# Multiplex PCR of Some Antibiotic Resistance Genes of Methicillin Resistant *Staphylococcus Aureus* (MRSA) Isolated from Infected Cat Fish *(*Clarias garipeneaus).

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**Abstract**

This study was designed to detect some antibiotic resistance genes of *S.aureus* by using PCR. A total of 150 Cat fish *(*Clarias garipeneaus*)* with a body weight ranged from200 – 250g. suffered from ulcerated skin and erosion. *S. aureus* were isolated in percent of 100% from infected fish by using Baird parker as selective media. By using multiplex PCR for investigation some antibiotic resistant genes, as *erm* A gen, *aac (6)-aph (2)* genes , *van* A gen and *mec* A gene. All isolated *S. aureus* were sensitive to Amikacin, Amoxicillin, Ampicillin and Ciprofloxacin while resistant to Gentamicin, Methicillin and Neomycin.

## 1-introduction

Fish and fishery products could be the major source and acts as a vehicles for many important species of food poising bacteria, which include in addition to *Salmonella, Staphylococcus, Cl. botulinum***,** the so-called nonspecific group of microorganisms such *as E. coli, Proteus spp., Enterocerus fecalis, Cl. perefringnes* and *B. cereus* ***(Lotfi, et al.,1972 and Roberts, 2001).*** In spite of Staphylococci are not a part of the normal part of fish microflora ***(Herrero et al., 1999***), Staphylococcus spp. May reach aquatic environments through fecal contamination and it has been isolated from fresh and brackish water fish culture pond in many countries.

*S. aureus* has been reported as the third major causative agent of food born illness by fish and fish products in the European Union ***(EFSA, 2010).***

Methicillin-resistant *S.aureus* (MRSA) are being increasingly found outside clinical settings **(*Popovich et al., 2007, Ribeiro et al., 2007, Stankovic et al., 2007).*** MRSA have thus been found in food animals ***(Lee, 2003)*** and in fishery products recently ***(Beleneva, 2011).*** Although there is currently no evidence that eating food contaminated with MRSA may lead to an increased risk of humans becoming healthy carriers or infected with this bacterium ***(EFSA, 2010)***.

So, for this reason, discussed molecular studies on Staphylococci infection in fresh water fish.

***2-Material and methods***

**1-Sample:-**

A total of 150 Cat fish ***(***Clarias garipeneaus***)*** with a body weight ranged from200 – 250g suffered from ulcerated skin, erosions and ulcers were collected from Sewages canal (Bahr EL baker) in Sharkia Governorate and fish marked in Abbo- Hamad sharkia, in summer season in 2016.

**2- Clinical & post mortem examination of fish:-**

Fish were examined clinically for any abnormal lesions according to ***Noga (1996)*** *and* ***Austin and Austin (2007),*** where the most common clinical signs were external hemorrhage and ulcer. Fish showed abscess formation on skin, loss skin and may extend to tail and part from fins.fig.(1)

Post mortem examination was done according to the methods described by ***Noga (1996)*** *and* ***Austin and Austin (2007).***

**3-Bacteriological examination of samples:-**

The isolation and identification of Staphylococcus infection was carried out using standard methods of ***USFDA (BAM, 2001).***

1-Collected samples(gills, ulcers, liver, kidney and intestine) table(1). were cultivated on Brain heart infusion broth at 37 $^0$C for 24 hrs. one ml from each tube was spread over a dry surface of BP agar plate. All plates were incubated at 37$^0$C for 24-72hrs and examined daily for bacterial growth.

2-The suspected colonies were examined for their colonial character, hemolytic activity on 5% Sheep blood agar, microscopical examination and biochemical character according to ***(Quinn et al.,1994)***

**4-Antibiotic susceptibility testing by disc diffusion method.**

The susceptibility to antibiotic was tested according to the procedures of NCCLS 2007 using disk diffusion technique. The susceptibility of the strain was determined according to inhibition zone diameter.

