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Bacteriological studies on some virulence factors in *Staphylococcus aureus* isolated from chicken and Nile tilapia

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Summary

Bacteriological examination of 300 samples from chicken and *O. niloticus* fish (150 from each) revealed isolation of 93 isolates of ***Staph. aureus***, 30 isolates from chicken and 63 isolates from *O. niloticus* fish with an incidence of (20% and 42%) respectively. All the isolates were subjected to different tests to detect some phenotypic characteristics related to virulence: coagulase, DNase, TNase, slime and hemolysin production. In the present study all examined isolates were coagulase positive and the production of DNase, TNase, slime and beta hemolysin was shown by (90 and 96.83), (83.33% -90.48%), (73.33%- 65.8%) and (80% - 92.06%) of chicken and fish isolates respectively. Coagulase gene was detected by PCR and the amplified products of the examined ***Staph. aureus*** strains isolated from chicken were analyzed at the band (570 bp), while in case of fish strains the amplified products were analyzed at the band (570 bp and 630 bp). All ***Staph. aureus*** isolated from chicken were (100%) sensitive to vancomycin followed by gentamicin (80%) , (36.67% and 40%) were sensitive to doxycycline and rifampicin respectively, while (36.67%) of the isolates were intermediately sensitive to doxycycline and rifampicin. ***Staph. aureus*** isolated from chicken were resistant to penicillin, spectinomycin, cefoxitin and ciprofloxacin at rates of (83.33%, 73.33%, 53.33% and 50%) respectively. ***Staph. aureus*** isolated from fresh water *O. niloticus* samples were (100%) sensitive to vancomycin and gentamycin. The majority of the isolates were (73% and 57.1%) sensitive to doxycycline and ciprofloxacin respectively. While (100%, 76.2% and 58.7%) of ***Staph. aureus*** isolates resistant to penicillin, spectinomycin and cefoxitin respectively.

Introduction

Staphylococcus aureus is one of the pathogens causing domestically acquired food borne illness**, (Abdalrahman and Fakhr 2015)**. In poultry industry, ***Staph. aureus*** is an important pathogen that causes economic losses **(Mosleh *et al.*, 2016)**. **Krupa *et al.*, (2014)** cultured and analyzed ***Staph. aureus*** isolates from chicken cloacae and chicken meat, they reported the possibility of transmission of ***Staph. aureus*** isolates from chicken to meat. **Marek *et al.*, (2016)** stated that in the pathology of poultry, infections caused by ***Staphylococcus spp.*** are taking on increasing significance. On the

other hand fish meat has excellent nutritional value being rich in proteins, vitamins and unsaturated fatty acid, it is also one of the most important feed stuffs as they are the cheapest source of animal protein during the last years **(Abdulla, 2003 and Albuquerque, *et al.*, 2007)**. One of the main factors affecting fish production and efficiency is the fish diseases and they represent a real danger for aquaculture in Egypt **(Aly, 2013)**. ***Staphylococcus spp.*** are one of the most important food borne opportunistic bacteria which isolated from fish samples and some of ***Staphylococcus spp.*** are potential pathogens and the high population of these bacteria indicates the general quality of fish and the degree of the spoilage it might have undergone **(Albuquerque *et al.*, 2007)**. **Soliman *et al.*, (2014)** isolated methicillin-resistant ***Staph. aureus*** (MRSA) from Nile tilapia (*Oreochromis niloticus*) during an outbreak in Egypt. Also (MRSA) was isolated from apparently healthy Nile tilapia (*O. niloticus)* **(Atyah *et al.*, 2010)**. ***Staph. aureus*** has a capacity to produce a large number of accepted virulence factors **(Salasia *et al.*, 2004)** and the disease process is influenced by various virulence factors possessed by these organisms, **(Yadav *et al.*, 2015)**, ***S. aureus*** has the ability to produce several exoenzymes such as coagulase, hemolysins, nuclease, acid phosphatase, lipase, protease, fibrinolysin, enterotoxins and toxic shock syndrome toxin that contribute to virulence **(Turkyilmaz and Kaya 2006)**.

