Epidemiology and antimicrobial activity of methicillin-resistant Staphylococcus aureus (MRSA) isolated from Nile tilapia *(Oreochromis niloticus)* during an outbreak in Egypt.

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Abstract: Swabs from kidney and brain of Nile tilapia *(Oreochromis niloticus)* were collected during an outbreak in Kafr-El-Sheikh governorate in August 2013. The collected fish were submitted for clinical, bacteriological, virological, parasitological and mycological examination. The isolated bacteria were subjected to phenotypic, molecular identification. Also, antimicrobial resistance to antibiotics was determined using disc diffusion method. In addition, DNA sequencing and phylogenetic analysis were performed for determination of epidemiological investigations. The isolated bacteria were identified as methicillin-resistant *Staphylococcus aureus* (MRSA) for the first time in Egypt based on phylogeneic and molecular identification. Antimicrobial sensitivity test of *Staphylococcus aureus* by disk diffusion method showed the bacteria was resistant to beta-lactam antibiotics (Ampicillin and Cephradine). These finding were confirmed by PCR which showed DNA bands at 528 bp using primers for detection of mecA gene which is responsible for methicillin resistance in *Staphylococcus aureus* (MRSA). Phylogeny showed that the source of bacteria was not from human or animal origin and its emergence was due to the excessive uncontrolable use of antimicrobials in fish farms. Since global spread of multi-drug-resistant bacteria has increased in the past decade. This result up to our knowledge is the first report of methicillin-resistant *Staphylococcus aureus* MRSA in *Oreochromis niloticus* in Egypt.
[Soliman, M.K., Ellakany, H.F., Gaafar, A.Y., Elbialy, A.K., Zaki, M.S. and Younes, A.M. **Epidemiology and antimicrobial activity of methicillin-resistant Staphylococcus aureus (MRSA) isolated from Nile tilapia *(Oreochromis niloticus)* during an outbreak in Egypt.** *Life Sci J* 2014;11(10):1245-1252]. (ISSN:1097-8135). http://www.lifesciencesite.com. 185

Key words: *Staphylococcus aureus*, Nile tilapia. Kafr-El-Sheikh governorate, PCR

1. Introduction;

Worldwide, Tilapia comes in the second place after Carp regarding production ***(FAO 2012)***.

In the last decade, as the production of farmed Tilapia has shown a tremendous increase jumping from 1,303,310 metric tons in 2001 to 3,497,391 metric tons in 2010 ***(FAO 2012)***. Global Tilapia production is dominated by three species: Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia *(Oreochromis mossambicus)* and Blue Tilapia *(Oreochromis aureus)* ***Rana, (1997).***

According ***FAO (2012)***, Nile tilapia *(O. niloticus)* is the most important farmed tilapia species, representing more than 73% of total tilapia production in 2010.

In Egypt, the total production of Nile Tilapia *(O. niloticus)* in 2010 was 557,049 tonnes or approximately 60.57 % of the total aquaculture production ***(FAO, 2012)***.

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)***.

Staphylococci are among the most widespread pathogenic and opportunist pathogenic Bacteria. It is an extraordinary versatile pathogen and the major causative agent of numerous hospital and community acquired infections ***(Doškař et al., 2010).***

Staphylococcus aureus has been reported as the causative agent of eye disease on Silver carp) ***Shah and Tyagi, 1986) and*** Channa marulius ***(Kumaraiah et al. 1977)***. Also methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from apparently healthy Nile tilapia *(O. niloticus)* ***(Atyah et al., 2010)***.

Staphylococcus aureus is well known for its ability to acquire antibiotic resistance, both historically in relation to penicillin, erythromycin and tetracycline and more recently methicillin and vancomycin resistance) ***Gaze et al., 2008).***

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a nosocomial pathogen in the early 1960s) ***Barber 1961).*** Methicillin, the first semisynthetic penicillin to be developed, was introduced in 1959 to overcome the problem of penicillin-resistant *Staphylococcus aureus* due to β-lactamase (penicillinase) production ***(Livermore 2000).***

Strains of MRSA have spread and become established as major nosocomial pathogens worldwide ***(Fluit et al., 2001).*** Afterwords, worldwide MRSA epidemic has occurred (***Wulf and Voss, 2008).*** Recently, these organisms have evolved and emerged as a major cause of community-acquired infections

(Lindsay and Holden 2004). These newly emerging community-acquired MRSA strains contain the mecA gene with or without additional antibiotic resistance genes and are more easily transferred to other strains of *S. aureus* ***(O'brien et al. 2004).*** The emerging spread of these community-acquired MRSA strains poses a significant threat to public health ***(Vandenesch et al. 2003)***

MRSA has also been reported in livestock, pet animals apparently healthy fish ***(Atyah et al., 2010)***. This study reports the presence of methicillin- resistant *S. aureus* (MRSA) associated with mortalities during an outbreak occurred in *Oreochromis niloticus*.

