



Efficacy of *Staphylococcus aureus* Vaccine in Chicken

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ABSTRACT

Staphylococcus aureus is considered one of the most important pathogens causing septic arthritis in poultry with significant economic losses. This study aimed to evaluate the efficacy of a locally prepared *S. aureus* vaccine against staphylococcal arthritis in poultry. Out of 78 samples collected from infected chickens showing clinical signs bumble foot, 10 field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of 12.82%. Molecular identification of clumping factor A (*ClfA*) and *blaZ* genes of *S. aureus* isolates revealed that the PCR amplification with *ClfA* and *blaZ* specific primers conducted with genomic DNA resulted in products of approximate size 638 bp and 833 bp, respectively. Phylogenetic tree for *S. aureus ClfA* virulence gene partial sequences was generated using maximum likelihood, neighbour joining and maximum parsimony in MEGA6. It showed clear clustering of Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank revealed 99.5% to 100% homology. Also, there was identity and homology in *S. aureus blaZ* gene nucleotide sequence in the Egyptian isolated strain (*S. aureus* ASM strain) and the different *S. aureus* strains uploaded from GenBank revealed 96.1% to 98.9% homology. Phylogenetic tree for *S. aureus blaZ* β -lactamases resistant gene partial sequences showed clear clustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. The results of humoral immune response revealed that the geometric mean antibody values against locally prepared *S. aureus* vaccine measured by indirect hemagglutination test increased from 1st week post vaccination gradually till reached maximum level (322.5) at 6th week post boosting. The results showed an increased humoral antibody production in vaccinated group that was capable of preventing establishment of new *S. aureus* infection in vaccinated group compared to control group. The mortality rates in unvaccinated group was higher than that of vaccinated group were (42.5%, vs. 7.5%) at 1st and 2nd week post challenge (39.1% vs. 5.4%). The protection % in challenge assay of the prepared *S. aureus* vaccine was (92.5% and 87.5%) at 1st and 2nd week post challenge respectively. It could be concluded that the prepared vaccine was safe, potent and protect birds against *S. aureus* infection.

Key words: *Blaz*, *ClfA*, PCR, Sequencing, *Staphylococcus aureus*, Vaccine.

INTRODUCTION

Staphylococcus aureus is regarded as one of the most prevalent pathogens that can cause great economic losses in poultry sector. In poultry, *S. aureus* causes many clinical syndromes as tenosynovitis, omphalitis, femoral head necrosis, bumble foot, infected hock and stifle joints (Sulemian et al., 2013). Different antimicrobial agents such as β -lactamases, macrolides, aminoglycosides and tetracyclines are extensively used in poultry for treating of staphylococci and other infections which lead to development of drug resistant strains of bacteria (Nemati et al., 2008). Resistance to penicillin as developed by staphylococci is mediated by two mechanisms: either by

the secretion of an β -lactamases enzyme, encoded by the *blaZ* gene in plasmid or chromosome, which inactivates the antibiotic by hydrolysis of its β -lactam ring, or by the production of a penicillin binding protein (PBP2A) encoded by gene *mecA* (Liu, 2009). The need for *S. aureus* vaccine was mainly determined by the economic loss in poultry farming resulting from arthritis in poultry Fluit et al. (2012). Prophylaxis via prevention of infection by using antibiotics is of low fee in case of antibiotic resistant strains, as penetration through the infected joints no way for therapy of arthritis caused by *S. aureus*. Vaccination is the solely way for protection against staphylococcal arthritis in poultry. The main objective of this study was to evaluate the efficacy of a

locally prepared *S. aureus* vaccine against staphylococcal arthritis in poultry.

Baird Parker agar media then incubated at 37°C for 24 h., according to Quinn et al. (2002).

MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care and Use Committee (IACUC) has approved animal use protocol used in this study (Vet. CU. 20022020146).

Samples collection and isolation of *Staphylococcus aureus*

A total of 78 samples (46 samples from layers farms and 32 samples from broiler farms) were collected from private poultry farms in Sharkia, Qalubia, Behira and Dakahlia governorates, Egypt. The samples taken from birds having swollen hock joints, wings and foot pads. These samples were taken under aseptic conditions according to Jordan et al. (2002). Isolation of *S. aureus* was obtained by culturing these samples on tryptic soy broth (TSB) containing 70 mg/ml NaCl, then culturing from this broth on blood agar, mannitol salt agar, and

Identification of *Staphylococcus aureus* isolates

Isolated colonies of *S. aureus* were identified by classical identification as microscopical examination, biochemical reactions using API-Staph system (Table 1) and pathogenicity test according to Quinn et al. (2002), Taponen et al. (2008), López-Malo et al. (2005), El- jakee et al. (2013), Kateete et al. (2010) and Topy and Wilsons (1993). Phenotypic characterization of the same isolation was applied to detect coagulase test according to Quinn et al. (2002), and hemolysis assay according to Koneman et al. (1997).

Molecular identification of *Staphylococcus aureus*

All the identified *S. aureus* isolates were examined by PCR for the presence of clumping factor A (*ClfA*) virulence associated gene then detected the presence of *S. aureus* (*blaZ*) gene in all field isolates. The primers sequences and PCR product sizes are shown in table 2.

Table 1. Results of biochemical identification of *Staphylococcus aureus* using API-Staph system

Test	ADH	URE	VP	GLU	MAN	SAC	MAL	FRU	MNE	LAC	TRE	XLT	MEL	NIT	PAL	RAF	XYL	MDG	NAG
Result	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+

(ADH) L-arginine, (URE) urea, (VP) VogusProskour, (GLU) D-glucose, (MAN) D-mannitol, (SAC) D-sucrose, (MAL) D-maltose, (FRU) D-fructose, (MNE) D-mannose, (LAC) D-lactose, (TRE) D-trehalose, (XLT) Xylitol, (MEL) D-melibiose, (NIT) Nitrate potassium, (PAL) L-B-naphthyl phosphate, (RAF) D-raffinose, (XYL) D-xylose, (MDG) methyl D-glucopyranoside, (NAG) N-acetyl-glucosamine.