The most important phenotypical features used in the identification of ***Staph. aureus*** its ability to produce coagulase, an enzyme that causes clotting of the blood plasma, the production of coagulase enzyme could be confirmed by the presence of coagulase gene which detected by PCR. **(Qing *et al.*, 2012)**. Treatment with antibiotic is one of the most important technique to control disease. Excessive and incorrect use of drugs considered the major selective force for the development of resistance **(Levy, 2002)**. The study was aimed for isolation and identification of ***Staph. aureus*** from chicken and fish, investigation of virulence factors including production of coagulase, DNase, TNase, slime and *hemolysin*, detection of coagulase gene by (PCR), determination of the antimicrobial sensitivity of the isolated ***S. aureus*** to some chemotherapeutic agents and comparison between isolates.

Material and Methods

Samples:-

Chicken samples:-Out of (150) samples were taken from foot lesions (bumble foot), (10 samples), internal organs (90 samples from lung and liver) of scarified diseased chickens

which showed respiratory disorders and / or diarrhea and 50 cloacal swaps from apparently healthy chickens. Samples were collected from different broilers farms in Beni Suef city.

Fish samples:- Out of (150) samples were taken from 150 fresh water *O. niloticus*, out of these, 30 apparent healthy and 120 diseased fish showed macroscopical clinical pathological lesions (external abscess , emaciation, erosions and eye lesions) were collected from fish farms in Beni Suef city. The samples were taken from gills, liver, eyes, kidneys and external lesions.

Isolation and identification of *Staph. aureus*:-

Samples from chicken and fish were streaked on Baired parker agar medium with sodium tolerate. The plates were incubated at 37^0C for 48hr, the isolated bacteria were identified morphologically and biochemically according to **Collee *et al.*, (1996)** and **Qunin *et al.*, (2002)**.

Phenotypic characterization of some virulence factors:- Coagulase activity of each isolate was tested by tube coagulase test using sterile human plasma and the production of hemolysins was also determined by cultivation of bacteria on sheep blood agar plates **(Qunin *et al.*, 2002)**. DNase activity was demonstrated by culturing organisms on DNase agar with toludine blue dye **(Kateete *et al.*, 2010)**. Thermostable nuclease activity was demonstrated by heating the organism in suspension to about *60°C* and then putting this suspension on DNase agar with toludine blue dye, which changes color in the presence of the degraded DNA **FDA (2001)**. Slime production by the organisms was determined by cultivation on Congo Red Agar **(Gundogan *et al*. 2006)**.

Antimicrobial sensitivity test:-

Using disc diffusion technique and interpretation was carried according to **Clinical and Laboratory Standards Institute (C L S I, 2013)**.

Polymerase Chain Reaction (PCR):-according to **Iyer and Kumosani, (2011)**

Six coagulase positive ***Staph. aureus*** isolated from chicken and fish (three from each) were confirmed by PCR.

Extraction of DNA from *Staph. aureus* isolates:- DNA extraction from ***Staph. aureus*** isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. The bacterial isolates were cultured on Baired parker agar plates and incubated for 24 hours at 37°C, then

colonies of each isolates were cultured on tryptic soy broth and incubated for 24 hours at 37°C, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}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 provided in the kit.

Oligonucleotide Primer: Primers used were supplied from **Metabion (Germany)** are listed in table (1).

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Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary Denaturation	Annealing	Extension		
Coa	F. ATA GAG ATG CTG GTA CAG G R. GCT TCC GAT TGT TCG ATG C	Four different types of bands may be detected **350 – 430 -570 – 630 pb**	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Iyer and Kumosani , 2011**

PCR amplification. For *coa* gene, 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 appliedbiosystem 2720 thermal cycler.

Analysis of the PCR Products. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products and were loaded in each gel slot. Gelpilot 100 bp and 100 bp plus DNA ladder (Qiagen, Germany, GmbH) were 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.

Results and Discussion

Bacteriological examination of 300 sample from chicken and *O. niloticus* fish (150 from each) revealed isolation of 93 isolates of ***Staph. aureus***, 30 isolates from chicken and 63 isolates from *O. niloticus* fish with an incidence of (20% and 42%) respectively as shown in table (2).

