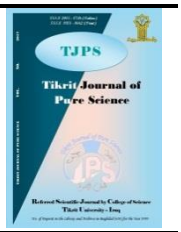

Prevalence of *PVL* gene in some methicillin- resistant *Staphylococcus sp.* isolated from frozen, non frozen chickens and slaughtering workers in Kirkuk and Erbil

Shameran M. Tawfiq

Department of Soil and Water, College of agriculture University of Sallahaddin Erbil, Kurdistan region, Iraq

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Corresponding Author:

Name: Shameran M. Tawfiq

E-mail:
dr.shamerantawfiq@gmail.com

Tel:

Abstract

The present study was performed on a total of 350 samples (145 freshly chiken, 20 frozen chiken, 30 skin wounds of slaughtering workers,55 nasal swaps of workers and 100 swap from healthy skins of slaughtering warkers. The specimen taken from Shops selling live chickens (local) in Kirkuk and Erbil Governorates that inspected for *Staphylococcus aureus.* Fifty nine *S. aureus* isolates were isolated after clinical assessment for bacteriological examination. The isolates were distributed as 15(25%) from heathy workers skins, 5(8.5%) from workers wounds, 20(33.9) from freshly chickens samples,4(6.8%)from frozen chiken and 15 (25%) nasal swaps. The antibiotic susceptibility showed that the most effective antibiotics was Norfloxacin followed Ciprofloxacin, Gentamycin, and trimethoprim.20 isolates were choosen for PCR test out of 59 depending on sensitivity test (20 hsolate appeared resistance to methecillin disc), PCR results showed that *mecA* was detected in 12(20.3%) isolate. Distributed to 5(8.5%) fresh chiken, 3(5%) wound, 2(3.4%) healthy workers skins and 1 (1.7%) to each of nasal swabs of workers and frozen chickens of studied isolates.While *pvl* virulence gene was detected in 6(30%) isolates out of the 12 isolates were positive to *mecA* gene, 3(25%) fresh chickens, 2(16.7%) healthy workers skins, 1 (8.3%) in wound workers skins, None of the MRSA isolates from nasal workers swabs and frozen chickens were found positive for the *PVL* genes.The aim of this study to detect *PVL* gene among MRSA isolates.

Introduction

Staphylococcus aureus (*S .aureus*) bacteria are G+, often found as normal human microbiota of the skin and nasal cavity. Generally no causing any problems for healthy people without cuts, abrasions, or breaks on their skin[1, 2]. But it can causes serious infections, especially in people weakened immune system, people who have scratches, cuts or wounds and chronic illnesses. Methicillin - resistant *S. aureus* (MRSA) is a strain of *S. aureus* that is resistant to methicillin. MRSA is the strain with a multi-resistance to beta-lactam antibiotics (cephalosporins &pencillins). It is any strain of *S. aureus* that has a mutation through horizontal gene transfer, MRSA strains carry an extra gene (*mecA*) that encodes a penicillin binding protein (PBP2a) that replaces the wildtype penicillin binding proteins[3,4]. Today MRSA has become more common in healthy people. MRSA is easily spread to other individuals by people with the organisms on their skin,by skin-to-skin contact or contact with towels, razors, doorknobs or benches. Some people (carriers) have MRSA strains on their body but have no symptoms, however, these people can still transfer MRSA to others through direct or indirect contact and who have close contact with one another. MRSA may cause deep infections in some people, because it is resistant to commonly used antibiotics. It is not easy to treat or become worse if the right treatment is delayed[5]. Cases of MRSA have increased in livestock animals,at the last decades a new variant of MRSA has emerged in animals and is found in intensively reared production animals, this type of MRSA is called LA-MRST (livestock -associated) where it can be transmitted to humans as LA-MRSA, though dangerous to humans often asymptomatic in food-producing animals(primarily pigs, cattle and poultry) and workers in poultry slaughtering [6], Panton – Valentine leukocidin (*PVL*) is a cytotoxin, virulence factor that has a major role in pathogenicity of this bacteria,this toxin form pores in the membrane of host defense cells, it increases MRSA virulence and

be able to cause severe necrotic pneumonia ,skin and soft tissue infections and disease progress till toxic shock syndrome in addition to it ability to cause life threatening[7], *PVL* is mostly associated with community-acquired MRSA infections, the aim of current study is to conferm that if *PVL* prevalence among methicillinresistant *Staphylococcus aureus* (MRSA) in chiken meats freshly and then froze plus slaughter emploe and estimate the impact of this animal reservoir on human healthcare[8,9] .