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences (5'-3')	Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>ClfA</i>	F:GCAAAATCCAGCACAAACAGGAAACGA	638	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Mason et al.(2001)
	R:CTTGATCTCCAGCCATAATTGGTGG							
<i>blaZ</i>	F:TACAACGTGAATATCGGAGGG	833	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 50 sec.	72°C 10 min.	Bagcigilet al. (2012)
	R:CATTACACTCTTGGCGGTTTC							

DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in Table 2.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Appllichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Phylogenetic and gene sequence analysis of *ClfA* and *blaZ* genes of *Staphylococcus aureus*

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed to establish sequence identity to Gen Bank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNA Star version 12.1 (Thompson et al., 1994) and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Vaccination and challenge test

Vaccine preparation

The vaccine was prepared according to the methods described by Giraud et al. (1997), Ahmad and Muhammad (2008) and Raza et al. (2015). From the complete identified isolated Egyptian strain (*S. aureus* ASM strain), 10 ml from the freshly prepared culture was streaked on brain-heart infusion agar and incubated at 37°C for 18 h. Then, the strain was separately subcultured in brain-heart infusion at 37°C for 24 h.

Preparation of *S. aureus* bacterin in liquid medium: A culture of well identified strain was prepared from single colony which was scaled up to one TSB medium. Also preparation of *S. aureus* bacterin on solid medium by cultivation of single colony on BHI agar or TS agar then incubated at 37°C for 24 h. The bacterial suspension was centrifuged at (3000 rpm at 4°C for 30 min) to pellet the bacterial cells, after that the bacterial pellets washed and resuspended in 0.15 mol/l PBS (pH 7.2). The culture suspension was examined for purity through Gram's stain

method and by streaked on blood agar. The colony forming unit was determined by plate counting and bacterial concentrations was adjusted to contain 1×10^9 cells/ml (Raza et al., 2015). The *S. aureus* toxin was prepared by taking 10 ml of freshly prepared working solution and incubated into 500 ml of BHI, then incubated at 37°C for 48 h. The supernatant was taken from broth culture by filtration. The bacterial culture was inactivated by adding 0.4% (v/v) formalin (Watson and Davies, 1993), at 37°C for 24-48 h with agitation for 24 hrs, Then, sodium bisulfite was added in a final concentration of 2% to stop the action of formalin. Samples from inactivated bacterial culture were tested for complete inactivation by cultivated on BHI agar media to assure complete inactivation where no growth was found on any of the inoculated media after incubation at 37°C for 24 h to 7 days of incubation, according to OIE (2014).

Determination of the Minimum Lethal Dose (MLD) of *Staphylococcus aureus* filtrate in mice

Double fold serial dilutions of the *S. aureus* filtrate were prepared in PBS, 0.1 ml of each dilution was injected into each of three experimental mice weighting about 25 grams. Mice were kept under observation for 3 days post inoculation and the MLD (which is the minimum amount of toxin that killed all mice in 3 days) was determined according to Smith (1975) and Smyth (1975).

Vaccine formulation according to (Ahmed 2012)

The inactivated *S. aureus* bacterin and toxoid vaccine was prepared as an oil emulsion vaccine using Montanidetm ISA 71 VG adjuvant (SEPPIC, France) in a ratio of 71 adjuvant: 29 antigen. Merthiolate (Thiomersal) was used in a final concentration of 1: 10,000 as a preservative. The dose of the prepared vaccine was 0.5ml contain 1×10^9 CFU and MLD50 of toxoid.

Quality control of the prepared *Staphylococcus aureus* vaccine

The prepared *S. aureus* inactivated oil emulsion vaccine was tested for sterility test, safety test, complete inactivation and potency according to the Standard International Protocols as described by the OIE (2017).

Experimental design

One hundred and sixty, 1-day-old SPF chickens were obtained from Nile- SPF farm, KomOshim project, El-Fayoum Governorate, Egypt. The chickens were housed in SPF isolator units in specific CLEVB animal care building with water and feed provided *ad-libitum*. At

3 weeks of age, blood samples were collected for serological examination to insure their freedom from maternally derived antibodies against *S. aureus*, 3 weeks old SPF broiler chickens were divided into 3 groups, chickens of group (1) of 80 birds injected S/C with 0.5ml of previously prepared oil adjuvanted *S. aureus* vaccine in the middle part of the neck two times with 3 weeks intervals, the groups 2 and 3 ,each group consisted of 40 birds, as control positive and negative groups were left unvaccinated.

Serum samples were obtained regularly from vaccinated and unvaccinated groups before immunization, weekly for 3 weeks after the primary vaccination and every week post boosting for 6 weeks, and stored at -20 °C until used.

Challenge test

Birds of groups 1 and 2 were challenged 4 weeks after the booster dose by oral administration of 1ml of broth culture containing 1×10^9 CFU of reference *S. aureus* virulent strain obtained from Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia. The inoculated chickens were observed for one month. The degree of protection was assessed according to the severity of the clinical signs, the mortality and the recovery of the challenge organisms from fecal samples were assayed according to Paiva et al. (2009).

Detection of the shedding of *Staphylococcus aureus* in fecal samples

One week after the challenge and for 4 weeks, cloacal swabs were collected from each of the infected as well as control groups and examined bacteriologically for the presence of *S. aureus* weekly during one month after challenge according to Ahmed (2012) and Raza et al. (2015). Suspected colonies were identified morphologically and biochemically.

Antibody titers

Indirect haemagglutination test for measuring antibody titers in vaccinated chickens were done according to Rahman et al. (2005).