Staph. aureus is one of the pathogens causing domestically acquired food borne illness**, (Abdalrahman and Fakhr 2015)**. **Marek *et al.*, (2016)** concluded that ***Staph. aureus*** was most frequently isolated from poultry. In case of *O.niloticus,* it is the most cultivated fresh water fish species due to its peculiar characteristics such as rusticity, resistance, productivity, and good sensorial properties of meat **(Maregoni, 2006),** despite of all benefits, there is a considerable risk of microbial contamination in this activity and, consequently, in the finished products, which represents critical concerns to public health questions **(Ababouch, 2006).**

In the present study ***Staph. aureus*** was isolated from diseased chicken at a rate of 20%, as shown in table (2) nearly similar result was recorded by **Bhargava*, et al.*, (2011)** who isolated ***Staph aureus*** from 25% of chicken samples but lower percentages were recorded by **Konicek *et al.*, (2016)** who isolated Methicillin resistant ***Staph. aureus*** (MRSA) from (0.3%) Of cloacal samples from wild birds, **Hajar Madehi*, et al.*, (2014)** isolated ***Staph. aureus*** from 6.42% of chicken nugget samples, **Marek *et al.*, (2016)** isolated 302 ***Staphylococcus*** strains from various species of poultry, (15.89%) of these strains were ***Staph aureus*** and **Zogg *et al.*, (2016)** isolated ***Staph aureus*** from 13 samples of chicken meat imported from Germany. Also higher results was recorded by **Gundogan *et al.*, (2005)** and **Buyukcangaz*, et al.*, (2013)** who isolated ***Staph aureus*** at rates of (53.3% and 67.6%) from chicken meat.

Regarding to fish samples a total of 63 ***Staph. aureus*** isolates were obtained from 150 fresh water *O. niloticus* with a percentage of 42% which come to agreement with the results recorded by **Obaidat *et al.*, (2015)** who isolated ***Staph. aureus*** from 47% of fresh fish samples, and nearly similar results were recorded by **Atyah *et al.*, (2010)** who isolated ***Staph. aureus*** from tilapia with percentage 35%. It was clear that incidence of ***Staph. aureus*** in fish samples was higher than that in chicken samples may be due to several factors as bacterial ecology of supply water, environment (air pollution and contamination by animal excrements), fish feed, soil and water table .

Coagulase production is an important phenotypic determinant of ***Staph. aureus*** which is associated with virulence as it resists phagocytosis and helps bacteria in virulence **(Bhanderi *et al.*, 2009)**, at the same time DNase is considered a virulence factor because of its ability to break down DNA. DNase activity is important to distinguish between pathogenic staphylococci and nonpathogenic resident flora **(Citak *et al.* 2003)**. Combination of mannitol fermentation and Dnase can be used along with coagulase for identification of S. *aureus* **(Kateete *et al.*, 2010** and **Gundogan *et al.*, 2013)**.

Table (3) showing that all ***Staph. aureus*** isolated from chicken and fish samples were coagulase positive and mannitol fermenter, 90 and 96.83, respectively were DNase positive, nearly similar results were recorded by **Yadav *et al.*, (2015)** who recorded that ***Staph. aureus*** isolated from mastitic milk were 100%, 93.7%, coagulase and DNase positive respectively and **Nashwa Ezzeldeen *et al.*, (2011)** recorded that ***Staph. aureus*** isolated from fish samples were 97.30 %, 95.2 % positive to mannitole fermentation and Dnase respectively. Lower percentage of coagulase production (65.5%) was recorded by **(Nashwa Ezzeldeen *et al.*, (2011)** and **Singh *et al.*, (20 11)** had reported coagulase production by only 78.5,88.3 and 90.7% ***Staph. aureus*** isolates obtained from intramammary infections in cattle, however, **Marques *et al.* (2013)** studied 36.84% coagulase positive S. *aureus* with DNase activity.

