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Molecular detection of antibiotic resistance and virulence genes in *staphylococcus* species isolated from human and poultry

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Abstract

Staphylococcus species are important potential pathogens that can give rise to acute and chronic diseases for poultry and humans. Since they carry virulence and antibiotic resistance genes which can be transmitted between food producing animals and humans during various modes, the major one being food chain and cause health hazards for the consumers. Therefore, the current study was intended to inspect the incidence rate of *Staphylococcus species* isolated from chicken and patients, detection the antimicrobial susceptibility of these isolates by using Vitek2 system and affirmation the attendance of genes coding for pathogenicity and antimicrobial resistance in detected isolates by PCR. To achieve these 200 samples were collected from poultry farms and patient (100 for each) in disparate districts in Assiut province, to be subjected to bacteriological examination. The results exposed that the incidence of *Staphylococcus* spp. was 35% and 45% in poultry and human samples respectively on mannitol agar. Vitek2 system differentiated the poultry isolates to 11 isolates as coagulase-positive *Staphylococci* (CoPS) and 24 isolates as coagulase-negative *Staphylococci* (CoNS). While 20 human isolates identified as CoPS and 25 isolates consigned as CoNS. Antibiogram refined that 45.7% and 53.3% of *Staphylococcus* isolates from poultry and human were identified as methicillin-resistant *Staphylococci* respectively, also *Staphylococcal* spp. clarified a resistance to different types of antimicrobials such as penicillin, tetracycline clindamycin. Vitek2 system showed a inordinate ability to differentiate *Staphylococcus species* and evaluate its antimicrobial susceptibility which was convoluted by conventional method. PCR results revealed that the *Staphylococcus* isolated from poultry and human were harbored genes encoding for pathogenicity (*coa, hld* and *pvl*) and antimicrobial resistance (*mec*A*, van*A*, cfr* and *blaZ*) while none of the isolates harbored *sei* and *seh* genes. The phylogenetic analysis constructed on *16SrRNA* sequencing of *Staphylococcus* spp. showed a relationship between these species isolated from poultry and human. So, the obtained results emphasized the importance of reducing the unwarranted use of antimicrobial agents and implementation of sanitary hygienic procedures in poultry production.

Keywords: Staphylococcus species, Vitek2 system, Virulence genes, antimicrobials resistance genes, *16SrRNA* sequencing, poultry, human

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INTRODUCTION

Staphylococcus are widely distributed in water, soil and air, in addition to its isolation from various animal species, including poultry and its genus is present in 70 species **(Götz *et al.*, 2006).**Poultry meat is the most important source of human food poisoning **(Kadariya *et al.*, 2014).***Staphylococcal* food-borne disease induced by poultry meat became an evident problem reflected negatively on the industry of poultry, causing drawbacks on public health and making difficulty for the medical and veterinary organization **(Teramoto *et al.*, 2016).**

Staphylococcus species, generally dubbed as coagulase-negative-*Staphylococci* (CoNS) and acquired its value as they have been responsible for multiple infections in humans and animals **(Vuong and Otto, 2002).**

Staph.aureus is the most critical species within this genus, recognized as coagulase-positive *Staphylococcus*(CoPS) as well as, be one of the causes of food intoxication **(Cunha, 2009).** This microorganism is the etiological factor causing several avian diseases such as arthritis, septicemia, omphalitis,..etc **(Smyth and McNamee, 2001).**These *Staphylococcal* infectious diseases of chickens are an economic threat and they are viewed as worldwide burden **(Lowder *et al.*, 2009).**

CoNS are causing nosocomial infections in neonatal intensive care units and uncommon food poisoning bacteria **(Tong *et al.*, 2015).**Various researches on CoNS and more than 15,000 references, reflecting the increasing of medical influence of these bacteria **(Becker et al., 2014)**due to the possible spreading of antimicrobial resistant bacteria and antimicrobial resistant genes **(Chajęcka-Wierzchowska *et al.*, 2015) ,** although, CoNS is documented as a very useful bacteria in the technology and hygiene of food production and preservation **(Šušković *et al.*, 2010).**

The virulence genes are communally responsible for the pathogenicity of this organism , like *Staphylococcal* protein A (*spa*), coagulase protein (*coa*), *Staphylococcal* enterotoxins A to E collagen adhesion gene (*cna*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins (*eta*, *atb*), and leucocidins **(Pereira *et al.*, 2009).**

The appreciation of antimicrobial resistance in food borne pathogens inventing from food producing animals such as chicken that has been recorded in developing countries **(Van *et al.*, 2007*).***The proclivity for *Staphylococci* to develop antimicrobial resistance is a reason for great worry in both human and animals **(Vanderhaeghen *et al.*, 2010).**

The undesirable antimicrobial resistance has mostly been risked as result of unreasonable use of antibiotics in producing animals **(Adesiji *et al.*, 2014).**Amongst all types of resistance, methicillin resistant *Staph.aureus* (MRSA) is considered as critical, as it had been confirmed as the source of acquired infections associated with high rate of bacterial mortality worldwide **(Tiemersma *et al.*, 2004).**

Automated systems VITEK 2 analyses have proven to be an accurate technique to differentiate *Staphylococcal* species **(Sukru *et al.*, 2018).** It is used for the identification of isolated colonies to the genus and species levels **(Jackson *et al.*, 2013).** Rapid and accurate methods for identification of food borne pathogens are important for microbiological safety. In previous recent years, polymerase chain reaction (PCR) was proven as the most suitable method for fast, sensitive and unrestricted detection of pathogenic bacteria in food **(Kim and Kim, 2017**).

The aim of the present study was isolation and identification the *Staphylococcus* species isolated from poultry and human samples by using VITEK2 system, also detection of genes encoding for pathogenicity (*coa,hld,sei,pvl* and *seh*) and antimicrobial resistance genes (*mec*A,*cfr*,*van*A *and blaZ*) in *Staphylococcus isolates* by PCR and evaluation the relationship between isolated species from poultry and humans by Phylogenesis of the sequenced isolates.

MATERIALS AND METHODS

Ethical approval

The study was carried out in line with all applicable regulations and legislation of Commission for Ethics in Scientific Research, faculty of medicine, Assiut University, Egypt. The date of ethical approval of the research was 1/1/2018.

Samples collection

A total of 200 samples were collected aseptically from poultry farms and university hospital in Assiut province (100 for each) during period between March to September 2018**.** Poultry samples included liver, tarsal joint and intestine were removed from each bird according to Monecke *etal*. (2013).Also patient swabs (Abscess, conjunctivitis, otitis, and urine) were gathered according Strommenger *etal*. (2008).