**5-DNA Extraction using QIA amp kit**

After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100ºC for 20 minutes. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and the steps were carried out according to ***(Shah et al., 2009)***

**6- PCR Amplification for MRSA gene (Pournajaf*et al.*, 2014):**

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The genomic DNA was amplified by PCR as a volume of 1 µL (0.5 µg) in a final volume of 25 µL PCR mixture containing 10 µL of 2×Master Mix, including 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L dNTP, and 1.25

IU *Taq* DNA polymerase, 0.7 µL of 0.8 µmol/L each primer and 12.6 µL of sterile distilled water. The thermal cycling protocol for PCR was comprised 95 °C for 3 min, followed by 33 cycles of 94 °C for 1 min, 53 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 6 min.

The PCR products were electrophoresed in 2% of agarose gel electrophoresis stained with ethidium bromide and visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

**7- PCR amplification for some antibiotic resistance genes as *erm* A gen , *aac (6)-aph (2*) genes and *van* A of *S.aureus (Perez et al., 2001):***

The multiplex PCR was performed in a total volume of 25 µL containing 80 mM $MgCl_2$, PCR buffer, 3.5 mM DNTP mix (Fermentas), 14L (10 p.mol)of each of primer and 1 unit of Taq polymerase with 1 µl of bacterial suspension.

**3-Result and discussion:-**

In the present study, as showen in table (1) 430/750 isolates from fresh water Cat fish samples (Clarias garipeneaus) were identified as *S. aureus* by colonical characters, microscopical examination and biochemical tests according to ***Quinn et al. (1994)***. In regarded to total prevalence of *S.aureus* in infected fishes, the present study recorded that 100% of infected fish were positive for Staphylococcal infection, these results go in barrel with ***Haifaa (2014)*** who recorded 100% from isolates (Carp, Cat fishes)were identified as Staphylococcal spp. But these results were relatively higher than those recorded by ***Noha (2015),*** who reported that 46.6% of infected Cat fishes were positive for Staphylococcal infection. ***Narges et al. (2013)*** who recorded that, *Staphylococcus aureus* were isolated in 37.2 % from 35 samples of schizothorax Zarudnyi. ***Bujjamma and Padmavath (2015),*** who reported high prevalence of *Staphylococcus aureus* (24.47%) in fishes of Guntur market. ***Daniel et al. (2010)*** recorded that, high prevalence of *Staphylococcus aureus* were isolated from retail outlets in Glaicia. But ***Vieira et al. (2001)*** isolated *Staphylococcus aureus* in percent of 30%from fresh fish**.** ***Eklund et al. (2004)*** isolated *Staphylococcus aureus* in percent of 20% from fresh fish and fish fillets (Cynoscion leiarchus). Disagreed with ***Athanassopoulou et al. (1999).*** who reported that the total prevalence of *Staphylococcus epidermidis* among diseased Puntazzo in marine aquaculture systems in Greece was 10%. The incidence of *Staphylococcus aureus* reported in the present study was relatively higher than those of freshwater fishes reported by ***Ali (2014) and El-olemy et al. (2014).*** The high incidence of

*Staphylococcus aureus* in the examined samples could indicate unhygienic conditions because the product contamination could be the results of a combination of improper handling, improper storage and cross contamination ***(Simon and Sanjev, 2007).***

The present result showed that, *Staphylococcus aureus* isolated in percent of 100%from skin and gills, 40% from liver, 25% from intestine. As in table (1).Which agreed with ***Oghondeminus (1993)*** who reported, fish intestine were found to be heavy loaded with large numbers of Staphylococcus species. And disagreed with ***Udeze et al. (2012)*** who found that *Staphylococcus aureus* present in skin and absent in their intestine of Cat fishes samples. These results go in parallel with ***Haifaa (2014)*** *and* ***Noha (2015),*** who isolated *Staphylococcus aureus* from Skin, gills, liver, and intestine of infected Cat fishes.

The skin part of fish was exhibit large number of bacteria and this is because the skin of fish is usually in direct contact with water**.** These results agreed with ***Ali and Hamza (2004)*** who recorded that, sixty hand swabs from fish sellers (35%) gave positive results for *Staphylococcus aureus,* fourty house wives hand swabs, (37.5%) gave positive for *Staphylococcus aureus*, this mean that spoilage bacteria penetrate only slowly from the skin and the gut, although when spoilage is well advanced their presence may be all too obvious in the term of unattractive bacterial slime and repulsive odors, respectively Just below the skin. Bacterial fish diseases and infection are very common and are one of the most difficult health problems, to deal with bacteria can enter the fish body through the gills or skin or it can stay on the surface of the fish body ***(Douglas and Hamel 2007)***.