Thermostable nuclease (TNase) is a specific, heat-stable DNase that breaks down DNA **(Gerceker *et al.*, 2009)** and Slime production may reflect the microorganism's capacity to adhere to specific host tissues and thereby to produce invasive microcolonies **(Necidová *et al*. 2009)**. In the present study TNase and slime production were produced by (83.33% - 73.33%) and (90.48% - 65.8%) of ***Staph. aureus*** isolated from chicken and fish samples respectively, the results agreed with **Sindhu *et al.* (2008)** who reported thermostable nuclease activity in 87.30% ***Staph. aureus*** isolated from mastitic cattle and buffaloes, and **Marques *et al.* (2013)** recorded that 176 out of 250 (76.8%) isolates from bovine mastitic milk were slime producers also **Singh *et al.*, (2011)** reported TNase

activity in 60.20% and 65.30% ***Staph. aureus*** isolates from cattle and buffaloes. Higher results of TNase and slime production (100% - 96.87%) were recorded by **Yadav *et al.*, (2015)**, however **Turkyilmaz and Kaya (2006)** recorded that ***Staph. aureus*** isolates from different animal clinical samples and chickens with various infections were (43.3% - 77.8%) positive for TNase and slime production respectively.

Hemolysin plays an important role in virulence, as it may increase the possibility of the occurrence of infection. Hemolysins of pathogenic microorganisms have been shown to have potent toxic effect on lymphocytes, macrophages, neutrophils, epithelial cells, fibroblasts and other cell lines **(Cariolato *et al*. 2008)**. Out of 80% of ***Staph. aureus*** isolated from chicken were beta hemolytic on sheep blood agar and 20% were non hemolytic while (4.76%, 92.06% and 3.18%) of ***Staph. aureus*** isolated from fish were Alpha, Beta and non hemolysine producer respectively. The results agreed with **Nashwa Ezzeldeen *et al.*, (2011)** who cleared that (13.8%, 82.80% and 3.40%) of ***Staph. aureus*** isolated from fish were Alpha, Beta and non hemolysine producer respectively while **Turkyilmaz and Kaya (2006)** and **Gundogan *et al.*, (2013)** recorded that 58.9% - 40% of ***Staph. aureus*** isolated from different animal sources were beta hemolytic.

The ability to rapidly and accurately distinguish between ***Staph. aureus*** (coagulase positive ***Staphylococcus***) and non– ***Staph. aureus*** bacteria (coagulase-negative ***Staphylococcus spp.*** [CoNS]) is essential for the appropriate therapeutic use of antibiotics and timely intervention for infection control. Therefore, the production of coagulase can confirmed by the presence of coagulase gene which detected by PCR **(Qing *et al.*, 2012)**. **Photo (1)** clear that the amplified product of coagulase gene in the examined ***Staph. aureus*** strains which isolated from chicken (A, 1 and 2) were analyzed at the band (570 bp). While the amplified product of coagulase gene in the examined ***Staph. aureus*** strains which isolated from fish (B, 3 and 4) were analyzed at the band (570 bp – 630 bp). The amplification of the *coa*-gene displayed four different size polymorphisms with about 350 – 430 -570 – 630 pb **(Iyer and Kumosani, 2011)**, the repeated polymorphic region can be used to measure relatedness among *S. aureus* isolates, **(Shopsin *et al.*, 2000 and Reinoso, 2004).** The ability to rapidly and accurately distinguish between ***Staph.aureus*** (coagulase positive ***Staphylococcus***) and non– ***Staph.aureus*** bacteria (coagulase-negative ***Staphylococcus spp.*** [CoNS] is essential for the appropriate therapeutic use of antibiotics and timely intervention for infection control. Therefore, the production of coagulase can confirmed by the presence of coagulase gene which detected by PCR **(Qing *et al.*, 2012)**.

On of the control measure is the use of appropriate chemotherapeutic agent. Antibiotic resistance is a worrisome phenomenon, although not new, because its occurrence is increasing and has a serious impact on public health. As shown in table (4) all ***Staph. aureus*** isolated from chicken were (100% and 80%) sensitive to vancomycin and gentamicin respectively, 36.67% and 40% were sensitive to doxycycline and rifampicin respectively, while 36.67% of the isolates were intermediately sensitive to doxycycline and rifampicin respectively. ***Staph. aureus*** isolated from chicken were resistant to penicillin, spectinomycin, cefoxitin and ciprofloxacin at rates of (83.33%, 73.33%, 53.33% and 50%) respectively. Nearly similar results was recorded by **Lubna Abdalrahman *et al.*, (2015)** who stated that ***Staph. aureus*** isolated from chicken were resistant to ciprofloxacin , cefoxitin and penicillin but the same isolates were susceptible to vancomycin, doxycycline, gentamicin and rifampin. Also **Abdalrahman, and Fakhr (2015)** concluded that the highest resistance in chicken isolates was in penicillin (53.8%). Different level of resistant of ***Staph. aureus*** isolated from chicken to different chemotherapeutic agents was recorded by **Kraushaar *et al.*, (2016)**, **Mosleh *et al.*, (2016)** and **Wei *et al.*, (2016)**.