Preparation of samples

Poultry samples:

Slices of liver , tarsal joints and were immersed in test tubes containing nutrient broth for overnight (Mkize, 2016) and nearly one gram of intestinal content was putted in a centrifuge tube containing 9 ml of sterile phosphate buffered saline (PBS) pH 7.4, and mixed by vortex with glass beads (4 mm in diameter) for 3 minutes. Debris was expelled by centrifugation at 700xg for 1 minute (Seidavi *et al*., 2010). One milliliter of supernatant was inoculated in a tube contained 9 ml Brain Heart Infusion broth (BHI) and incubated at 37°C for 24h

Patient samples:

The patient swabs were immersed in a tube contained 9 ml Brain Heart Infusion broth (BHI) and incubated at 37°C for 24h.

Isolation and identification of *Staphylococcus species*

Aloopful of BHI broth was streaked on mannitol salt agar at 37°C for 24h **(Gharajalar and Shahbazi, 2018).**The obtained colonies were plated onto Sheep blood agar , Baird-Parker agar for identification of *Staph. aureus* from other species **(Son *et al*., 2010)** and Oxacillin Resistance Screening Agar Base(ORSAB) agar for identification of methicillin-resistant *Staphylococcus* isolates of poultry and human**(Nahimana *et al*., 2006).**

Morphological examination of the suspected colonies was done by using Gram staining and biochemical identification of isolates on the base of catalase activity, coagulase (rabbit plasma) and oxidase test **(Fijałkowski *et al*., 2016)**.

Identification of *Staphylococcus species* and antibiotic susceptibility using VITEK 2 compact®

The identification of *Staphylococcus spp.* was done by VITEK GP (Gram Positive)on all obtained isolates from mannitol salt agar according to the cards with reference number 21342, also antimicrobials susceptibilities were done conferring to antibiogram cards AST-GP 67 with reference number 22226 (Sukru *et al.*, 2018)**.**

Detection of virulence and antimicrobial resistance genes in *Staphylococcal* species by PCR

DNA was extracted from ten *Staphylococcal* isolates belonging to different species according to Mansour et al., (2017), after refreshment of these isolates in 5 ml of BHI broth at 35ºC (±2ºC) for 24hrs by using QIAamp DNA

Mini kit (Qiagen, Germany, GmbH) on bases of manufacturer's instruction.

Different primers were used as*16SrRNA* primer derived from *Staphylocoocus* genome for confirmation our isolates, in addition to (*coa*, *sei*, *seh*, *hld* and *pvl*) for detection virulence and (*mec*A, *van*A, *cfr* and *blaZ*) for antimicrobial resistance (Table 1). Uniplex PCR amplification reaction was performed in a final volume of 25µl, composed of 6µl DNA, 12.5 µl Mastermix(Emerald Amp GT PCR), 1µl for each primer and 4.5 µl of PCR grade water. Reactions were implemented in thermal cycler (MJ Research, Inc. Watertown, MA) with the following program: initial denaturation at 95ºC for 5 min followed by 45 cycles of 95ºC for 45sec, 50ºC for 45sec and 72ºC for 1min with a final extension at 72ºC for 10 min. The amplicons size (bp) were detected by electrophoresis on 1.5% agarose gel (BioshopR, Candainc.) stained with ethidium bromide, then visualized in a UV transilluminator.

Amplification and sequencing of universal *16S rRNA* gene

The amplification of universal *16S rRNA* gene was done in 5 isolates of *Staph. aureus* (2 from poultry and 3 from human) and 2 isolates of *Staph. lentus* (one isolates from each poultry and human) by using the universal primers27F (5′-AGAGTTTGATCCTGGCTCAG-3′) And 1492R (5′ TACGGTTACCTTGTTACGACTT-3′) at 1,500-bp (Liu et al.,2009). The reaction mixture was combined of 1 µl of bacterial DNA, l µl each primer 12.5 PCR master mix (Emerald Amp Max PCR Master) the mixture was completed by PCR water in a final reaction volume of 25 µl after that, the program was run in thermal cycler (MJ Research, Inc. Watertown, MA). as follows: 30 cycles were done in a thermocycler; denaturation 95 °C for 1 min, annealing 54 °C for 1 min, extension 72° C for 3 min and final extension time of 72 °C for 5 min (Alfatih *et al*.,2018). Amplified products were analyzed by product detected by electrophoresis on 1.5% agarose gel then visualized in a UV transilluminator. PCR products were purified using QIA quick PCR Product purification kit (Qiagen, Valencia, CA). For the sequencing reaction, a prism Big Dye terminator V3.1 Kit (applied Bio system) on DNA automated sequencer (applied Bio systems) were analyzed the sequence of *16S rRNA* gene in both directions forward and reverse. The sequence results of our isolates were analyzed by blasting on gene bank https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM= blastn&PAGE_TYPE=BlastSearch& LINK_LOC=blasthome. Sequence alignment was done by multiple alignment algorithms in megalign (DNASTAR, Window version 3.12e).

Phylogenetic analysis

Phylogenetic tree was based on *16S rRNA* gene nucleotides sequence which was performed on our five *Staph.aureus* isolates (2isolates from poultry and 3isolates from human) and two *Staph. lentus* isolates (one for each) to inspect the identity of amplified fragment of *16S rRNA* gene in our isolates with *Staphylococcus* isolates reference strains registered with Gene bank using MEGA version2.1 (Kumar *et al*.,2001).

Statistical analysis

Statistics were done using IBM SPSS19.0. The person chi-squar test was used to inspect the relation between variables Statistically significant was defined as $P<0.05$

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Table (1): Nucleotidesequences, operative protocols and amplicons size (bp) of primers

Gene	Oligonucleotide sequences (5-3)	Amplion size (bp)	PCR conditions	References
16s rRNA	F: AAC TCT GTT ATT AGG GAA GAA CA R: CCA CCT TCC TCC GGT TTG TCA CC	250	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:50◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Zhang et al., 2004)
coa	F:ACCACAAGGTACTGAATAACG R:TGCTTTCGATTGTTCGATGC	987	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:55◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Veras et al., 2008)
sei	F:CAACTCGAATTTTCAACAGGTACC R:CAGGCAGTCCATCTCCATCTCCTG	466	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:50◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Pereira et al., 2009)
seh	R: CAA CTG CTG ATT TAG CTC AG F:GTC GAA TGA GTA ATC TCT AGG	360	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:50◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Monday and Bohach, 1999)
hld	F: AAGAATTTTTATCTTAATTAAGGAAGGAGTG R: TTAGTGAATTTGTTCACTGTGTCGA	111	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:60◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Jarraud et al., 2009)
pvl	F:ATCATTAGGTAAAATGTCTGGACATGATCCA R:GCATCAAGTGTATTGGATAGCAAAAGC	433	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:50◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(McClure et al., 2006)
***mec*A**	F:GTAGAAATGACTGAACGTCCGATAA R:CCAATTCCACATTGTTTCGGTCTAA	480	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:52◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Spanu et al., 2004)
***van*A**	F: GCGCGGTCCACTTGTAGATA R: TGAGCAACCCCCAAACAGTA	314	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:54◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Nam et al., 2012)
cfr	F:TGAAGTATAAAGCAGGTTGGGAGTCA R:ACCATATAATTGACCACAAGCAGC	746	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:60◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Kehrenberg and Schwarz, 2006)
blaZ	F ACTTCAACACCTGCTGCTTTC R TGACCACTTTTATCAGCAACC	173	Initial denaturation:95◦C for5min Amplification(45 cycles of) Denaturation:95◦C for45s Annealing:55◦C for45s Extension:72◦C for1min Final extension: 72◦C for10min	(Martineau et al., 2000)