It has reported that, the gills act as the first barrier to combat infection by trapping and sloughing off pathogen, since they contain numerous mucous cells, mast cell and leukocytes. ***(Corrales et al., 2010, Pan et al., 2010).***

In this study,430 tested samples were collected from different organs of infected cat fish. All isolates cultivated on BPA which is selective media for *S. aureus* were found black dot colony surrounded by hallow zone.

These positive colonies were tested for further biochemical characteristics, (Morphological, culture character, biochemical identification, coagulase production. *mec* A gen to determine methicillin resistant *S.aureus* **(MRSA**) by PCR and determine antibiotic inhibiting gen. These positive colonies were Gram +ve cocci arranged in cluster, to produce bubbles from H2O2 due to production of O2 by the action of catalase enzyme produced by them. When urea hydrolysis test was performed with these isolates, they were found to form red to pink

colonies. It may be due to liberation of ammonia, the product of urea hydrolysis by the enzyme urease secreted from their cell wall. Production of coagulase is an important phenotypic feature used worldwide to identify *S.aureus* reported by ***Da Silva and Da Silva (2005) and Aslantas et al. (2007).*** Coagulase reacts with prothrombin in blood and causes blood clot by converting fibrinogen to fibrin. The coagulase is tightly bounded to the surface of *S.aureus* and coats its surface with fibrin upon contact with the blood. So the result of coagulase test confirmed that those bacterial isolates which give positive results were *S. aureus*.

In these results isolated *S. aureus* were sensitive to Amikacin, Amoxicillin, Ampicillin and Ciprofloxacin; intermediated sensitive to Erythromycin and Vancomycin while resistant to Gentamicin, Methicillin and Neomycine. These results go in parallel with ***Daniel et al. ( 2010)*** who recorded that, *S. aureus* isolated from products purchased from retail outlets were susceptibil to a range of antibiotics (Cephalothin, Clindamycin, chloramphenicol, erythromycin, gentamycin, oxacillin, penicillin G, tetracycline, vancomycine, methicillin , ciprofloxacin and trimethoprim–sulfamethoxazole ). Disagreed with ***Albuquerque et al. (2007)*** who reported that**,** All *S. aureus* isolates from fish stalls and hands, nasal, oral cavities of fish handlers of the Mucuripe Fish Market were resistant to Ampicillin and 44 % were multi-drug resistant. And disagreed with ***Haifaa (2014)*** who recorded that, most species of Staphylococcus isolated from fresh water fish, isolates were resistant to Ampicillin but sensitive to Ciprofloxacin. Partial agreed with ***Al-Obaidy and Al-Dabahg (2011)*** who recorded, most species of Staphylococcus were sensitive to Ciprofloxacin.

Nucleic acid amplification by PCR has applications in many fields of biology and medicine, including the detection of viruses, bacteria and other infectious agents. In the present study, oligonucleotide primer set were synthesized which recognized sequences of the MRSA **(***mec***A,** *co***A,** *sp***A )** genes.

PCR approaches using (*co* **A**, *sp* **A**, *mec* A) genes targeted primers, have proved specific and combined with growth techniques may improve detection of both *S.aureus* and MRSA in different types of food ***(Alarcon et al., 2006*** *and* ***Hata et al., 2006).***

In the present study, *mec* **A** gen for characterization of Methicillin Resistant *S. aureus*(MRSA) were detected in re-isolated sample at 533bp. These results agreed with ***Noha (2015)*** who detected that, **mec A** gene at 310 bp from isolates identify as *S. aureus* isolated from infected Cat fish. ***Zamri-Saad et al. (2010)***. Who mentioned the report that, first documented finding of

presence of MRSA in an aquatic animal cage-culture tilapia (***Oreochromis niloticus*****). *Tadashi Shimamoto (2012)*** who isolate MRSA from retail ready-to-eat raw fish in Japan. Nevertheless, antibiotic-resistant *S.aureus* has been isolated from cases of fish handlers disease, therefore, the issue of preventing antibiotic-resisrant *S.aureus,* including MRSA suggests regular inspection of Tilapia and perhaps other fish at points of sale is necessary ***(Albuquerque et al., 2007).*** But disagreed with ***Daniel Vázquez-Sánchez et al. (2010)*** who reported that, no MRSA was isolated from fishery products and no isolate carried the *mec* **A** gene, though intermediate resistance to methicillin was detected in all isolates from fishery products marketed in Galicia (Northwest Spain).