Regarding to fish isolates data in Table 3 it was showed that all ***Staph. aureus*** isolated from fish samples were sensitive to vancomycin. This result completely agreed with **Eok *et al.*, (2010)** who reported that all isolates of ***Staph. aureus*** were sensitive to vancomycine. Moreover, **Hiramatsu *et al.*, (2001)** suggested that vancomycin has been the most reliable therapeutic agent against infections caused by ***Staph. aureus***. Gentamycine sensitivity revealed that all the strains of ***Staph. aureus*** isolates were sensitive (100%), this result was nearly similar to what achieved by **Nashwa Ezzeldeen *et al.*, (2011)** who recorded that 98% of ***Staph. aureus*** isolates were sensitive to gentamycin. The majority of isolates showed high sensitive to doxycycline (73%) followed by ciprofloxacin (57.1%)these results recorded by **Soliman *et al.*, (2016)**. All ***Staph. aureus*** isolates were highly resistant to penicillin (100%), spectinomycin (76.2%) and cefoxitin (58.7%).These finding were supported and nearly coincides with the observation stated by **Obaidat *et al.*, (2015) and Haifaa Hussien, (2014)**.

In conclusion bacteriological examination of 300 chicken and fish samples revealed isolation of 93 isolates of coagulase positive ***Staph. aureus***, there were clear differences in the incidence, phenotypic and genotypic features and ***Staph. aureus*** isolated from chicken were more resistant to the used antibacterial agents.

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Table (2): Incidence of *Staph. aureus* in Chicken and fresh water *O. niloticus* samples

***Staph. aureus* isolated from chicken (n=30)**				***Staph. aureus* isolated from *O. niloticus* (n=63)**				**Total (n=93)**			
Samples from apparent healthy birds (n=50)		**Samples from diseased birds (n=100)**		**Samples from apparent healthy fish (n=30)**		**Samples from diseased fish (n=120)**		**Chicken samples (n=150)**		**Fish samples (n=150)**	
No	**%**	**No.**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
4	**8**	**26**	**26**	**9**	**30**	**54**	**45**	**30**	**20**	**63**	**42**

Table (3): Biochemical and some enzyme production characteristics of *S. aureus* isolates

Biochemical and enzymes production	**No of *Staph. aureus* isolated from chicken (n=30)**		**No of *Staph. aureus* isolated from *O. niloticus* (n=63)**	
	+ ve	%	+ ve	%
Mannitol fermentation	30	100	63	100
Coagulase (Human plasma)	30	100	63	100
DNase	27	90	61	96.83
TNase	25	83.33	57	90.48
Slime production	22	73.33	41	65.08
Hemolysine				
Alpha	-	-	3	4.76
Beta	24	80	58	92.06
No hemolysis	6	20	2	3.18

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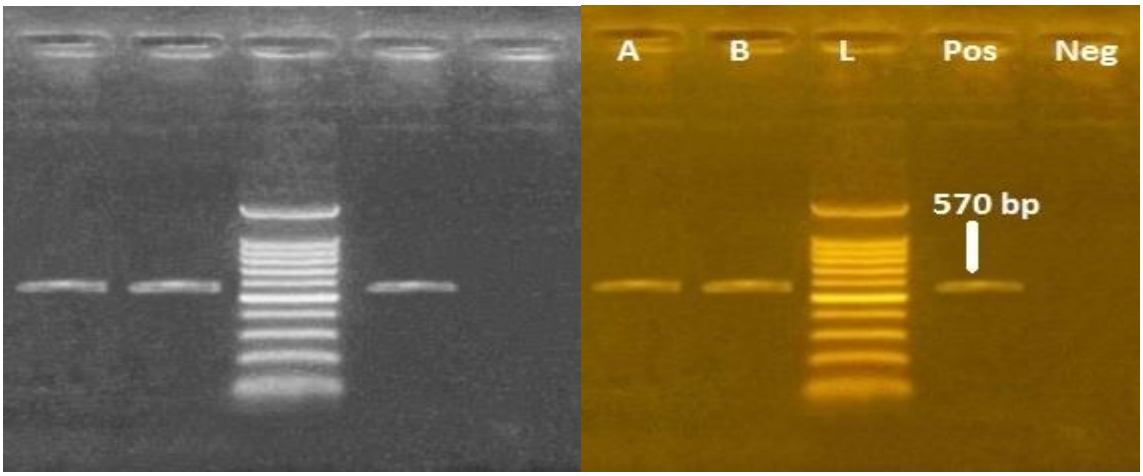