2. Materials and methods

Fish:

In August 2013, the fish were collected from an outbreak in farm in Kafr-El-Sheikh governorate with mortalities reached approximately 20%. The examined fish were Nile Tilapia (*Oreochromis niloticus*) show clinical signs, with 300 to 400 gms average weight. They were transferred alive to the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhur University, and kept in glass aquaria until examined.

Fish were kept in glass aquaria (90 X 50 X 35 Cm) which supplied with chlorine free tap water. The continuous aeration was maintained in each aquarium using an electric air pump. Water temperature was kept at 26 ± 1° C.

The fish were fed on a commercial fish diet containing 25% crude protein. Feeding ratio was 5% of body weight. The daily amount of food was offered on two occasions over the day (at 9 AM and 12 PM) **(Eurell *et al.*, 1978)**.

The fish were subjected to clinical, bacteriological, mycological, virological and parasitological examinations. Samples were collected under aseptic conditions from liver, spleen, kidney and brain under septic condition. Inoculated on Tryptone Soya Broth (TSB) tubes for bacterial isolation, Sabouraud Dextrose Agar (SDA) plates for fungal isolation. The inoculated plates and tubes were incubated at 37 °C for 24hr. Loopfuls were taken from the inoculated tubes and streaked onto the surface of Tryptone Soya Agar (TSA) plates, Mannitol Salt Agar (MSA) plates and MacConkey agar plates. The inoculated plates were incubated at 37 °C for 24hr **(Noga, 2010).**

Skin scraping, gill biopsy and wet mount were performed according to **(Noga, 2010**) for detection of any external parasites. For viral isolation, primary tissue culture from ovaries of healthy *Oreochromis niloticus* and subculture were performed according to method described by **(Wolf and Quimby, 1976a, b)**. Samples from kidney, spleen and liver inoculated of monolayer cell line according to method described by **(Heil, 2009)**.

Phenotypic examination of bacterial isolates:

The isolated bacteria was grown on specific media for *Staph aureus* mannitol salt agar and Triple sugar iron agar for 24hr at 37°C. Oxidase and Catalase test were done) **Buller, 2004).**

Extraction of bacterial DNA:

A number of colonies obtained after overnight growth on tryptic soy agar or mannitol salt agar were suspended in 1 ml TE buffer and centrifuging it at 3,000 g for 5 min, and the pellet was resuspended in 100 ul of TE buffer. The suspension was boiled for 10 min before centrifugation at 3,000 g for 5 min. The supernatant served as the PCR. DNA concentration was calculated by measuring at OD260 and OD280 using the U.V. fiber-optic spectrometer. Pharmacia-LKB. Ultra-spect. Cuvette-quartez England) **Hanssen et al., 2004).**

Polymerase Chain Reaction amplification (PCR):

The PCR used for detection of mecA gene using primers in (Table 1), according to **Predari et al. (1991)** with modification. The PCR was performed in a 50ul volume DreamTaq Green PCR Master Mix 2X (ThermoScientific #K1081) containing the following: 25ul DreamTaq Green PCR Master Mix 2X, 40 pmol of each primer and 1 ug DNA. PCR amplifications were performed in a MyGenie 32 Thermal Block (BIONEER) with predenaturation for 5 min at 96°C; 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 45 s. Postextension for 5 min at 72°C; and soaked at 4°C.

Amplification products were separated on Agarose 1% **[** (biobasic, CAT NO D0012) (agarose 1.0g + 50X TAE buffer 2 ml + Distalled water till 100 ml)**]** at 30 V/cm for 15 min. The gels were stained with ethidium bromide (1 ug/ml) for 30 min and photographed under UV illumination.