RESULTS

Isolation and identification of *Staphylococcus aureus* field isolates

Out of a total number of 78 samples were collected from infected chicken showing clinical signs of planter

abscess or bumble foot, ten locally field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of (12.82%). Gram's staining revealed that the colonies morphology of *S. aureus* were small to medium sized and golden yellow on BHI agar, while the colonies appeared surrounded by a double zone of beta haemolysis on blood agar plates, on mannitol salt agar, they were yellow color surrounded by yellow halo with yellow colored medium the colonies were typically black smooth with entire margin on Baird Parker agar media.

Phenotypic characterization of some virulence factors as haemolysin production assay and coagulase production.

All ten *S. aureus* isolates were coagulase positive and produce beta hemolysis.

Molecular identification of *ClfA* and *blaZ* genes of *Staphylococcus aureus* isolates

The PCR amplification with *ClfA* specific primers was conducted with genomic DNA, which resulted in a product of approximate size 638bp (Figure 1). *ClfA* gene was found in all ten (100 %) *S. aureus* isolates. The PCR amplification with *blaZ* gene specific primers was conducted with genomic DNA, resulted in a product of approximate size 833 bp (Figure 2). *BlaZ* gene was present in ten (100 %) *S. aureus* isolates. Sequence identities between the isolated Egyptian strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank revealed that 99.5% to 100% homology. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank revealed that 96.1% to 98.9%.

Quality control of the prepared *Staphylococcus aureus* vaccine

assured that the locally prepared vaccine free from any bacterial (aerobic or anaerobic contaminants) or fungal contamination and safe as there was no local reaction found in all injected chickens.

Antibody titers in vaccinated chickens

The results of humoral immune response revealed that the geometric mean antibody titers against *S. aureus* values of both groups as shown in table 3. The GMT antibody titer was (16) in the 1st week post vaccination,

and increase gradually at the 2nd week (20.16), till reach to maximum level at 6th week was (322.5) as shown in Table 3.

Challenge test

The vaccine showed a considerable survival rate in challenged vaccinated group compared to control group. The mortality rates and survival percentages in groups control and vaccinated are summarized in table 4, the NO. of dead chickens in the 1st week post challenge in the control and vaccinated group were 17 (42.5%) and 3 (7.5%), respectively. The mortality rate in the 2nd week in the control group 9 (39.1%) and vaccinated group 2 (5.4%). Protection or survival (%) till day 7 post challenge (20%) in control group and vaccinated group

(92.5%). Protection or survival (%) till 15 day post challenge (0%) in control group and (87.5%) in vaccinated group as shown in table 4. The challenge protection assay showed a considerable protective immune response of prepared *S. aureus* vaccine.

Detection of the shedding of *Staphylococcus aureus* in fecal samples

The results in Table 5 showed that the fecal shedding of chickens challenged with virulent *S. aureus* strain in group 1 vaccinated with locally prepared oil adjuvanted *S. aureus* vaccine was 10.8%, 8.5% and 0% in 1st, 2nd, 3rd week post challenge, respectively. Shedding disappeared by the 4th week post challenge.

Table 3. Geometric mean of *Staphylococcus aureus* antibody titers in sera of chickens vaccinated with locally prepared oil adjuvanted *S. aureus* vaccine and non-vaccinated groups measured by indirect haemagglutination test.

Time intervals	Geometric mean anti- <i>S. aureus</i> antibody titers
Pre-vaccination	0
1 st wpv	16
2 nd wpv	20.16
3 rd wpv	32
1 st wpb	40.3
2 nd wpb	50.79
3 rd wpb	161
4 th wpb	256
5 th wpb	256
6 th wpb	322.5

wpv: week post-vaccination; wpb: week post-boostering

Table 4. Comparison of mortality rate in chicken groups unvaccinated and vaccinated with the locally prepared oil adjuvanted *Staphylococcus aureus* vaccine post challenge with virulent reference *Staphylococcus aureus* strain

Groups	Total birds	No. of dead birds				Mortality rate			
		1 st wpc	2 nd wpc	3 rd wpc	4 th wpc	1 st week	2 nd week	3 rd week	Total
Vaccinated	40	3	2	0	0	7.5%	5.4%	0%	12.5%
Unvaccinated	40	17	9	8	3	42.5%	39.1%	57.1%	92.5%

wpv: week post- challenge

Table 5. Comparison of rate of fecal shedding in chicken groups unvaccinated and vaccinated with the locally prepared oil adjuvanted *Staphylococcus aureus* vaccine post challenge with virulent reference *Staphylococcus aureus* strain

Groups	No. of birds positive for <i>S. aureus</i> isolation/ total No. of living birds			
	1 st wpc	2 nd wpc	3 rd wpc	4 th wpc
Vaccinated	10.8% (4/37)	8.5% (3/35)	0% (0/35)	0% (0/35)
Unvaccinated	47.8% (11/23)	57.1% (8/14)	33.3% (2/6)	33.3% (1/3)