In the present study, gave PCR product with specific band at 139 bp which confirmed the presence of *erm* **A** gen in 5 isolates, at 174 bp confirmed the presence of *aac (6)-aph (2)* genes in 3 isolates, at 1030 bp confirmed the presence of *van* **A** gen in 2 isolates. These results confirm presences of antibiotic resistance genes to erythromycin, vancomycin and gentamycin. These results disagreed with ***Tadashi Shimamoto (2012)*** who recorded that, Antibiotic resistance genes that conferm resistance to aminoglycosides, tetracyclines, β-lactams, macrolides, lincosamides and streptogramin B (MLS(B)) antibiotics were detected isolated samples from retail ready-to-eat raw fish in Japan

**Table(1);** *Total incidence of S.aureus among examined different organs and tissue of infected Cat fish;-*

| Organ | Total number of examined sample | Positive / 150 | | Negative / 150 | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| **gills** | 150 | 150 | 100 | 0 | 0 |
| **ulcers** | 150 | 150 | 100 | 0 | 0 |
| **liver** | 150 | 60 | 40 | 90 | 60 |
| **kidney** | 150 | 45 | 30 | 105 | 70 |
| **intestine** | 150 | 25 | 17 | 125 | 83 |
| **total** | 750 | 430 | 57 | 320 | 42.6 |

*Table (2): Interpretation of antibiotic sensitivity test for S.aureus isolates .*

| Antimicrobial agent | Symbol | Disc conc. | Diameter of inhibition zone (m m) | | | Diameter | Interperet ation |
|---|---|---|---|---|---|---|---|
| | | | R | I | S | | |
| **Amikacin** | AK | 10 µg | ≤ 11 | 12-13 | ≥ 14 | **19** | **S** |
| **Amoxicillin** | Ax | 25 µg | ≤ 19 | | ≥ 20 | **22** | **S** |
| **Ampicillin** | Am | 10 µg | ≤ 11 | 12-14 | ≥ 15 | **21** | **S** |
| **Ciprofloxacin** | CIP | 5 µg | ≤ 15 | 16-20 | ≥ 21 | **22** | **S** |
| **Erythromycin** | E | 15 µg | ≤ 13 | 14-22 | ≥23 | **18** | **I** |
| **Gentamicin** | GM | 10 µg | ≤ 12 | 13 -14 | ≥ 15 | **12** | **R** |
| **Methicillin** | ME | 5 µg | ≤ 9 | 10-13 | ≥ 14 | **8** | **R** |
| **Neomycin** | N | 30 µg | ≤ 12 | 13-16 | ≥ 17 | **10** | **R** |
| **Vancomycin** | VA | 30 µg | ≤ 9 | 10 -11 | ≥ 12 | **10** | **I** |

Fig .(1): Diseased Cat fish showing skin lesion at tail

Fig. (2): ***S. aureus*** disc diffusion sensitivity pattern showing sensitive to Amikacin, Amoxicillin, Ampicillin and Ciprofloxacin while resistant to Gentamicin, Methicillin and Neomycine

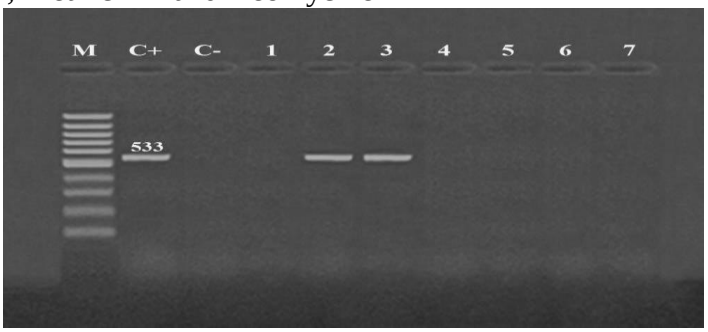


Fig. (3):Agarose gel electrophoresis of PCR amplification products of mecA gene for characterization of Methicillin Resistant *Staphylococcusaureus* (MRSA).