Table 1. Primers used for detection of mec gene

Primer	Primer sequence (5–3)	Amplicon size (bp)	Reference
Mec F	GGGATCATAGCGTCATTATTC	528 bp	**(Predari et al., 1991)**
Mec R	AACGATTGTGACACGATAGCC		

DNA sequencing:

DNA sequencing was done according to method described by **Bergeron et al., 2011** After purification of PCR products by QIAquick PCR Purification Kit (Qiagen Cat no: 28104) according to manufacturer instructions, Sequencing reactions were

performed in a total volume of 20 µL containing 8.0µL Terminator Ready Reaction Mix* (Bigdye terminator v3.1 cycle sequencing kit, Applied Biosystems Cat no: 4337455), 3.2 pmol sequencing primer. The sequencing products were purified by using the Centri-Sep™ Spin Columns (Invitrogen Cat no: 401762) and analyzed with the 3500 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic analysis:

The sequences were compared with other *Staph aureus* mecA gene in the database using the BLAST 2 algorithm **(Myers et al., 1990).**

Nucleotide sequence alignments were performed by AlignX contained within Clustal X program version1.83 **(Thompson et al., 1997).** Pairwise and multiple alignment gap opening and gap extension settings were 15.00 and 6.66, respectively. A phylogenetic tree was constructed using the 528 nucleotide region of mecA gene. Phylogenetic analyses were performed using MEGA version 3.1 **(Kumar et al., 2004).**

Antimicrobial profile:

Antimicrobial susceptibility test for isolated bacteria was performed by disc diffusion method **(Hudzicki, 2009)** using Mueller–Hinton agar (Difco). Diameter of inhibition zone was measured and interpreted as susceptible (S), intermediate (I) or resistant (R)

Experimental infection of methicillin- resistant *Staphyloccocus aureus (MRSA)*:

a) Preparation of the inoculums:

The isolate was subcultured on Tryptone Soya agar plates and inoculated at 37 ^{o}C for 24hr. A typical isolated colony was picked up and inoculated into Tryptone Soya Broth and inoculated at 37 ^{o}C for 24hr. then washed in PBS for 3 times by centrifugation at 4000 rpm. The sediment were resuspended in sterile saline and standardized for 5 OD at wave length 650 using the U.V. fiber-optic spectrometer (each 1 ml contain approximately 10^{10} bacterial cells).

b) Pathogenicity:

Pathogenicity was done according to method by **Chang and Plumb, 1996**, One ml of inoculums contain approximately 10^{10} CFU/ml was diluted in 1 liter distilled water to get final concentration of bacteria 10^{7} CFU/ml. then, fish (about 200 gm body weight) were removed from their home aquaria and scraping approximately 1.5 cm of the lateral body surface with a scalpel. The fish were then immersed for 10 min in *Staph aureus* suspension containing approximately 1X 10^{7} CFUs/ ml and then returned to their home aquaria. Fish were divided into 4 groups each group contain 5 fish. Group 1 contain injured, infected fish, group 2 contain injured, non-infected fish, group 3 contain non-injured, infected fish and Control group contain non-injured, non-infected fish.

3. Results;

Clinical signs and PM lesions of naturally infected fish:

Naturally infected Nile tilapia *O. niloticus* showed haemorrhages on the skin (fig. 1), erosions at the dorsal region (fig. 2).

Postmortem examination showed swollen congested kidney, congested gills and paleness of liver (fig. 3).

Bacteriological, mycological, virological and parasitological examination.

The mycological and parasitological examinations were negative in all examined samples. No cytopathic effect has been noticed on tissue culture.

Phenotypic examination of bacteria

Phenotypic analysis showed that bacterial colonies were golden yellow on TSA and MSA media, gram positive cocci accumulated in clusters(fig. 5), catalse positive, oxidase negative, Growth on mannitol salt agar media showed presence of yellow halo surrounding the colonies (fig. 4). Bacteria growth on TSI showed yellow slant, yellow butt, no H2S and gas production.

Phenotypic characteristics of *Staphylococcus aureus* are shown in table 2.

Table 2: Phenotypic characteristics of *Staphylococcus aureus* strain

Test	Result
Gram stain	Gram positive cocci
Catalase test	+
Oxidase test	-
Motility	-
Mannitol fermentation	+
Acid from glucose	+
Acid from lactose	+
Acid from sucrose	+
H_2S production	-
Gas production from sugars	-
Growth on NaCl 0%	+
Growth on NaCl 7.5%	+
Growth on NaCl 10%	+
Citrate utilization	-

PCR amplification:

Polymerase chain reaction showed positive band at 528 bp using two primers internal to mecA gene (fig 6).

Phylogenetic tree

Phylogenetic tree generated using the 582 bp of mecA gene of the bacteria of this study with other mecA genes of other MRSA on database after

performing BLAST search and genetic aligment. Phylogeny showed that MRSA of this study is distantly related from other MRSA of human or animal origin (fig 7).