Photo (1)

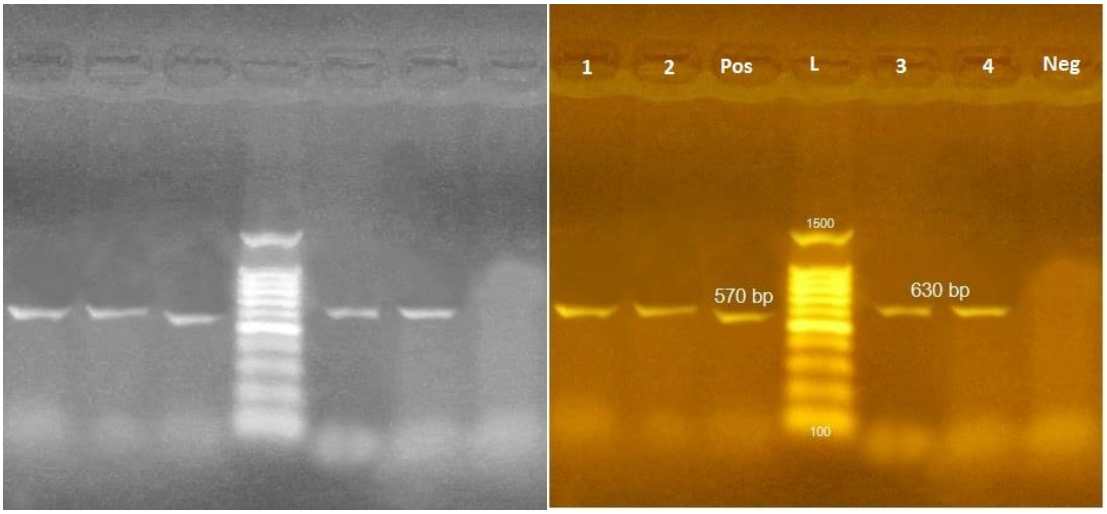

Photo (2)

Photo (1&2) Lane (L): 100 bp plus DNA ladder, Lane (pos): positive sample, Lane (neg): negative sample, Lane A and B coagulase positive (570 bp) ***Staph. aureus*** isolated from Chicken and fresh water *O. niloticus* samples. Lane (1) and (2) ***Staph. aureus*** strains isolated from Chicken samples: Lane 3 and 4 ***Staph. aureus*** strains isolated from Fish samples.

Table (4): Antimicrobial susibtability of *Staph. aureus* isolated from Chicken and fresh water *O. niloticus* samples

Antimicrobial agents	Chicken isolates (n=30)						Fish isolates (n=63)					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
	No	%	No.	%	No.	%	No.	%	No.	%	No.	%
Vancomycin **(30 µg)**	30	100	-	-	-	-	63	100	-	-	-	-
Ciprofloxacin **(5 µg)**	5	16.67	10	33.33	15	50	36	57.1	21	33.3	6	9.5
Doxycycline **(30µg)**	11	36.67	11	36.67	8	26.67	46	73	11	17.5	6	9.5
Gentamicin **(10 µg)**	24	80	4	13.33	2	6.67	63	100	-	-	-	-
Rifampicin **(5 µg)**	12	40	11	36.67	7	23.33	27	42.8	11	17.5	25	39.7
Cefoxitin **(30 µg)**	8	26.67	6	20	16	53.33	26	41.2	-	-	37	58.7
Penicillin **(10 IU)**	3	10	2	6.67	25	83.33	-	-	-	-	63	100
Spectinomycin **(100 µg)**	0	0	8	26.67	22	73.33	6	9.5	9	14.2	48	76.2

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