Results

Results of conventional method

The data illustrated in table (2) showed the result of bacterial examination for 200 samples were taken from poultry and human samples (100 for each) on mannitol salt agar, as followed, *Staphylococcus* spp. isolated from 35% and 45% of poultry and human samples

respectively.94% of poultry isolates were obtained from intestinal content and 6% obtained from joints while 60% of human isolates obtained from urine samples and 40% from abscesses. On the other hand, tube coagulase test divided the poultry and human isolates into 31.4% and 44.4% coagulase positive and 68.6% and 55.6% were coagulase negative respectively. While ORSAB agar detected methicillin-resistant *Staphylococci* in 37.14% (13/35) and 42.22% (19/45) from *Staphylococcal* isolates respectively**.**

Table (2): Incidence of *Staphylococcus spp.* isolated from poultry and human

Source	Number of examined samples	Suspected isolates on mannitol agar	Coagulase test		ORSAB agar positive *Staphylococci*
			Positive	Negative	
Poultry	100	35(35%)	11(31.4%)	24(68.8%)	13(37.14%)
Human	100	45(45%)	20(44.4%)	25(55.6%)	19(42.22%)
Total	200	80(40%)	31(38.75%)	49(61.25%)	32(40%)

Result of VITEK 2 Compact

By using VITEK 2 system, 35 poultry isolates could be differentiated into17 *Staphylococcal* species, the highly identified species were *Staph.aureus*14.3% (5/35) followed by *Staph. lentus* 8.5% (3/35), whereas the least species were *Staph. lugdunensis*, *Staph. simulans* and *Staph. capitis* (2.86% for each). On the other hand, from 45 human isolates could differentiate 19 *Staphylococcal species*, the main species were *Staph. aureus* 31.1% (14/45) followed by *Staph. haemolyticus* and *Staph. cohnii* (3/45 for each) (6.7%) (Table3) Statistical analysis showed a significant positive correlation between poultry and human isolates at ($R=0.66$ at $p< 0.05$)

Antibiogram for VITEK 2 Compact

Compact antibiogram device was used for detection the antimicrobial resistance. The result of antibiogram showed in table (4) reveled that antimicrobial resistance profile of the 35*Staphylococcus* isolates from poultry samples to different antibiotics was investigated; none of the isolates were completely sensitive to the13 tested antibiotics. High percentage of resistance was observed in tetracycline 28 (80%), clindamycin 26 (74.3%), penicillin 22(62.8%) and erythromycin18 (51.4%). While, low resistance was noticed to gentamicin 6(17%) and trimethoprim / sulfamethazole7(20%) between tested antibiotics.

Table (3): Results of *Staphylococcus* identification by using Vitek system

Staphylococcus Species	Source	Number	Coagulase		Percentage (*)
			Positive	Negative	
Staph.aureus	Human	14	14	0	31.11
	Poultry	5	5	0	14.29
Staph.chromogens	Human	0	0	0	0.00
	Poultry	2	0	2	5.71
Staph.hyicus	Human	2	2	0	4.44
	Poultry	2	2	0	5.71

Staph.hominis	Human	2	0	2	4.44
	Poultry	2	0	2	5.71
Staph.haemolyticus	Human	3	0	3	6.67
	Poultry	2	0	2	5.71
Staph.arlettae	Human	1	0	1	2.22
	Poultry	2	0	2	5.71
Staph.warneri	Human	2	0	2	4.44
	Poultry	2	0	2	5.71
Staph.pseudointermedius	Human	2	2	0	4.44
	Poultry	0	0	0	0.00
Staph.lentus	Human	2	0	2	4.44
	Poultry	3	0	3	8.57
Staph.epidermidis	Human	2	0	1	4.44
	Poultry	0	0	0	0.00
Staph.capitis	Human	1	0	1	2.22
	Poultry	1	0	1	2.86
Staph.vitulinus	Human	1	0	1	2.22
	Poultry	2	0	2	5.71
Staph.simulans	Human	0	0	0	0.00
	Poultry	1	0	1	2.86
Staph.auricularis	Human	1	0	1	2.22
	Poultry	0	0	0	0.00
Staph.cohnii	Human	3	0	3	6.67
	Poultry	2	0	2	5.71
Staph.saprophyticus	Human	2	0	2	4.44
	Poultry	0	0	0	0.00
Staph.schleiferi	Human	1	1	0	2.22
	Poultry	2	2	0	5.71
Staph.sciuri	Human	2	0	2	4.44
	Poultry	2	0	2	5.71

Staph.xylosus	Human	2	0	2	4.44
	Poultry	2	0	2	5.71
Staph.lugdunensis	Human	1	0	1	2.22
	Poultry	1	0	1	2.86
*Staph.intermedius**	Human	1	1	0	2.22
	Poultry	2	2	0	5.71

(*) Percentage of each *Staphylococcus species* was calculated from the total number of isolates*:* poultry isolates(n=35) and human isolates(n=45)

*the obtained isolates from poultry and human showed a significant correlation between each other's R=0.6 and P<0.05

Further that the antibiogram results showed that the least resistant species was *Staph. simulans* that was resistant to3 antibiotics (tetracycline, clindamycin and quinupristin/dalfopristin). While, *Staph. aureus* and *Staph. lentus* were the most resistant species to the 13tested antibiotics. While, methicillin-resistant *Staphylococci* were identified in 16isolates (8 as methicillin resistant coagulase positive *Staphylococci* (MRCoPS) and the other 8 were methicillin resistant coagulase negative *Staphylococci* (MRCoNS)). Vitek2 system also cleared that vancomycin resistant *Staphylococcus species* was detected in14 isolates **(**Table 4**)**

The result of antibiogram showed in table (5) reveled that antimicrobial susceptibility profile of the 45 *Staphylococcus* isolates from human samples to 13 types of antibiotics. The high ratio of resistance to penicillin (84.4%), clindamycin (73%), tetracycline (66.7%) and rifampicin (64.4%). Also, penicillin and clindamycin recorded the high percentages of resistance (84.4%) and (73%) respectively. Whereas gentamicin and trimethoprim/sulfamethazole showed the low resistance (26.7%) for both from tested antibiotics.