wpv: week post- challenge

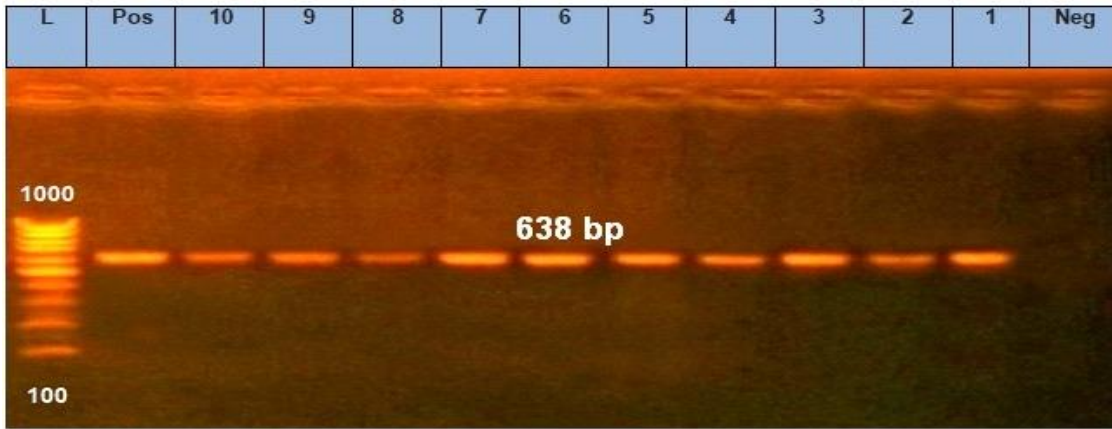


Figure 1. Agarose gel showing PCR amplified product of 638 bp of clumping factor A (*ClfA*) virulence gene for *Staphylococcus aureus*, lanes (1) to (10): samples positive for *ClfA* gene, Lane (Pos.): positive control, Lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

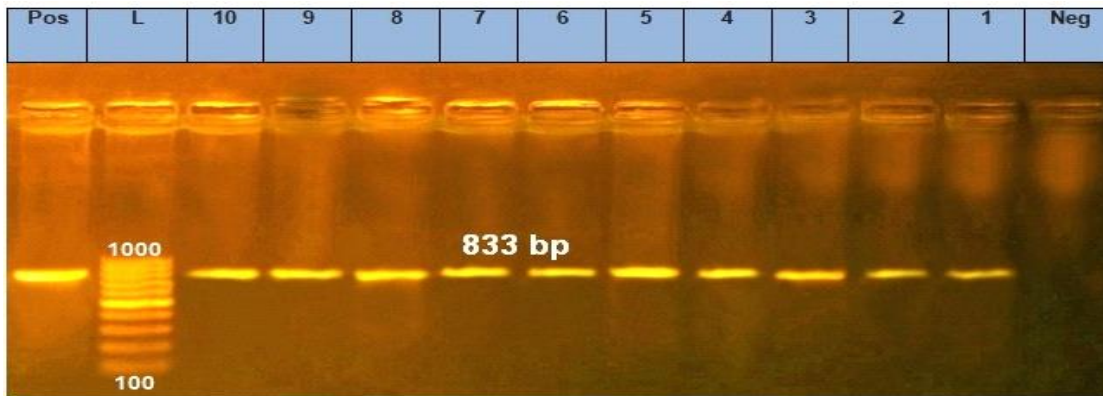


Figure 2. Agarose gel showing PCR amplified product of 833 bp of β -lactamase resistant (*Blaz*) gene for *Staphylococcus aureus*, lanes (1) to (10): samples positive for *Blaz* gene, lane (pos.): positive control, lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

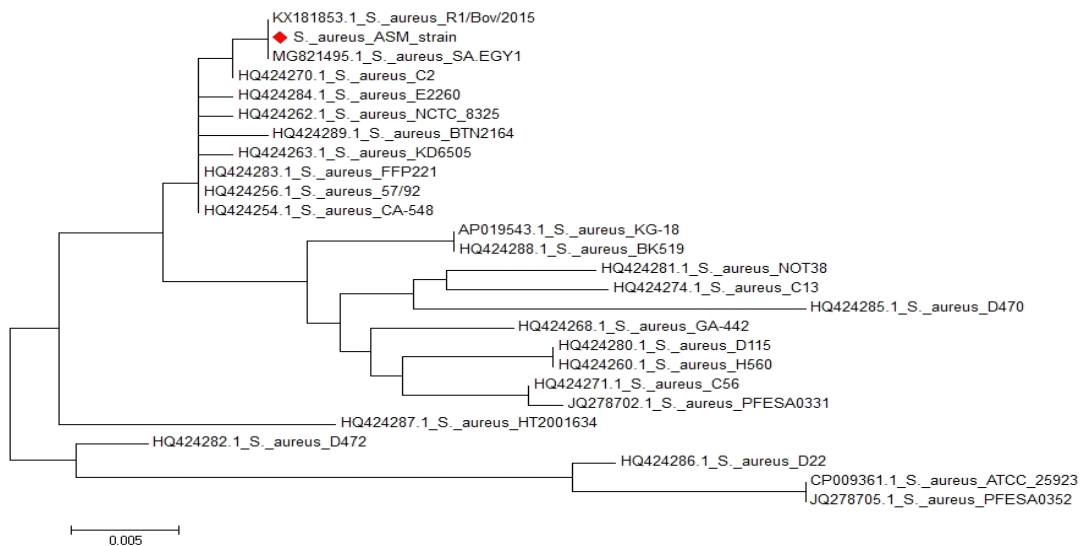


Figure 3. Phylogenetic tree for *Staphylococcus aureus* clumping factor A (*ClfA*) virulence gene partial nucleotide sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. It shows clear clustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank.