**Lane M :**100 bp ladder as molecular size DNA marker.

**Lane C+:** Control positive *S. aureus* for *mec* A gene.

**Lane C- :** Control negative.

**Lanes 2 and 3:** Positive *S. aureus* strains for *mec* A gene.

**Lanes 1, 4, 5, 6 & 7:** Negative *S. aureus* strains for *mec* A gene.

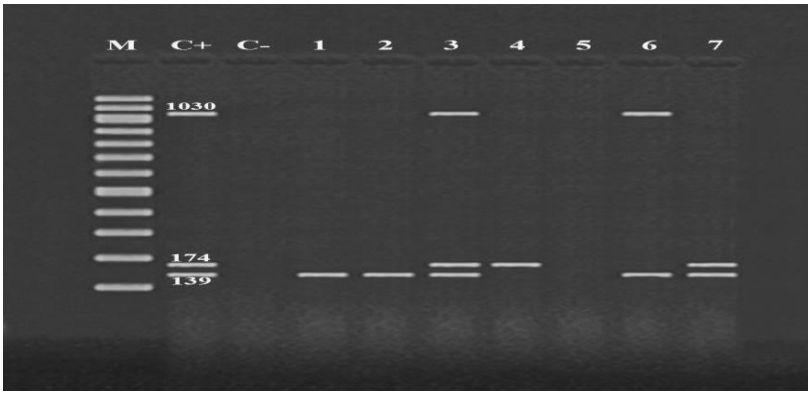


**Fig. (4):Agarose gel electrophoresis of multiplex PCR of *erm* A (139 bp), *aac (6)-aph (2)* (174 bp) and *van* A (1030 bp) as antibiotic resistance genes of *S. aureus*.**

**Lane M :** 100 bp ladder as molecular size DNA marker.
**Lane C+:** Control positive for *erm* A, *aac (6)-aph (2)* and *van* A genes.
**Lane C- :** Control negative.
**Lanes 1 and 2:** Positive *S.aureus* strains for *erm* A gene.
**Lane 3 :** Positive *S.aureus* strain for *erm* A, *aac (6)-aph (2)* and *van* A genes.
**Lane 4 :** Positive *S.aureus* strain for *aac (6)-aph (2)* gene.
**Lane 6 :** Positive *S.aureus* strain for *erm* A and *van* A genes.
**Lane 7 :** Positive *S.aureus* strain for *erm* A and *aac (6)-aph (2)* genes.
**Lane 5 :** Negative *S.aureus* strain for *erm* A, *aac (6)-aph (2)*and *van* A genes.

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**الملخص العربى**

يعتبر عدوى المكورات العنقودية احد أنواع البكتيريا الموجبة الواسعة الإنتشار و التى تصيب الاسماك بالجلد و فى الغدد و الأغشية المخاطية , حيث تعتبر الجروح و التهابات الجلدية موبئ للعدوى . حيث يعد من اهم ثالث مسبب بكتيرى لتسمم الغذائى للانسان عن طريق الاسماك، علاوة على أهميته الكبرى فى الأسماك وتأثيره على الثروه السمكية. ولذلك فقد تناولت الدراسة عزل وتصنيف المكورات العنقودية فى عينات أخذت من أسماك مصابه إكلينيكا ، و بعمل دراسة لتحديد ضراوة العترات المعزولة على الاسماك ,اظهرت التهابات فى الجلد، فقر دم فى الخياشيم، انتفاخ فى القناة الهضمية وبالكشف عن بعض الجينات المسببة لمقاومة الميكروب للمضادات الحيوية بإستخدام تفاعل البلمرة المتسلسل تم عزل( *van* A, *mec* A, *erm* A، *aac (6)-aph (2*) genes ) واخيرا بإجراء اختبار الحساسية لميكروب المكورات العنقودية المعزولة من الاسماك وجد أنه اظهر حساسيه لجميع المضادات الحيوية المستخدمة. ماعدا المثيثلين والنيوميسين والجنتاميسين.