Further that the antibiogram results showed that the least resistant specie was *Staph. auricularis* that was resistant to 6 antibiotics (penicillin, clindamycin, vancomycin, ciprofloxacin, gentamicin and trimethoprim/sulfamethazole). On the other hand, *Staph. aureus* was the most resistant specie to the 13tested antibiotics. Methicillin-resistant *Staphylococci* were detected in 24 isolates, (11were (MRCoPS) and the other 13 were (MRCoNS)). Vitek2 system also detected vancomycin resistant *Staphylococcus* species in 12 isolates.

Table (4): Distribution of *Staphylococcus species* isolated from poultry samples according to their species diversity and multidrug resistance pattern

Staphylococcus sp.(n = isolates)		**Antibiotics**												
		Ox acillin	**Penicilin**	**Gentamycin**	**Sulfamethoxazole/ Trimethoprim**	**Clindamycin**	**Erythromycin**	**Tetracycline**	**Vancomycin**	**moxifloxacin**	**levofloxacin**	**rifampicin**	**Quinupristin/dalfopristin**	**ciprofloxacin**
Resistance Pattern														
	Staph.aureus(5)	4	4	1	2	5	4	4	2	2	2	5	2	3

Coagulase Positive	***Staph.hyicus***(1)	2	2	1	1	2	0	1	0	1	1	0	0	1
	Staph.schleiferi(2)	1	0	0	0	0	2	1	1	2	2	1	1	2
	Staph.intermedius (2)	1	2	1	1	1	0	2	1	0	0	0	2	0
Coagulase Negative	***Staph.lentus*** (3)	2	3	1	1	3	2	3	2	1	1	2	2	1
	Staph.hominis(2)	0	0	0	0	0	0	1	0	1	1	0	0	1
	Staph.chromogens (2)	0	2	0	0	0	2	2	1	0	0	2	0	0
	Staph.warneri(2)	1	1	0	0	2	0	1	1	2	2	0	0	2
	Staph.haemolyticus(2)	0	2	0	0	2	2	1	0	0	0	2	1	0
	Staph.arlettae(2)	1	0	0	0	2	0	2	1	0	0	0	0	0
	Staph.sciuri(2)	0	2	1	1	2	2	2	1	0	0	2	1	0
	Staph.xylosus(2)	1	0	0	0	2	0	2	0	0	1	0	0	0
	Staph.vitulinus(2)	1	2	1	1	0	0	2	2	1	1	0	0	1
	Staph.cohnii(2)	0	0	0	0	2	2	1	0	0	0	2	1	1
	Staph.capitis(1)	1	1	0	0	1	1	1	1	1	1	1	0	0
	Staph.simulans(1)	0	0	0	0	1	0	1	0	0	0	0	0	0
	Staph.lugdunensis(1)	1	1	0	0	1	1	1	1	1	1	0	1	1
Total	**35**	**16**	**22**	**6**	**7**	**26**	**18**	**28**	**14**	**12**	**13**	**17**	**11**	**13**

Table (5): Distribution of *Staphylococcus species* isolated from human samples according to their species diversity and multidrug resistance pattern

Staphylococcus sp.(n = isolates)		**Antibiotics**												
		Ox acillin	**Penicilin**	**Gentamycin**	**Sulfamethoxazole/ Trimethoprim**	**Clindamycin**	**Erythromycin**	**Tetracycline**	**Vancomycin**	**moxifloxacin**	**levofloxacin**	**rifampicin**	**Quinupristin/dalfo pristin**	**ciprofloxacin**
Resistance Pattern														
Coagulase Positive	***Staph.aureus(14)***	8	13	2	3	11	11	11	6	4	5	12	3	5
	Staph.hyicus(2)	2	2	1	1	2	1	1	2	0	1	1	1	1
	Staph. pseudintermedius (2)	0	2	1	1	2	2	1	0	1	1	2	1	1
	Staph.intermedius (1)	0	1	1	0	1	1	0	1	0	1	1	1	1
	Staph.schleiferi (1)	1	0	0	1	1	0	1	1	1	1	0	0	1
Coagulase Negative	***Staph.haemolyticus(3)***	3	2	1	0	3	2	3	2	0	2	2	2	2
	Staph.cohnii(3)	1	2	0	0	2	0	2	2	2	1	0	1	2
	Staph.hominis(2)	1	1	0	0	0	1	1	0	1	1	1	1	0
	Staph.warneri(2)	1	1	0	0	2	1	1	1	1	1	1	1	1
	Staph.lentus (2)	1	1	1	1	1	1	1	1	0	0	1	1	0
	Staph.saprophyticus(2)	1	2	0	0	1	2	1	0	1	1	2	1	1
	Staph.sciuri(2)	1	2	1	1	0	1	1	1	1	0	1	0	0
	Staph.xylosus(2)	1	2	1	1	1	1	2	0	1	1	0	1	1

	Staph.arlettae(1)	1	1	1	1	1	1	1	1	0	1	1	1	1
	Staph.epidermidis(1)	1	2	0	0	2	0	2	1	1	0	1	1	0
	Staph.capitis(1)	0	1	0	0	1	1	0	1	1	0	1	0	1
	Staph.vitulinus(1)	0	1	1	1	1	1	0	0	1	1	1	1	0
	Staph.auricularis(1)	0	1	1	1	1	0	0	1	0	0	0	0	1
	Staph.lugdunensis(1)	1	1	0	0	0	1	1	0	0	1	1	1	0
Total	**45**	**24**	**38**	**12**	**12**	**33**	**28**	**30**	**12**	**16**	**19**	**29**	**18**	**19**

Results of PCR

Ten *Staphylococcus* isolates were selected and classified into;5 isolates from poultry (*Staph.lentus* (1), *Staph.aureus*(2), *Staph. lugdunensis*(1) and *Staph. warneri*(1)) and 5 from human (*Staph.cohnii*(1), *Staph.lentus*, (1) *Staph. aureus* (2) and *Staph. haemolyticus*(1)) for testing by PCR. *16SrRNA* gene primers confirmed the presence of *Staphylococcal* DNA in these isolates (Table 6&Fig.1), also On the other hand,different antimicrobial resistance genes were detected in our isolates(ten isolates).*mecA* gene detected in 6 isolates devided into *Staph. aureus*(2), *Staph. lugdunensis*(1),*Staph. warneri*(1), *Staph. cohnii* (1)and *Staph. lentus*(1) and *vanA* gene were detected in 6 isolates (*Staph aureus*(4), *Staph. lentus*(1) and *Staph. haemolyticus*(1) while *blaZ* gene was detected in all isolates except *Staph. lugdunensis* and *Staph haemolyticus*, also *cfr* gene detected in 3 isolates (*Staph aureus*(2) and *Staph lentus*(1) (Table 6 and Fig.5,6,7and8) respectively. While *sei* and different virulence genes were detected in *Staphylococcal* isolates. Out of ten isolates *coa* gene was detected in four *Staph. aureus* isolates, *hld* gene detected in 6 isolates belonging to 3 *Staph. aureus* ,2 *Staph.lentus* and 1 *Staph.haemolyticus* and *pvl* gene detected in two *Staph.aureus* isolates (Table 6 and Fig.2,3 and4) respectively.

seh genes not detected in the tested samples. *Staph. aureus,Staph.lentus* and *Staph. chonii* harbored most of virulance and antimicrobial resistance genes respectively.