Conclusion

This study showed the spread of *PVL* among MRSA isolates in chiken meats that gave results clearly a Probability of potential public health threat to consumers and prevalence of MRSA in the community, Outputting from contamination of chiken meats with pathogenic bacteria is mainly due to unhygienic processing, handling and unhygienic environment.

Materials and Methods

sampling

Three hundred fifty swabs were taken from nasal and skin of slaughter workers,wounds freshly slaughtered chicken carcasses and frozen chicken portions (breast, leg quarters, drumsticks, thighs, ground) in a chicken shops located in Kirkuk and Erbil governorates in 2016. The samples originated from 33 local poultry slaughter shops. One hundred fourty-five chicken meat samples (breast, leg quarters and drumsticks), twinty from frozen chickens. Thirty swabs from wonds of workers and one hundred fifty five swab from each of healthy skins and nasal of slouter shops workers respectively.All meat samples packaged in sterile plastic bags and taken to the laboratory for bacteriological tests maintaining low temperature then frozed In the freezer, Isolation of *S.aureus* .

The *S. aureus* was isolated from chiken samples and workers as follow: 25 g meat samples were collected from all chicken meat after trimmed from bones before sampling, and mixed in to 225 ml Buffered Peptone Water (BPW) (Oxoid Ltd UK),the samples were incubated at 37 °C for 18-24 hours, (0.1 ml) in BPW were spread on the surface of Mannitol salt Agar (Oxoid Ltd UK) and incubated at 37 °C for 24-48 hours (hr),while the swabs samples were resuspended in 5 ml of pepton water broth and incubated at37°C for 24 hr, A loopful from incubated pepton water broth was streaked onto Mannitol salt agar, and further incubated at 37°C for 24-48 hr, (wound samples we rotate swab across open wound, approximately 3 times [10]. Positive isolates on mannitol salt agar medium were identified as *S. aureus* by (growth is present, tiny pin head golden yellow colonies, Gram-positive, cluster forming cocci (using Gram Staining), non-motile (on mannitol motility medium appeared negative growth along the stab line) and conventional biochemical tests (catalase positive, coagulase positive) and formed β-hemolysis on Bloob base agar (Oxoid Ltd UK) [11,12,13,14].

Antibiotic susceptibility test was performed using disc diffusion method on Mueller Hinton agar(LAB UK) by Clinical and Laboratory Standards Institutes and the result of the susceptibility test was interpreted in accordance with susceptibility break point as previous described [15]. The antibiobitics disc that were used includes; Ciprofloxacin (5µg), Gentamycin (10µg), Norfioxacin (10µg), Methicillin (10µg), Trimethoprim (5µg), Cephotaxime (30µg), cefoxitin (FOX, 30µg), ceftazidime (CAZ, 30µg) and Erythromycin (15 µg), Mueller-Hinton agar (LAB, UK)used for susceptibility Test,all study media was prepared in accordance with manufacturer's instruction.

DNA Extraction and Identification

DNA was extracted using the phenol-chloroform method [16]. DNA samples were dissolved in trisacetate - Ethylene diaminetetraacetic acid (Tris-EDTA)(TE) buffer and DNA concentration was determined by spectrophotometer at A260nm based on µg/ml concentration, The samples were then resolved on agarose gel (0.8%) with 1 ml of template DNA mixed with 3 ml of loading dye (EDTA + KOH +glycerol+ bromophenol blue)[17]. Samples were electrophoresed at 45 volts and 60 amper for 15 minutes in the first stage and 90V,60A for 30 minutes,then the gel was stained with ethidium bromide, DNA samples showing intact bands were used for PCR amplifications In this study, DNA obtained was preserved at -22°C.[18].

Detection study genes by Polymerase Chain Reaction

PCR was applied by using two sets of primers for detection of tow virulence genes that may play a role in virulence of *S. aureus*. These genes were (*mecA*) methicillin resistant and leukocidine (*pvl*) by using specific primers for *S. aureus* as shown in Table 3. It was applied on 20 *S.aureus* isolates selected depending on the resistance to methicillin antibiotic in sensitivity test, especially to methecillin, isolates isolated as following :5 isolates from each of(nasal swabsof workers,fresh chickens, wounds and healthy workers skins),while 4 isolates were isolated from frozen chickens, Amplification *mecA* and *pvl* genes by using specific primers 533 bp and a 433 bp fragments, respectively, the mixture 50 µl reaction volume of PCR mastermix, 2X (corporation promega, UAS) was used and Composed of DNA polymerase 50mM Tris-HCl (pH 9.0), 50mM NaCl, 5mM MgCl2, 200µM each of dATP, dGTP, dCTP, dTTP, upstream primer1.5µl, downstream primer, 1.5µl, DNA template 2.5µl, deionized Sterile distilled water was added to make a final volume of 50 µl for *mecA* gene while for*PVL* the differences was in DNA template 3.4µl, The thermocycler was programmed to *mecA* gene with the following: 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 sec. and extension at 72°C for 1 minutes