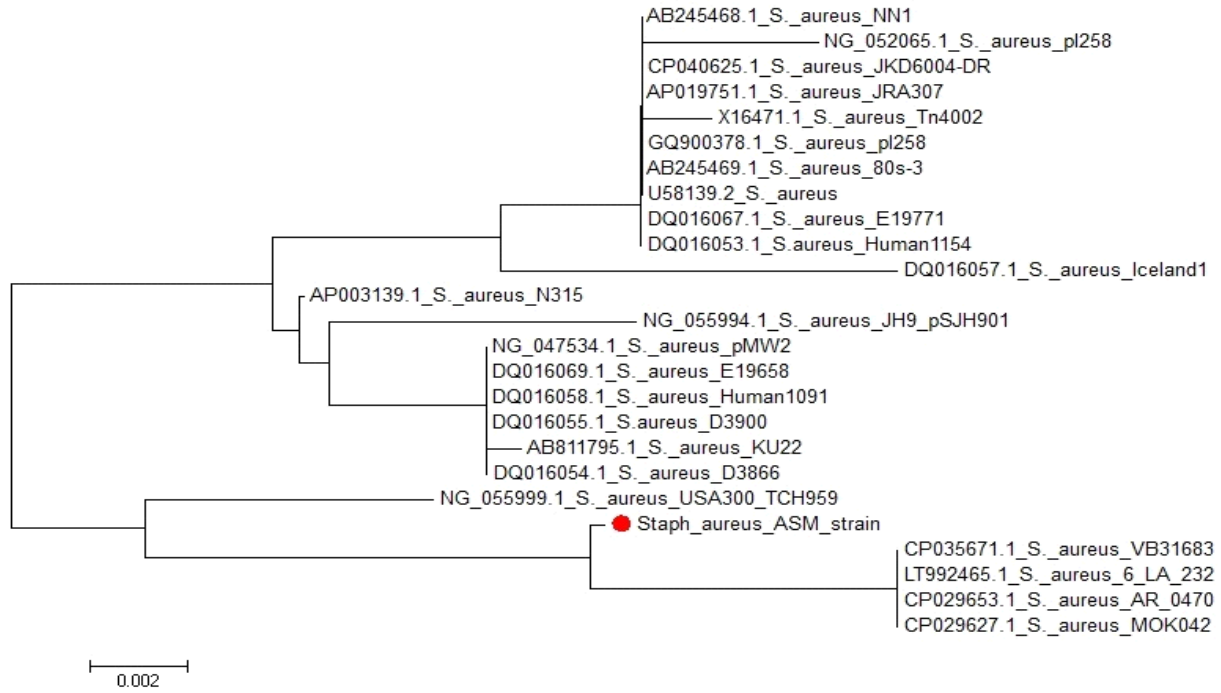


Figure 4. Phylogenetic tree for *Staphylococcus aureus*β-lactamase (*Blaz*) resistant gene partial nucleotide sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. It showed clearclustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank.

		Percent Identity																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
Divergence	1	█	100.0	99.8	99.7	99.7	99.7	99.5	99.5	99.5	99.4	98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	100.0	1	MG821495.1 <i>S. aureus</i> SA_EGY1
	2	0.0	█	99.8	99.7	99.7	99.7	99.5	99.5	99.5	99.4	98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	100.0	2	KX181853.1 <i>S. aureus</i> R1/Bow2015
	3	0.2	0.2	█	99.8	99.8	99.8	99.7	99.7	99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.8	3	HQ424270.1 <i>S. aureus</i> C2
	4	0.3	0.3	0.2	█	100.0	100.0	99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	4	HQ424283.1 <i>S. aureus</i> FFP221	
	5	0.3	0.3	0.2	0.0	█	100.0	99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	5	HQ424256.1 <i>S. aureus</i> 57/92	
	6	0.3	0.3	0.2	0.0	0.0	█	99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	6	HQ424254.1 <i>S. aureus</i> CA-548	
	7	0.5	0.5	0.3	0.2	0.2	0.2	█	99.7	99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	7	HQ424284.1 <i>S. aureus</i> E2260	
	8	0.5	0.5	0.3	0.2	0.2	0.2	0.3	█	99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	8	HQ424263.1 <i>S. aureus</i> KD6505	
	9	0.5	0.5	0.3	0.2	0.2	0.2	0.3	0.3	█	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	9	HQ424262.1 <i>S. aureus</i> NCTC 8325	
	10	0.6	0.6	0.5	0.3	0.3	0.3	0.5	0.5	0.5	█	98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	10	HQ424289.1 <i>S. aureus</i> BTN2164	
	11	1.6	1.6	1.4	1.3	1.3	1.3	1.4	1.4	1.4	1.6	█	97.3	97.3	96.7	96.7	96.5	96.5	96.4	96.4	96.4	96.2	95.8	96.9	96.2	96.2	11	HQ424282.1 <i>S. aureus</i> D472	
	12	1.8	1.8	1.6	1.4	1.4	1.4	1.6	1.6	1.6	1.8	2.7	█	100.0	98.1	98.1	98.3	98.0	97.8	96.2	97.8	97.6	97.2	94.5	93.9	93.9	12	AP019543.1 <i>S. aureus</i> KG-18	
	13	1.8	1.8	1.6	1.4	1.4	1.4	1.6	1.6	1.6	1.8	2.7	0.0	█	98.1	98.1	98.3	98.0	97.8	96.2	97.8	97.6	97.2	94.5	93.9	93.9	13	HQ424288.1 <i>S. aureus</i> BK519	
	14	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9	█	100.0	98.1	97.8	98.6	97.5	98.3	98.1	96.7	95.1	95.1	95.1	14	HQ424280.1 <i>S. aureus</i> D115	
	15	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9	0.0	█	98.1	97.8	98.6	97.5	98.3	98.1	96.7	95.1	95.1	95.1	15	HQ424260.1 <i>S. aureus</i> H560	
	16	2.6	2.6	2.4	2.2	2.2	2.2	2.4	2.4	2.4	2.6	3.5	1.8	1.8	1.9	1.9	█	97.8	98.6	96.2	98.0	97.8	97.3	94.3	93.9	93.9	16	HQ424281.1 <i>S. aureus</i> NOT38	
	17	2.1	2.1	1.9	1.8	1.8	1.8	1.9	1.9	1.9	2.1	3.1	1.6	1.6	1.8	1.8	1.8	█	97.6	96.9	98.9	98.7	96.4	94.3	93.6	93.6	17	HQ424268.1 <i>S. aureus</i> GA-442	
	18	2.7	2.7	2.6	2.4	2.4	2.4	2.6	2.6	2.6	2.7	3.7	2.2	2.2	1.4	1.4	1.4	1.9	█	96.7	98.1	98.0	97.5	94.7	94.5	94.5	18	HQ424274.1 <i>S. aureus</i> C13	
	19	2.2	2.2	2.1	1.9	1.9	1.9	2.1	2.1	2.1	2.2	3.2	3.4	3.4	2.1	2.1	3.4	3.2	2.9	█	97.3	97.2	95.8	95.8	95.8	95.8	19	HQ424287.1 <i>S. aureus</i> HT2001634	
	20	2.2	2.2	2.1	1.9	1.9	1.9	2.1	2.1	2.1	2.2	3.2	1.8	1.8	1.3	1.3	1.6	1.1	1.4	2.7	█	99.8	96.2	94.5	94.0	94.0	20	HQ424271.1 <i>S. aureus</i> C56	
	21	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9	1.4	1.4	1.8	1.3	1.6	2.9	0.2	█	96.1	94.3	93.9	93.9	21	JQ278702.1 <i>S. aureus</i> PFESA0331	
	22	3.4	3.4	3.2	3.1	3.1	3.1	3.2	3.2	3.2	3.4	4.4	2.9	2.9	3.4	3.4	2.7	3.2	2.6	3.9	3.4	3.6	█	93.6	93.4	93.4	22	HQ424285.1 <i>S. aureus</i> D470	
	23	4.1	4.1	3.9	3.7	3.7	3.7	3.9	3.9	3.9	4.1	2.7	5.2	5.2	4.6	4.6	5.4	4.9	5.1	3.4	4.8	4.9	6.3	█	98.3	98.3	23	HQ424286.1 <i>S. aureus</i> D22	
	24	5.2	5.2	5.0	4.9	4.9	4.9	5.0	5.0	5.0	5.0	5.0	5.2	3.9	6.4	6.4	5.0	5.0	6.4	6.3	5.7	5.9	6.9	1.3	█	100.0	24	CP009361.1 <i>S. aureus</i> ATCC 25923	
	25	5.2	5.2	5.0	4.9	4.9	4.9	5.0	5.0	5.0	5.2	3.9	6.4	6.4	5.0	5.0	6.4	6.3	5.7	3.9	5.7	5.9	6.9	1.3	0.0	█	25	JQ278705.1 <i>S. aureus</i> PFESA0352	
	26	0.0	0.0	0.2	0.3	0.3	0.3	0.5	0.5	0.5	0.6	1.6	1.8	1.8	1.4	2.4	2.6	2.1	2.7	2.2	2.2	2.4	3.4	4.1	5.2	5.2	26	<i>S. aureus</i> ASM strain	