Despite of the harmony between aresults of Vitek system and PCR than conventional method as recorded in table(6) but PCR cleared a high accuracy in detection of *mecA* and *vanA* gene in human isolate no.(8), however Vitek system identified this isolate as vancomycin resistant strain only

Table (6): The distribution of virulence genes and antimicrobial resistance genes in the

Isolates no.	Source	Vitek 2		Conventional		PCR						
		Species	Resistance genes	ORSAB	Coagulase	*coa*	*hld*	*pvl*	*mecA*	*blaZ*	*vanA*	*cfr*
1	Poultry	*Staph.lentus*	vancomycin resistant	+ve	-ve	-	+	-	-	+	+	+
2	Poultry	*Staph.aureus*	vancomycin resistant	-ve	+ve	+	+	+	+	+	+	-
3	Poultry	*Staph.aureus*	vancomycin resistant	-ve	+ve	+	-	-	-	+	+	-

4	Poultry	*Staph.lugdunensis*	methicillin resistant	+ve	-ve	-	-	-	+	-	-	-
5	poultry	*Staph.warneri*	methicillin resistant	-ve	-ve	-	-	-	+	+	-	-
6	human	*Staph.cohnii*	methicillin resistant	+ve	-ve	-	-	-	+	+	-	-
7	human	*Staph.lentus*	methicillin resistant	-ve	-ve	-	+	-	+	+	-	-
8	human	*Staph.aureus*	vancomycin resistant	-ve	+ve	+	+	+	+	+	+	+
9	human	*Staph.aureus*	vancomycin resistant	+ve	+ve	+	+	-	-	+	+	+
10	human	*Staph.haemolyticus*	vancomycin resistant	-ve	-ve	-	+	-	-	-	+	-

tested isolates

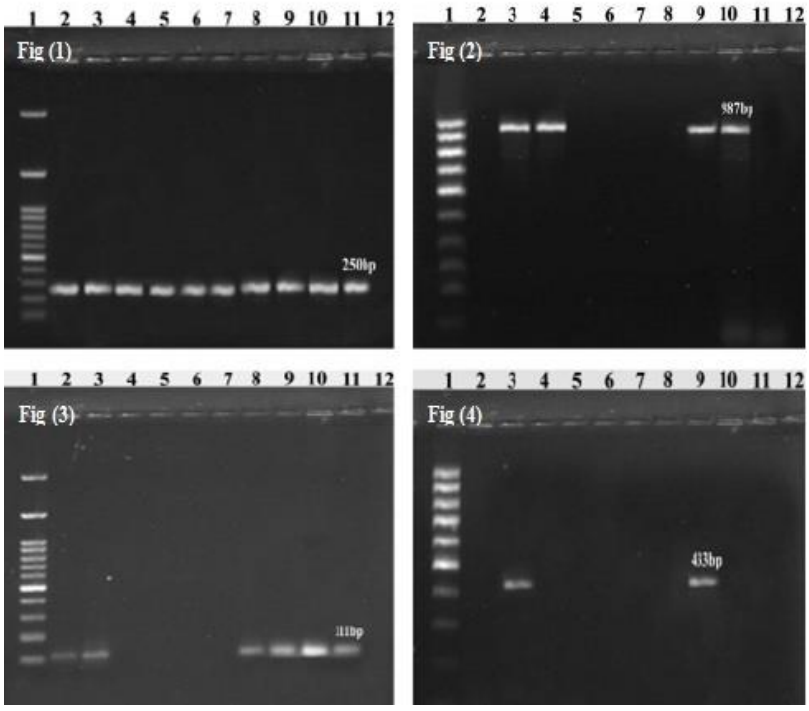

Fig (1): Agarose gel electrophoresis of *16s rRNA* gene amplification at 250b, Lane 1: Ladder, (100bp), Lanes 2 to 6: positive poultry isolates, Lanes 7 to 11: positive human isolates.

Fig (2): Agarose gel electrophoresis of *coa* gene amplification at 987bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 3 and 4 were positive isolates, Lanes 7 to 11: human isolates, included lanes 9 and 10 were positive isolates.

Fig (3): Agarose gel electrophoresis of *hld* gene amplification at 111bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 2and 3were positive isolates, Lanes 7 to 11: human isolates, included lane 8,9,10,11 were positive isolates.

Fig (4): Agarose gel electrophoresis of *Pvl* gene amplification at433bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 3 was positive isolate, Lanes 7

to 11: human isolates, included lane 9 was positive isolate

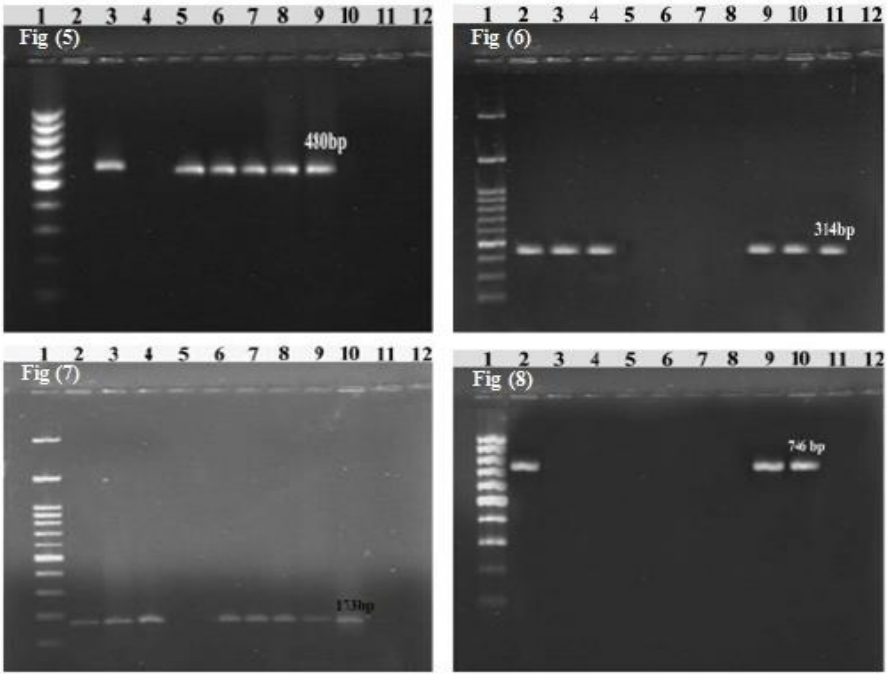

Fig (5): Agarose gel electrophoresis of *mecA gene* amplification at 480, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 3,5 and 6 were positive isolates, Lanes 7 to 11: human isolates, included lane 7, 8 and 9 were positive isolates

Fig (6): Agarose Gel electrophoresis of *vanA* gene amplification at314bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, lane 2,3and 4 positive, Lanes 7 to 11: human isolates, lane 9,10 and 11postive.