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and a final extension step at 72°C for 10 min [19], whereas *PVL* amplification was carried out under the following conditions: an initial 30-sec denaturation step at 94°C, followed by 30 cycles 30sec of annealing at 55°C, and 1 min of extension at 72°C and a final extension step at 72°C for 7 min, The PCR products were purified by using the kit (Promega, USA) and sequenced with the primers used for PCR (alpha,Canada) The PCR products were detected by gel electrophoresis using 1.5% agarose gel (Sigma, France) and run in 1X TBE buffer (pH 8.3) at 60V for 2 hours, 100 bp plus DNA ladder was used to determine the size of the PCR products (Promega, USA). The gels were stained with ethidium bromide and visualized under UV light.

Table (1) Source of *S.aureus* isolates

Source	No. of sample	No.of S.aureus (+) Samples (%)
Nasal	78	15(19.2)
healthy skins	77	15(19.5)
wound skins	30	5(16.7)
Frozen chiken	20	4(20)
Fresh chiken (leg quarters)	30	6(20)
Fresh chiken (drumsticks)	30	2(6.7)
Fresh chiken (thighs)	30	5(16.7)
Fresh chiken (ground)	30	4(13.3)
Fresh chiken (breast)	25	3(12)
Total	**350**	**59(16.9)**

Table (2) Antibiotics Suceptibilty of *S.aureus* isolates(N=59)by disk diffusion method

Antibiotic	No. of isolates resistant to antibiotic agents (%)
Norfloxacin	5 (8.5)
Ciprofloxacin	8 (13.6)
Gentamycin	12 (20.3)
Trimethoprim	12 (20.3)
Cephotaxime	15 (25.4)
Ceftazidime	16 (27.1)
cefoxitin	19 (32.2)
Methicillin	20 (33.9)
Erythromycin	30 (50.8)

Table (3) Nucleotide sequence and primers used for identification *mecA* & *PVL* among *S. aureus* by PCR.

Primer	Gene	Sequence (5'-3')	Length bp	Reference
MecA	*mecA*F1	AGTTCTGCAGTACCGGATTG	533	(Alli *etal*.,2011)
	*mecA*B1	AAAATCGATGGTAAGGTTCGC	533	(Alli *etal*.,2011)
LukS/F-PV	*PVL* F1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	(Motamedi *etal*.,2015)
	PVL B1	GCATCAAGTGTATTGGATAGCAAA AGC	433	(Motamedi *etal*.,2015)

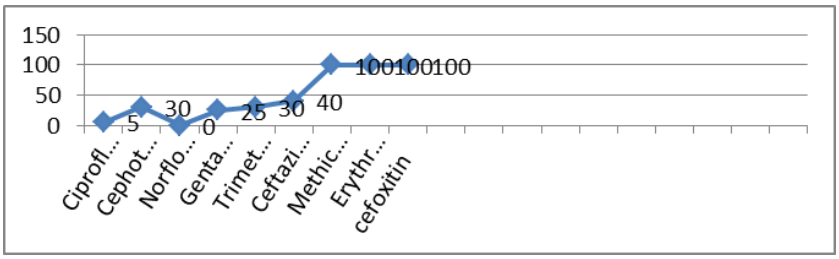

Figure 1: Pattern of Resistance to Antimicrobial agents among 20 *S. aureus* isolates

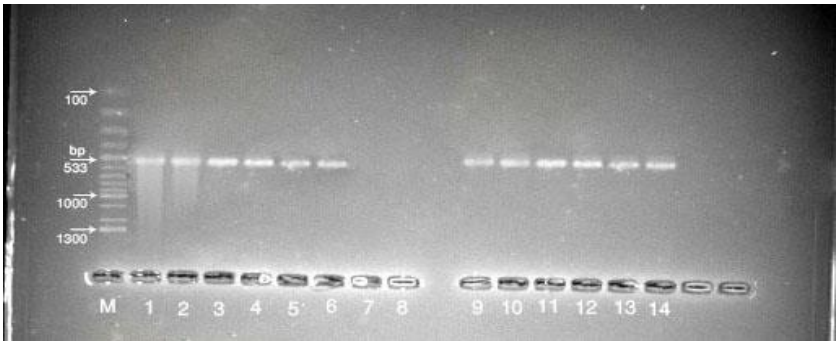

Figure 2: investigation of *mecA*(533bp) gene amplification from chiken isolates of S. aureus on 1.5% agarose gel: M: 100bp DNA ladder (marker); Lane 1-6,9-14:(mecA amplicon bands) positive ; Lane7, 8: negative bands appears