Antimicrobial sensitivity:

Antimicrobial sensitivity showed that the bacteria were resistant to Ampicillin, Cephradine and Erythromycin. The bacteria were sensitive to Gentamicin, Streptomycin, Lincomycin, Doxycycline, Oxytetracycline, Ciprofloxacin, Norfloxacin, Chloramphenicol according to)**Cockerill, 2011(.**

Pathogenicity test:

One week after experimental infection with 3X 107 (CFUs)/ ml *Staph aureus* with skin scraping and sinking in *Staph aureus* suspension, some fish of group 1 showed erosion of the skin all over the body surface especially at nuckal region with redness of some parts (fig. 8), other showed corneal opacity (fig. 8). PM examination revealed congested spleen and enlarged gall bladder (fig. 10). No clinical signs were noticed in other groups. *S. aureus* was reisolated from fish showed clinical signs.

Fig. (1): Naturally infected *O. niloticus* with *Staph aurues* showing haemorrhages on the skin (arrows).

Fig. (2): Naturally infected *O. niloticus* with *Staph aurues* showing erosions at the Nuckal region (arrow).

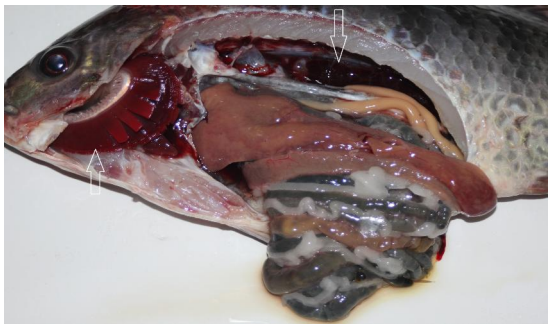

Fig. (3): Postmortem examination of naturally infected *O. niloticus* with *Staph aurues* showing swollen, congested kidney (arrows).and paleness of liver.

Fig. 4. Growth on mannitol salt agar media showing presence of yellow halo (arrows) Surrounding the colonies which is specific for *Staph aureus* colonies.

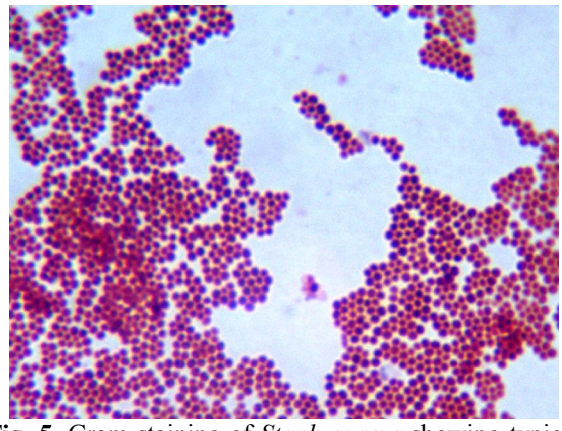

Fig. 5. Gram staining of *Staph aureus* showing typical gram positive cocci.

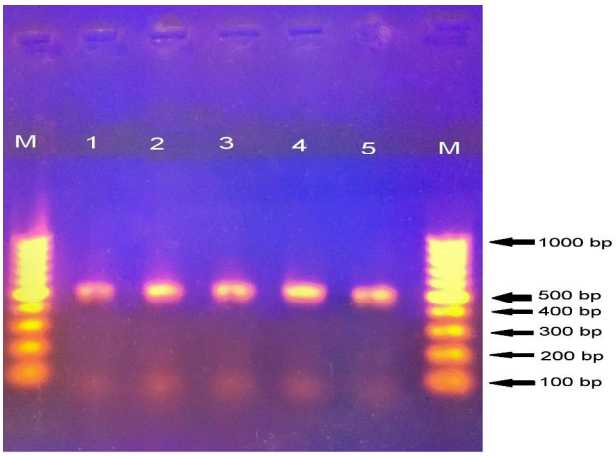

Fig. 6. Agarose Gel electrophoresis of the PCR product (528 bp) generated by using two oligonucleotide primers internal to mecA gene. (lane 1, 2, 3) from natural infected fish. (Lane 4, 5) from experimental infected fish. (lane M) is ladder.