Fig (7): Agarose gel electrophoresis of *BlaZ g*ene amplification at 173 bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 2,3,4,6 were positive isolates, Lanes 7 to 11: human isolates, included lane 7,8,9,10were positive isolates.

Fig (8): Agarose gel electrophoresis of *cfr* gene amplificationat746bp, Lane 1: Ladder (100bp), Lanes 2 to 6: poultry isolates, included lane 2 positive, Lanes 7 to 11: human isolates, included lane 9 and lane 10 positive.

Table (7): Accession number of isolated *Staphylococcus* spp

Isolate No.	Isolate species	Source	Accession number
1	*Staph lentus*	Poultry	OM976507
2	*Staph.aureus*	poultry	OM920073
3	*Staph.aureus*	poultry	OM920074
7	*Staph lentus*	human	OM976506
8	*Staph aureus*	human	OM920070

9	*Staph.aureus*	human	OM918375
12	*Staph.aureus*	human	OM918376

Results of phylogeny

The blasting of our sequncing results on gene bank confirmed the identification results of Vitek2 system.As well,the results of phylogenetic analysis showed that our *Staph.aureus* strains isolated from both poultry and human cleared high similarities between each other ranged from (99.7-99.9%)(Tabe.7& Fig.9) and grouped with refernce isolates obtained from gene bank; (LN871053) from wound in India , (MN078268) water in India, (KR085871) water with range (94.5-99.9%)(Fig.9&Supp.tabel.1) . In addition to, our *Staph.lentus* isolates showd identity with percentage(96.6%) (Tabe.7 Fig.9),also group with refernce isolates from poultry houses in Bangaladesh (MN701067) and soil in Korea (MF948914) with percentage(95.6-99.2%)(Fig.9&Supp.tabel.1)

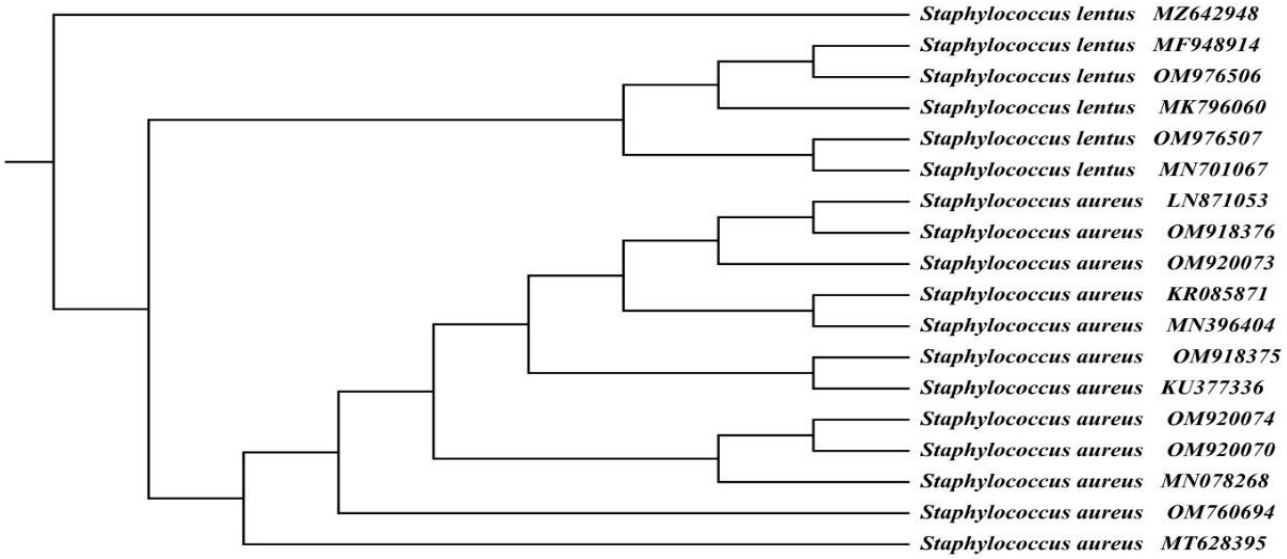

Figure9: Phylogenetic Tree of the entire nucleotide sequence of *16SrRNA*gene of our *Staphylococcus* spp. isolates compared with reference strains regained from Gene Bank

Discussion

Earlier studies have revealed that *Staphylococci* are a common inhabitant of the chicken intestinal tract (Lee *et al*.,2018). Though, their presence in the intestines can have dual roles as commensals and pathogens (Rosenstein and Gotz, 2013). Similarly *Staphylococci* can be found as natural inhabitants of the skin of humans and other animals, they may also cause infections in those same tissues as well as other diseases. In poultry, researches have also proved an association of both coagulase positive *Staphylococci* as well as coagulase negative *Staphylococci* with poultry diseases (Al-Rubaye *et al*., 2015; Nazia *et al*., 2015).The problem of infections caused by these micro-organisms cannot be treated with common antibiotics (Phillips *et al*., 2004)

There are a different traditional diagonistic methods for identification of

Staphylococcus spp. such as growing on mannitol salt agar, coagulase and acetoin production (Kateete *et al*., 2010), in this study *Staphylococcus* species recovered from35%and 45% in poultry and human samples respectively on mannitol salt agar (Table 2). Higher percentage of *Staphylococcus* spp. in poultry farms (52.5%) were observed by Onaolapo *et al*. (2017)lower incidence (10.8%)were recorded by Marek *et al*.(2016). Ghias *et al*.(2016)isolated *Staphylococcus* with high rate (55%) from pus samples of patient's skin.

Coagulase test divided the poultry and human isolates into 31.4% and 44.4% coagulase positive and 68.6% and 55.6% were coagulase negative respectively. Higher results were observed by Islam *et al*. (2014)and Ghias *et al*. (2016).

Oxacillin Resistance Screening Agar Bas (ORSAB) improved the recovery of methicillin-resistant *Staphylococci* in 37.14% and 42.22% from *Staphylococcal* isolates from poultry and human respectively (Table 2). In comparison to our result, Simor et al.(2001) recorded a higher predictive value for isolation of MRSA from different clinical samples (76%).

The elevation in ratio of contamination by pathogenic species of *Staphylococcus* might have resulted to the contamination of hatcheries , farm surroundings and by tools used in the farms, also it has been recorded that the isolation of *Staphylococci* in poultry and its products are often connected to low hygienic methods during slaughtering, cutting, storage and shopping(Karmi, 2013).