Figure 5. Nucleotide distance of *Staphylococcus aureus*clumping factor A (*CfA*) virulence gene between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank

		Percent Identity																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25			
Divergence	1	█	100.0	100.0	100.0	100.0	100.0	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	1	GO900378.1 <i>S. aureus</i> pl258	
	2	0.0	█	100.0	100.0	100.0	100.0	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	2	AP019751.1 <i>S. aureus</i> JRA307	
	3	0.0	0.0	█	100.0	100.0	100.0	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	3	CP040625.1 <i>S. aureus</i> JKD6004-DR	
	4	0.0	0.0	0.0	█	100.0	100.0	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	4	AB245469.1 <i>S. aureus</i> 80s-3	
	5	0.0	0.0	0.0	0.0	█	100.0	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	5	AB245468.1 <i>S. aureus</i> NN1	
	6	0.0	0.0	0.0	0.0	0.0	█	99.8	99.4	100.0	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	6	U58139.2 <i>S. aureus</i>	
	7	0.2	0.2	0.2	0.2	0.2	0.2	█	99.2	99.8	99.8	98.5	97.9	97.7	97.9	97.3	97.9	97.9	97.9	97.9	96.3	95.0	95.0	95.0	95.0	95.8	7	X16471.1 <i>S. aureus</i> Tn4002	
	8	0.6	0.6	0.6	0.6	0.6	0.6	0.8	█	99.4	99.4	98.1	97.5	97.4	97.5	96.9	97.5	97.5	97.5	97.5	97.5	96.0	94.6	94.6	94.6	94.6	95.5	8	NG_052065.1 <i>S. aureus</i> pl258
	9	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6	█	100.0	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	9	DQ016067.1 <i>S. aureus</i> E19771	
	10	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6	0.0	█	98.7	98.1	98.0	98.1	97.5	98.1	98.1	98.1	98.1	96.5	95.2	95.2	95.2	95.2	96.1	10	DQ016053.1 <i>S. aureus</i> Human1154	
	11	1.3	1.3	1.3	1.3	1.3	1.3	1.6	1.9	1.3	1.3	█	99.4	99.3	99.4	98.8	97.5	99.4	99.4	99.4	97.6	96.1	96.1	96.1	96.1	96.9	11	AP003139.1 <i>S. aureus</i> N315	
	12	1.9	1.9	1.9	1.9	1.9	1.9	2.2	2.6	1.9	1.9	0.6	█	99.9	100.0	98.5	96.9	100.0	100.0	100.0	97.0	95.5	95.5	95.5	95.5	96.5	12	NG_047534.1 <i>S. aureus</i> plW2	
	13	2.1	2.1	2.1	2.1	2.1	2.1	2.3	2.7	2.1	2.1	0.7	0.1	█	99.9	98.3	96.8	99.9	99.9	99.9	96.9	95.4	95.4	95.4	95.4	96.4	13	AB811795.1 <i>S. aureus</i> KU22	
	14	1.9	1.9	1.9	1.9	1.9	1.9	2.2	2.6	1.9	1.9	0.6	0.0	0.1	█	98.5	96.9	100.0	100.0	100.0	97.0	95.5	95.5	95.5	95.5	96.5	14	DQ016069.1 <i>S. aureus</i> E19658	
	15	2.6	2.6	2.6	2.6	2.6	2.6	2.8	3.2	2.6	2.6	1.2	1.6	1.7	1.6	█	97.3	98.5	98.5	98.5	97.0	95.4	95.4	95.4	95.4	96.2	15	NG_055994.1 <i>S. aureus</i> JH9 pSJH901	
	16	1.8	1.8	1.8	1.8	1.8	1.8	2.1	2.4	1.8	1.8	2.4	3.1	3.2	3.1	2.7	█	96.9	96.9	96.9	95.7	94.3	94.3	94.3	94.3	95.1	16	DQ016057.1 <i>S. aureus</i> Iceland1	
	17	1.9	1.9	1.9	1.9	1.9	1.9	2.2	2.6	1.9	1.9	0.6	0.0	0.1	0.0	1.6	3.1	█	100.0	100.0	97.0	95.5	95.5	95.5	95.5	96.5	17	DQ016058.1 <i>S. aureus</i> Human1091	
	18	1.9	1.9	1.9	1.9	1.9	1.9	2.2	2.6	1.9	1.9	0.6	0.0	0.1	0.0	1.6	3.1	0.0	█	100.0	97.0	95.5	95.5	95.5	95.5	96.5	18	DQ016055.1 <i>S. aureus</i> D3900	
	19	1.9	1.9	1.9	1.9	1.9	1.9	2.2	2.6	1.9	1.9	0.6	0.0	0.1	0.0	1.6	3.1	0.0	0.0	█	97.0	95.5	95.5	95.5	95.5	96.5	19	DQ016054.1 <i>S. aureus</i> D3866	
	20	3.6	3.6	3.6	3.6	3.6	3.6	3.8	4.2	3.6	3.6	2.4	3.0	3.2	3.0	3.0	4.3	3.0	3.0	3.0	█	97.0	97.0	97.0	97.0	97.1	20	NG_055999.1 <i>S. aureus</i> USA300_TCH959	
	21	4.9	4.9	4.9	4.9	4.9	4.9	5.2	5.6	4.9	4.9	4.1	4.7	4.8	4.7	4.8	5.9	4.7	4.7	4.7	3.0	█	100.0	100.0	100.0	98.9	21	CP035671.1 <i>S. aureus</i> VB31683	
	22	4.9	4.9	4.9	4.9	4.9	4.9	5.2	5.6	4.9	4.9	4.1	4.7	4.8	4.7	4.8	5.9	4.7	4.7	4.7	3.0	0.0	█	100.0	100.0	98.9	22	LT992465.1 <i>S. aureus</i> 6_LA_232	