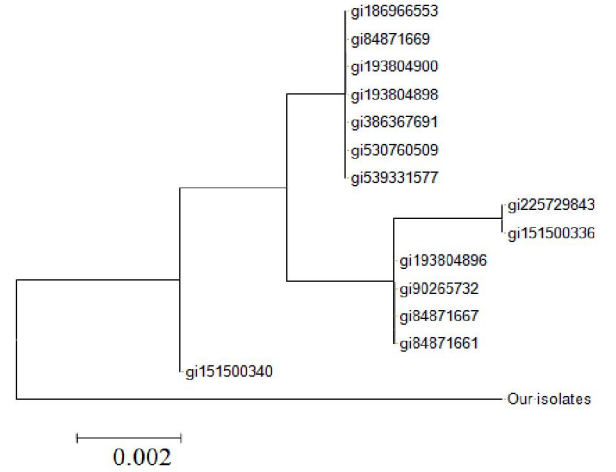

Fig. 7. Phylogenetic tree generated by neighbor-joining analyses of the 582 bp of mecA gene sequence showing that MRSA of this study is distantly related from other MRSA of human and animal origin.

Fig. (8): *O. niloticus* after 1 week of experimental infection with *Staph aureus* showing erosion of the skin all over the body surface especially at nuckal region (arrows).

Fig. (9): *O. niloticus* after 1 week of experimental infection with *Staph aureus* showing erosion of the skin and corneal opacity (arrows).

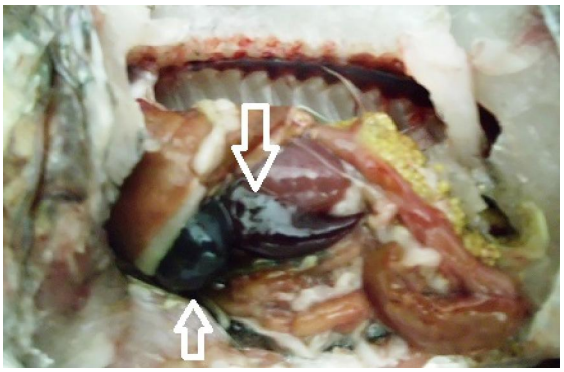

Fig. (10): Postmortem examination of experimentally infected *O. niloticus* with *Staph aurues* showing congested spleen and enlarged gall bladder (arrows).

4. Discussion

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a tremendous problem in human medicine because it has a low affinity for all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) and its widespread throughout the world since its emergence in the early 1960s.Recently, MRSA have emerged as a significant problem in veterinary medicine, including both animal and public health standpoints. In 2010 it was recorded for the first time in aquaculture in apparently healthy *O. niloticus*.

According to phenotypic and genotypic identification, it was found that the bacteria at this study was *Staphylococcus aureus* because the gram staining showed typical gram positive cocci, oxidase negative, catalase positive and its growth on mannitol salt agar media, showed yellow halo surrounding the yellow colonies ***(Buller, 2004)***. Moreover, ***Atyah et al. (2010)*** isolated *Staph aureus* from tilapia and used bacterial growth on mannitol salt agar and polymerase chain reaction (PCR) for specificity of the bacteria.

From the results it was found that *Staphylococcus aureus* in this study was penicillin and

methicillin-resistant (MRSA) because the antimicrobial sensitivity testing of *Staphylococcus aureus* by disc diffusion method showed the bacteria was resistant to Ampicillin (penicillins group) which belongs to beta-lactam antibiotics and Cephradine (cephalosporin group) which also from beta-lactam antibiotics. This finding was confirmed by PCR and sequencing results which showed that DNA bands at 528 bp using primers for detection of mecA gene which responsible for methicillin resistant in (MRSA) ***(Hanssen and Sollid 2006).*** Also the BLAST search and phylogenetic tree of this study confirmed these investigations.

The results of antimicrobial sensitivity testing of *Staphylococcus aureus* of this study were similar to those of ***(Najiah et al., 2012)*** who found that the antimicrobial sensitivity testing of *Staphylococcus aureus* of fish origin was susceptibe to Lincomycin and Tetracyclines and resistance to Ampicillin.

In Egypt, most of fish farmers operate on a small scale basis with little technical support and apply treatment rather than prevention as the antibiotics are largely available with little regulation on the use in aquaculture. This can exert selective pressure on bacteria and stimulate the proliferation of strains having resistance to drugs and probably evolution of *Staphylococcus aureus* to methicillin-resistant *Staphylococcus aureus* (MRSA))***Gaze et al., 2008).***

According to Egyptian law it's allowed only to irrigate fish farms by agricultural drainage water which contain high amount of antibiotics due to wide use of antibiotics in agriculture ***(Tenover and Hughes, 1996).*** That may be the cause of emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in Egypt.