The phenotypic differentiation of *Staphylococcus* spp. is considered a complicated case due to the absence of precise biochemical markers. Nevertheless, phenotypic analyses can't reach to complete identification for *Staphylococcus* species, also, this method are relatively time consuming and most importantly, difficult to analyze results. So, the use of automated devices such as Vitek 2 system has become routine in human and veterinary microbiology fields to overcome the traditional methods problems(Sasaki *et al*., 2010).

In the current study, by using VITEK 2 system, poultry isolates could be differentiated into 17 *Staphylococcal* species, the highly identified coagulase positive specie was *Staph.aureus* (14.2%) and coagulase negative specie was *Staph.lentus* (8.5%) (Table 3),this result in accordance with Wieliczko *et al*.(2002)who found that the most frequently coagulase positive specie was *Staph.aureus* and among coagulase-negative species were *Staph.lentus, Staph.xylosus* and *Staph.cohnii*. On the other hand,Marek et al.(2016)detected the most isolated species were *Staph.cohnii* (23.50%), *Staph.aureus* (15.89%) and *Staph.lentus* (13.90%).However Coagulase negative *Staphylococci* are less pathogenic than *S. aureus* (Livermore,2000) but they was were isolated from infected chickens with cellulitis, granulomas in the liver and lungs , gangrenous dermatitis and abscesses in chickens(Linares and Wigle 2001 and Stępień-Pyśniak*et al*.,2017)

Human isolates could be differentiated into 19 *Staphylococcal* species, the highly identified species were *Staph.aureus* 14isolates (31.1%) followed by *Staph.haemolyticus* and *Staph.cohnii* 3 isolates for each species (6.7% for each) by VITEK 2 system (Table 3).Higher results were obtained by Abd El-Tawab *et al*.(2017)who isolated *Staph.aureus* from human samples with percentage 67.5%. On the other hand, Delmas *et al*.(2008)detected the most frequently occurring species were *Staph.epidermidis* (20%) followed by *Staph.saprophyticus* and *Staph.warneri* (10% for each).The difference in percentage of *Staphylococcus*

isolation may be due to different reasons consist of (human and animal sources), geographical situation, numbers of samples and a variation routine of isolation (Fagundes and Oliveira, 2004).

The data recorded in table (4 and 5) demonstrated that the isolates of poultry and human showed multidrug resistance (resistant to ≥ 3 class of antibiotics) high percentages of resistance were observed to tetracycline, clindamycin, penicellin and gentamycin. The high most resistant specie was *Staph.aureus*, it showed a resistance for 13 types of antimicrobials, this result reinforced by Onaolapo *et al.* (2017)who used different biochemical parameters such as disk diffusion, microgen *Staph*. kit and other tests and found their isolates resisted more than 3 family of antibiotics. Nearly related results of antimicrobial susceptibility have been recorded by Leonard and Markey (2008); Otalu *et al*.(2011); Pesavento *et al*.(2007) andWaters *et al*.(2011).

Vitek2 system detected methicillin-resistant *Staphylococci* in 45.7% of poultry isolates and 53.3% of human isolates (Table 4&5) while ORSAB detected methicillin-resistant *Staphylococci* in 37.1% of poultry isolates and 42.2% of human isolates (Table 2). This result confirmed that Vitek2 system is more accurate in the detection of resistant strains of *Staphylococcus* than ORSAB(Sukru *et al.*, 2018).Moreover, it was more rapid in getting of the results (12hrs) and overcome the false negative results than ORSAB(24hrs)(Malaviolle et al., 2008).

PCR considered a gold stander in identification *Staphylococcus* spp. and became more essential to overcome the difficulties of conventional methods. In our results *16SrRNA* gene confirmed the presence of *Staphylococcal* DNA in our isolates (Table 6 and Fig.1). The role of this gene was reinforced by many authors (Ghebremedhin *et al*.,2008 and Johnson *et al.*,2016), also, coagulase gene (*coa* gene) has a title role in identification of these species in this study,it detected 4 *Staph.aureus* isolates (Table 6 and Fig.2),this result supported by Vintov *et al.* (2003) who found that *coa* gene can be used for research purposes to explored diversity and polymorphism of *Staphylococcus*, also, Bharadwaz *et al*. (2015)decided that *coa* gene was considered as a marker for identification of *Staph.aureus* strains and other novel species for instance *Staph.intermedius, Staph.delphini, Staph.shleiferi* as coagulase positive species.

Different virulence genes were harbored by *Staphylococcus* spp., one of the most important virulence gene was haemolysin gene(*hld*) which is exoproteins that are produced by *Staphylococci*, haemolysin is responsible for the increased dissemination and virulence of these species. In present study, *hld* gene detected in 6 isolates belonging to different *Staphylococcus* spp. *Staph. aureus* and *Staph. lentus* the most species harbored this gene (Table 6 and Fig.3). Abdalrahman *et al.* (2015) found the incidence of *hld* gene was (75.6%) in the 168 *Staph. aureus* isolates from poultry. Also, Rossato *et al.* (2018) detected *hla* gene in 87.6% from 177 nosocomial MRSA strains isolated from patients.

Panton-Valentine leukocidin (*pvl*) was a cytotoxin gene and has a major role in the pathogenicity of this bacteria, this toxin form pores in the membrane of host defense cells, and be able to cause severe necrotic pneumonia, tissue infections furthermore to its ability to cause life threatening and associated with community-acquired MRSA infections (Motamedi *et al*., 2015).Kraushaar and Fetsch(2014)elucidated *pvl* gene incidence among (MRSA) in retail poultry meat and slaughter employee and emphasized the

impact of this animal reservoir on human health care. In this study, *pvl* gene was detected in *Staph.aureus* (one isolate from each poultry and human isolates)(Table 6 and Fig.4), Tawfiq (2018)detected *pvl* gene in three isolates from fresh chicken and Jackson *et al.* (2013)detected *pvl* gene in one *Staph.aureus* isolated from retail beef, in addition to Durand *et al*.(2006) and Thabit *et al.* (2017) detected *pvl* gene in community-acquired infection isolates.

In the existing study multidrug resistance was perceived, a number of genes have been clarified for detection of antimicrobial resistance in different species of *Staphylococcus* (Table 6). Resistance to methicillin is intended by the existence of the *mecA* gene encoding PBP2a which has a very low affinity to β-lactam antibiotics (Rice, 2012).*Wendlandt et al*.(2015) reported the implication of methicillin resistant *Staph.aureus* (MRSA) in poultry, as it was the utmost consumed protein responsible for wide spreading of MRSA among humans, that could be fatal and associated with multi-drug resistance.