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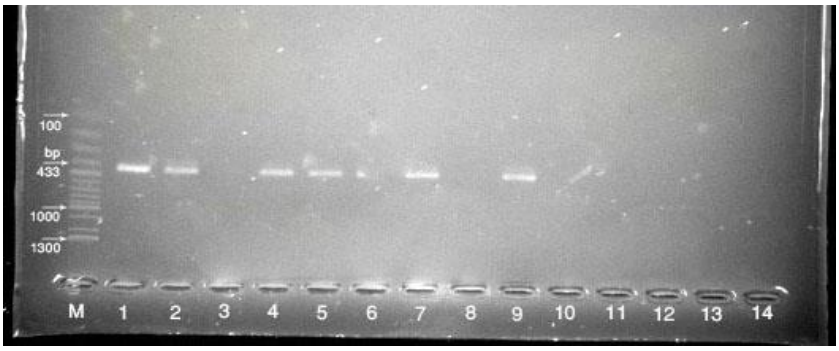

Figure 3: investigation of *PVL* gene(433bp) amplification from chiken isolates of S. aureus on 1.5% agarose gel: M: 100bp DNA ladder(marker); Lane 1,2,4,5,7,9:(*PVL* amplicon bands) positive ; Lane3,6,8- 8: negative bands appear.

Result and discussion

59 isolates diagnosed were Subordinate to *S. aureus* bacteria from workers nasal, skins and wounds, different portions of freshly slaughtered chicken carcasses and frozen chickens as showen in table(1) according to the standard methods described by[10] results were 15(15%) from heathy workers skins, 5(16.67%) wound workers skins,20(14.81) from freshly chickens samples, 4 (8.0%) frozen chiken and 15(42.88%)nasal isolates, susceptibility examination tested for(9) antibiotics, Isolates showed a clear variation in resistance to antibiotics, resistance ratios, highest rate of resistance was recorded for Erythromycin (50.8%), Methicillin (33.9%), and cefoxitin (32.2%) and the lowest rate was for Norfloxacin (8.5%), Ciprofloxacin (13.6.%), and Gentamycin, Trimethoprim (20.3%), table(2), 20 isolates selected for detecting *mecA* gene according to their resistance towards betalactam antibiotics especially Methicillin which shown 20(100%) to Methicillin, cefoxitin and Erythromycin, 8(40%)to Ceftazidime, 6 (30%) to Cephotaxime and Trimethoprim, 5 (25%) to Gentamycin, 1(5%) to Ciprofloxacin while all 20 isolates appeared sensitivity to Norfloxacin figure (1), *mecA* gene was present in 60% (12/20) of the isolates distributed to 5(25%) fresh chiken, 3(15%) wound skins of workers, 2(10%) healthy workers skins and 1 (5%) to each of nasal swabs of workers and frozen chickens of the studied isolates Figure(2). In the same isolates (those with *mecA*), *PVL* gene was present in 50% (6/12) of the isolates, (figure 3), which were exist in the 25% (3/12) fresh chikens,16.7%(2/12) healthy workers skins, 8.3% (1/12) wound skins of workers while PVL gene did not exist In the nasal swabs of workers and frozen chickens Figure(3).

Most previous studies showed the prevalence MRSA among retail meats as astudy conducted in Japan and reported that some samples of commercially sold meat products in Japan were found to harbor MRSA strains [20], whereas other studies showed existence of *PVL* between MRSA strains in(CA-MRSA) ,So in a study conducted in Swiss (2002) showed prevelance of *PVL* gene among community-acquired (CA-MRSA), the gen was present in ratio 70%(7/10) from patients with skin infections [21] and they attributed that most CA-MRSA isolates expressed *PVL*, a highly potent toxin previously implicated in these types of infections, in another study by[22] they found in their study that both methicillin-sensitive *S. aureus* (MSSA) and methicillin – resistant *S. aureus* (MRSA) may carry the *PVL* and they proved Community-acquired necrotizing pneumonia caused by *PVL* secreting *S. aureus* which is a highly lethal infection that mainly affects healthy children and young adults,while little studies that deliberated prevelance *PVL* among livestock-associated (LA)-MRSA, The current study virtually agreed with [23] Study, they found 25% (7/28) of MRSA isolates harbouring *PVL* genes from wild boar meat samples but they disagreed with present study on the source type, they returned this prevelance into people handling the food were the probably general source of contamination with these isolates, our study also nearly agreed with the [9] they found in chiken meat samples that positive for *mecA* prevalence *PVL* gene lukS-lukF which was 8/12 (66.7%)in study carried out in Tulsa, Oklahoma and they reported that *PVL* gene lukS-lukF was detected only in chicken and MRSA isolates, furthermore their results indicating a human origin rather than livestock association. These results proved that infections caused by *PVL*-MRSA have been increased in recent years that require seeks about the prevalence of this virulent marker among MRSA strains in community acquired infections and livestock association [23],besides of their resistance to most betalactams antibiotics [24,23]. MRSA play an important role in the development of antibiotics resistance and such resistant isolates can be difficult to treat and if accompanied by a *PVL* increase it ferocity and difficulty, Most of the studies attributed the reason behind this wide spread such as contamination through slaughter procedures, splitting of carcasses, trimming and washing of surface, and handling of carcasses, contamination of the meat during the slaughter route,contamination may occur as a result of direct or indirect contact with fasces, skin, contaminated tools and clothing, the hands of workers, the nares, throat, hands and nails of food handling personne and hygienic practices during handling [23], some other reasons for example exposure to dust in infected animal housing,, contact