The observed behavioral signs on tilapia due to methicillin-resistant *Staphylococcus aureus* (MRSA) infection were erratic swimming which also reported by)***Shah and Tyagi, 1986)*** in silver carp due to *S. aureus*, and the observed clinical signs were skin erosion at different parts of the body in both naturally and experimentally infected fish, haemorrhages near peduncle area and corneal opacity in eye which also reported by)***Shah and Tyagi, 1986)***. Postmortem examination revealed congested kidney, and congested gills, enlarged spleen in naturally infected fish and swollen gall bladder, congested spleen in experimental infected fish. Clinical signs and PM lesions were more sever in experimentally infected; this may be due to exposure of fish to high concentration of bacteria.

Clinical signs and PM lesions may be due to the toxins produced by bacteria. ***Doškař et al. (2010)*** stated that *S.aureus* secret many toxins like toxic shock syndrome toxin-1 (TSST-1), the Staphylococcal enterotoxins (SEs), the exfoliative toxins (ETs) and leukocidin.

To analyze the genetic diversity and to know the source of methicillin-resistant *Staphylococcus aureus* (MRSA) of this study, Phylogenetic analysis using 528 bp of mec A gene was conducted with other *Staphylococcus aureus* on databases after BLAST search. The results proved that Staphylococci of this study are distantly related from others on databases. Actually this was expected because all other *Staph aureus* on databases was from human or animal origin unlike Staphylococci of this study that isolated from fish. **Atyah et al. (2010)** reported the presence of MRSA in fish but did not conduct phylogenetic analysis. Also this gives a clear evidence that the source of MRSA isn't animals or human but it was due to the selective pressure on *Staph aureus* that is present normally in aquatic water and aquatic environment due to high amount of antibiotics in wastewater and/or in ponds by fish farmers without regulations and/or in agriculture These finding are similar to those of (**Kusuda and Sugiyama, 1981)** who showed that *Staphylococcus epidermidis* which present in the aquatic environment originated from water (or fish) rather than from human beings, because of pronounced antigenic differences from human strains. Also, **Lloyd (2007)** reported the presence of antimicrobial resistant bacteria in pets as methicillin-resistant *Staphylococcus aureus* due to increasing amounts of antimicrobials used. ***Graveland et al. (2010)*** stated that calves were more often carrier for MRSA when treated with antibiotics. Additionally ***Tanaka et al. (1995)*** reported that the extensive use of a group of beta-lactam antibiotics to counteract MRSA infection seems to be responsible for transition of MRSA types.

From the results of our investigations, we can say that the only way for prevention of this disease is to limit antimicrobials use, encourage use of probiotics, prebiotics and herbal remedies in prevention and treatment of fish diseases by immunostumulation effect. (***Galina et al., 2009)***.

This study gives high interest to epidemiological investigation because it is the first record of methicillin-resistant *Staphylococcus aureus* (MRSA) in fish in Egypt according to the best knowledge of the authors. It's very important to know where it comes from to perform preventive measures rather than treatment. Thus, prevent spreading of these bacteria.

Our results proved that Nile tilapia *Oreochromis niloticus* are susceptible to (MRSA) infection. ***Atyah et al., (2010)*** isolated MRSA from Nile tilapia *(O. niloticus).*

From the results it was found that the isolated bacteria (MRSA) was associated with morbidities and mortalities in Nile tilapia in Egypt for the first time in outbreak in summer of 2013.

From the results of our study, we can say that**,** the danger of this disease isn't only its effect on aquaculture, but its zoonotic importance. This

bacterium is primarily human pathogen that is hard to treat. Similar clarifications were raised by) ***Harrison et al., 2013 (***who reported transmission of MRSA from livestock reservoir to human who confirmed their results by DNA sequencing and phylogenetic tree. Transmission of these bacteria isn't only to fish handlers, but it may be also to water, thus increasing contamination of MRSA in the environment. These observations agreed with ***Elmir et al. (2007)*** and ***Plano et al. (2011)*** who stated that bathers are a potential source of MRSA contamination in water.

We can conclude that methicillin-resistant *S. aureus* (MRSA) have emerged as significant problems in aquaculture associated with morbidities and mortalities in Nile tilapia in Egypt. This is so far, the first report of MRSA in aquaculture in Egypt.

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10/15/2014