	23	4.9	4.9	4.9	4.9	4.9	4.9	5.2	5.6	4.9	4.9	4.1	4.7	4.8	4.7	4.8	5.9	4.7	4.7	4.7	3.0	0.0	0.0	█	100.0	98.9	23	CP029653.1 <i>S. aureus</i> AR_0470	
	24	4.9	4.9	4.9	4.9	4.9	4.9	5.2	5.6	4.9	4.9	4.1	4.7	4.8	4.7	4.8	5.9	4.7	4.7	4.7	3.0	0.0	0.0	0.0	█	98.9	24	CP029627.1 <i>S. aureus</i> MOK042	
	25	4.1	4.1	4.1	4.1	4.1	4.1	4.3	4.7	4.1	4.1	3.2	3.5	3.7	3.5	3.9	5.0	3.5	3.5	3.5	2.9	1.1	1.1	1.1	1.1	█	25	Staph aureus ASM strain	

Figure 6. Nucleotide sequence distance of *Staphylococcus aureus*β-lactamase (*Blaz*) resistant gene between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank

DISCUSSION

Among the most important diseases of poultry, *S. aureus* infection, also called bumble foot, is a common bacterial disease of commercial broilers and layers. It causes significant economic losses through mortality (0-15%) and reduce production performance of birds. Out of a total number of 78 samples were collected from infected chicken showing clinical signs of planter abscess or bumble foot, ten locally field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of (12.82%), this findings agree with Marek et al. (2016) (15.89%) and disagree with Mamzaet al. (2010) (52.5%), (Suleiman et al., 2013) (54%), Abd El-Tawab et al. (2017) (66%), Rasheed (2011) (50.98%). The colonies morphology of *S. aureus* were small to medium sized and golden yellow on BHI agar, while the colonies appeared surrounded by a double zone of beta haemolysis on blood agar plates, on mannitol salt agar, they were yellow color surrounded by yellow halo with yellow colored medium the colonies were typically black smooth with entire margin on Baird Parker agar media. For the biochemical results, strains revealed that all were identified as *S. aureus* as shown in table 2. This finding was in accordance with the findings of Topley and Wilson (1990), Selim et al. (1984) and Paul et al. (2014).

Phenotypic characterization of some virulence factors as haemolysin production assay and coagulase

production. All ten *S. aureus* isolates were coagulase positive and produce bata hemolysis, these two factors are important in phagocytosis and infection due to toxins production respectively (Bhanderi et al., 2009 and Cariolato et al., 2008).

Molecular identification of *ClfA* and *blaZ* genes of *S. aureus* isolates revealed that the PCR amplification with *ClfA* specific primers was conducted with genomic DNA, which resulted in a product of approximate size 638bp. clumping factor A (*ClfA*) gene was found in all ten (100 %) *S. aureus* isolates these results agree with Nemati et al., (2009) (100%), Erfan and Marouf (2015) (100%) and disagree with Mohamed A. Lebdah et al., (2015) (20%). *S. aureus* expresses several different proteins including clumping factors A (*ClfA*) that play an important role in the ability of *S. aureus* to cause disease Perkins et al., (2001) and Walsh et al., (2008). Clumping factor A (*ClfA*) is a microbial surface protein that promotes *S. aureus* binding to fibrinogen, and is associated with septic arthritis and infective endocarditis Elkhatib et al., (2015). The PCR amplification with *blaZ* gene specific primers was conducted with genomic DNA, resulted in a product of approximate size 833 bp. *blaZ* gene was present in ten (100 %) *S. aureus* isolates these finding nearly agree with Bakheet et al. (2018) (74%) and disagree with Ganugula Mohana Sheela (2017) (57.69%).