with contaminated meat products, meat packers that probable spread into the larger community for instance hospitals or spread by means of environmental routes counting air, water, or fertilizer in areas in nearness to live animal farms or crop farms where manure has been used as a fertilizer, other studies have attributed the cause of this spread is the use of antibiotics in animal husbandry as feed in addition for growth support in poultry may be leaded to antibiotic resistance initiated during animal husbandry possibly will be helper in Livestock-associated LA-MRSA and after that be raise to the broad human inhabitants, MRSA are normal inhabitants of the skin, mucous membranes and nares of healthy birds ,it has a short incubation period for infection with chicks showing signs usually within 48-72 hours and have the potential to cause disease if it enters the body of the bird, through a wound, inflammation, trimming of toe nails or beak, MRSA have been found in human, food-producing animals and retail meat, the concern about the exposure for humans through the food chain is increasing day by day that is a potential health risk for consumers [25,26,6]. finally result in this study human-originated outbreaks of LA-MRSTand to prevent this outbreak is provide a balanced nutritional diet quickly and suitably attend to and treat any wounds Decrease risk of injury by eliminating birds' access to sharp surfaces or objects Practice good sanitary practices [27].

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انتشار الجين *PVL* في بعض عزلات المكورات العنقودية الذهبية والمعزولة من الدجاج الطازج والمجمد ومن العاملين في ذلك الحقل

شاميران محمود توفيق

قسم التربة والمياه ، كلية الزراعة ، جامعة صلاح الدين ، اربيل ، اقليم كردستان ، العراق

الملخص

تضمنت الدراسة الحالية جمع (350) عينة من الدجاج الحي والمجمد ومن مسحات (الجروح, الانف والجلد) للعاملين بنسب (55,30,20,145و100) على التوالي من محلات بيع اللحوم الدجاج الحي المحلي في مدينتي كركوك واربيل في 2016.اعطت (59) عينة نتيجة موجبة لبكتريا المكورات العنقودية الذهبية اعتمادا على الخصائص المظهرية والزرعية والكيموحياتية توزعت الى (25%)15 من الجلد السليم, (8.5%)5 من الجروح, (33.9%) 20 من الدجاج الطازج, (6.8%)4 من الدجاج المجمد, (25%)15 من مسحات الانف,كما اظهرت الدراسة حساسية العزلات تجاه مضاداتNorfloxacin ,Ciprofloxacin, Gentamycin , Trimethoprim, 20 عزلة اختيرت لفحص PCR من اصل 59 عزلة اعتمادا على نتائج فحص الحساسية(20 عزلة ابدت مقاومة للميثيسلين), كشفت نتائج التحري عن وجود الجينين *mecA* و*pvl* باستخدام التفاعل التسلسلي لانزيم البلمرة الدنا PCR امتلاك (33.9%)12 من اصل 20 عزلة نتيجة موجبة للجين *mecA* بنسب (8.5%)5 من الدجاج الطازج, (5%)3 من الجروح, (3.4%) 2 من الجلد السليم و (1.7%)1 لكل من الجروح والدجاج المجمد,بينما اعطت (30%)6 من عزلات *S.aureus* من اصل 12 عزلة موجبة للجين *mecA* نتيجة موجبة للجين *pvl* توزعت على النحو التالي (25%)3 دجاج طازج, (16.7%)2 من الجلد السليم, (8.3%)1 من الجروح بينما اعطت عزلات مسحات الانف والدجاج المجمد نتيجة سالبة للجين *pvl* .