Our results cleared that *mecA*gene was detected in 3 isolates out of 5 from each of poultry and human samples belonging to different species as *Staph.aureus*, *Staph. lugdunensis*, Staph. warneri, Staph. cohnii and *Staph. lentus* by PCR. *Staph.aureus* was the most specie harbored this gene (Table 6and fig.5). This results supported by Ali *et al*. (2017) and Abdalrahman et al. (2015) who recorded that incidence of *mecA* gene in *Staph.aureus* isolates was 1.2%. Osman *et al.* (2016) conveyed the *mecA* gene in different species (*Staph. lugdunensis*; *Staph. haemolyticus*; *Staph. hominus* and *Staph. lentus*) isolated from poultry. Al-Muhanna (2014) confirmed by PCR that all CoNS isolated from poultry carried *mec A* gene, also, Mulders *et al.* (2010) detect *mecA*gene in 26 out of 466 (5.6%) *Staph.aureus* isolates of individuals. Coelho *et al.* (2007)found that 12 out of 80 *Staph.aureus* isolates (15%) of human have *mecA* gene.

The presence of MRSA in both poultry and human isolates is a zoonotic issue among animals and humans through direct contact, environmental contamination, and contaminated animal products (Aqib *et al*.,2017 and Erwin et al.,2014).

Molecular detection became a necessary tool because methicillin resistance is often heterogeneously expressed in vitro and provides consistent results because the protocol is basically standardized and has progressed as an proficient tool for epidemiological investigations(Strommenger *et al.*, 2006),these result supported our results which cleared that PCR detected the *mec*A gene in isolate that not detected as methicillin-resistant *Staphylococci*by in the vitek2. Also, these results mean that *mac*A gene was present but hasn't any expression (Hoopes, 2008). However, Shan *et al*. (2016) asserted the role of Vitek in predicting by MRSA even if the accuracy rate is not perfectly reached 100%.

Higher mortality, greater morbidity were recorded in patients infected with methicillin-resistant *Staphylococc*i, they utilize more healthcare resources compared with those who have infections instigated by methicillin-susceptible *Staphylococci*(Itani, 2016).The previous data emphasized the increasing role of *Staphylococci* in poultry infections, which recommended that the safety risks associated with their occurrence in the food consumed by humans, induced hospital infections with a high mortality rate (De Silva *et al*., 2002; Piette and Verschraegen, 2009).

Vancomycin resistant *Staph.aureus* (VRSA) were a protuberant pathogens that cause a wide range of infections in different hosts(Grundmann *et al*., 2010). These strains convey the *vanA* gene that responsible for depressing the cell wall affinity for Vancomycin(Sibbald et al., 2006).By using PCR assay, we detected *vanA* genein3out of 5 isolates from each of poultry and human samples(Table 6and fig.6).Martins *et al.* (2013)detected *vanA* gene in 3 samples out of 15 of chilled industrialized uncooked chicken parts andOkolie et al.(2015)found *van*A in 22isolates out of 155 from chicken carcasses. On other hand,Saadat et al. (2014)detected *vanA* in 34% of clinical isolates in hospital, while Khudaier(2018)detected *vanA* in 4 human isolates out of 163.

Taponen and Pyörälä(2009)reported that the utmost communal mechanism of *Staphylococcus* resistance to antibiotics is the production of β-lactamases due to the presence of *blaZ*genethat coded for an alteration of penicillin-binding protein 2a which reduced the affinity for β-lactam antibiotics. The Clinical and Laboratory Standards Institute (CLSI) recommending the detection of *blaZ* gene specially in infected cases treated previously with penicillin (Testing and Testing, 2016).Results obtained in this study indicate the presence of *blaZ* gene indifferent *Staphylococcal* spp. obtained from poultry and human samples(Table 6 and Fig.7), this results related to Ferreira *e tal*.(2017);Mkize(2016); Pyzik *et al*.(2019) andWhichard *et al.* (2007).

Other antimicrobial resistant gene was *cfr* gene was detected in different *Staphyloccous* spp. in our isolates as *Staph.aureus* and *Staph.haemolyticus*(Tabel 6 and Fig 8). A different mechanism of linezolid resistance has recently been described in veterinary staphylococcal isolates. The mechanism is non mutational and includes attainment of a natural resistance gene, *cfr* (Kehrenberg *et al*.,2007).This gene encodes an rRNAmethyl–transferase which modifies the adenine at position 2503 in 23SrRNA(Kehrenberg *et al.*, 2005).It confers a resistance not only to linezolid, but also to phenicols, lincosamides, pleuromutilinsand streptograminA antibiotics (Long *et al*., 2006). Toh *et al*. (2007) stated the first *cfr*-mediated, linezolid-resistant clinical isolate of MRSA. Wang *et al*. (2013) detected the *cfr* gene in *Staph.haemolyticus*

It is worth to record that surprisingly a high percentage of strains were resisted to several types of antibiotics used in this study. From a clinical view this is a vital observation, as resistant bacteria can transmit genes coding for antibiotic resistance to other bacteria by transduction, conjugation or transformation. This may lead to a spreading antibiotic resistance rapidly in the *Staphylococcus* population(Marek *et al*., 2016).These information can be used to inform public health official to enforce judicious use of antimicrobial agents in human and veterinary medicine (Cummings *et al*., 2013).Otto (2013) recorded that coagulase negative *Staphylococcus* supposed to act as reservoirs for antibiotic resistance genes.

Several studies confirmed pathogenicity of coagulase positive *Staphylococcus*(Livermore *et al.*,2000, Youssef and Hamed, 2012) but the most we can talk about in this study multidrug drug resistance of coagulase negative and its possession to different virulence and antimicrobial resistant genes, these results supported by Becker *et al*.(2014) who cleared that Strains of coagulase negative of both animal and human origins are supposed to attend as main reservoirs of antimicrobial resistance genes . These genes are frequently cited on mobile genetic elements, permitting their

horizontal transfer to pathogenic Staphylococci (Resch *et al.* 2008).

The results of *16SrRNA* gene sequencing in *Stah.aureus* and *Staph.lentus* isolated from both poultry and human showed that a high similarity in sequence of the same species obtained from poultry and human isolates with identity range (99.9-100%)and the surprise is that most of our isolates were grouped with reference strains obtained from environment like soil and water with identity(95.6 -100%) and this may be elucidated these resemblances between our isolates of the same spp because they have the same source. Pan and Yu (2014) supported these results and clarified that several types of microorganisms existent in poultry intestines largely depend on their diet and the environment in which they live besides *Staph.ylococcal* spp. were have a double roles as commensals and pathogen for both poultry and human (Rosenstein and Gotz, 2013) so the transmission between two hosts can be occurred.

Conclusion

Finally, we concluded that ViteK system played important role in identification of *Staphylococcus* spp and their susptability to different antimicrobials. PCR gave a good screening for virulance and antimicrobials genes in ,also sequencing of *16SrRNA* cleared the releation between our isolates. So, we recommended decreasing the unwarranted use of antimicrobials in poultry production and educate people about the use of antibiotics

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Conflict of interest

The authors declare that they have no conflict of interest.

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