Phylogenetic and partial gene sequence analysis of *ClfA* and *blaZ* genes of *S. aureus* that was generated using maximum likelihood, neighbour joining and maximum

parsimony in MEGA6, showed clear clustering of isolated Egyptian strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Sequence distance of *S. aureus ClfA* virulence gene (Figure 3) was created by the MegAlign module of Laser gene DNA Star. Sequence identities between the isolated Egyptian strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank (Figure 5) revealed that 99.5% to 100% homology. When analyzing nucleotide sequence of *ClfA* gene of the Egyptian isolated strain (*S. aureus* ASM strain) in the current study showed 100% nucleotide identity with locally isolated Egyptian *S. aureus* strain SA.EGY1 recovered by Erfan and Marouf (2018) (accession No. MG821495.1). The Egyptian isolated strain (*S. aureus* ASM strain) showed also 100% identity with the Indian *S. aureus* strain R1/Bov/2015 by Vaidya et al., (2016) (GenBank accession No. KX181853.1). Also showed 99.8% identity with the American *S. aureus* strain C2 by Murphy et al., (2011) (GenBank accession No. HQ424270.1), and showed 99.7% identity with the American *S. aureus* strain FFP221 by Murphy et al. (2011) (GenBank accession No. HQ424283.1), American *S. aureus* strain 57/92 by Murphy et al. (2011) (GenBank accession No. HQ424256.1) and American *S. aureus* strain CA-548 (GenBank accession No. HQ424254.1). Concerning *S. aureus blaZ* gene nucleotide sequence analysis revealed great homology and identity between the Egyptian isolated strain (*S. aureus* ASM strain) and the different *S. aureus* strains uploaded from GenBank. Phylogenetic tree for *S. aureus blaZ*β-lactamase resistant gene (Figure 4) partial sequences showed clear clustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Figure 6 shows that sequence distance of *S. aureus blaZ* antibiotic resistant gene was created by the MegAlign module of Laser gene DNA Star. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank revealed that 96.1% to 98.9%. When analyzing nucleotide sequence of *blaZ* B-lactamase resistant gene of the Egyptian isolated strain (*S. aureus* ASM strain) in the current study it showed 98.9% identity with the Ireland *S. aureus* MOK042 strain recovered by Keane and Cormican 2018), (GenBank accession No. CP029627.1), the American *S. aureus* strain AR_0470 recovered by Benahmed et al., (2018) (GenBank accession No. CP029653.1) and the German *S. aureus* isolate 6_LA_232 recovered by Schleimer et al. (2018) (GenBank accession No. LT992465.1).

Quality control of the prepared *S. aureus* vaccine assured that the locally prepared vaccine free from any bacterial (aerobic or anaerobic contaminants) or fungal contamination and safe as there was no local reaction found in all injected chickens.

The results of humoral immune response revealed that the geometric mean antibody titers against *S. aureus* values of both groups as shown in table 3. The GMT antibody titer was (16) in the 1st week post vaccination, and increase gradually at the 2nd week (20.16), till reach to maximum level at 6th week was (322.5) as shown in Table 3. These findings agree with (Raza et al., 2015). The vaccine showed a considerable survival rate in challenged vaccinated group compared to that of control group. The mortality rates and survival percentages in groups control and vaccinated are summarized in table 4, the NO. of dead chickens in the 1st week post challenge in the control and vaccinated group were 17 (42.5%) and 3 (7.5%), respectively. The mortality rate in the 2nd week in the control group 9 (39.1%) and vaccinated group 2 (5.4%). Protection or survival (%) till day 7 post challenge (20%) in control group and vaccinated group (92.5%). Protection or survival (%) till 15 day post challenge (0%) in control group and (87.5%) in vaccinated group as shown in Table 4. The challenge protection assay showed a considerable protective immune response of prepared *S. aureus* vaccine. The findings were in agreement with Giraudo et al. (1997) and Ahmad and Muhammad (2008) with significantly higher survival percentage in vaccinated group compared to that of in control group. The results of challenge protection assay suggested that vaccine is capable of eliciting protective immune response and prevent further new infections of *S. aureus*. Fecal shedding post challenge was also significantly reduced in the vaccinated chickens compared with those in the unvaccinated suggesting that the vaccine could be effective against *S. aureus*. Table 5 showed that the fecal shedding of chickens challenged with virulent *S. aureus* strain in group 1 vaccinated with locally prepared oil adjuvanted *S. aureus* vaccine was (10.8%, 8.5% and 0%) in 1st, 2nd, 3rd week post challenge, respectively. Shedding disappeared by the 4th week post challenge. These results agree with previous studies showed that bacterin-toxoid prevent development of new *S. aureus* infection probably due to increased opsonization, increased phagocytic activity of polymorphonuclear cells as a result of augmented specific IgG antibodies against *S. aureus* (Pellegrino et al., 2010 and Raza et al., 2015). From this study it could be concluded that the locally prepared vaccine was safe, potent and could be recommended to be used in poultry farms to prevent *S. aureus* infections in chicken.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists.

Authors' contribution

Abeer El-Maghraby designed the concept of the article, wrote the manuscript. SherenAzez and AbeerMwafy prepared the vaccine. AbeerMwafy revised the manuscript. All authors designed and performed the experiments and reviewed and approved the manuscript.

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