Molecular typing and evaluation of Sidr honey inhibitory effect on virulence genes of MRSA strains isolated from catfish in Egypt

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Abstract: Fish represent a worldwide significant source of animal protein. In order to investigate the prevalence of MRSA in catfish as well as the inhibitory effect of Sidr honey on virulence genes of MRSA, fish were collected from Bahr Elbaker canal at Sharkia Governorate, Egypt. Swab samples were collected under complete aseptic conditions from internal organs (pancreas, liver, kidney and intestine), gills and skin then subjected to bacteriological examination. A total of 70 *S. aureus* strains were isolated from catfish, out of them 15 (21.42%) strains were identified as MRSA as a first record in Egypt. PCR was used for detection of *meca*, *coa* and *spa* genes in the isolated MRSA strains before and after the exposure to sidr honey. Before exposure to sider honey, all the selected MRSA strains showed positive results for *meca*, *coa* and *spa* genes with specific amplicon size of 310 bp, 430 bp and 226 bp, respectively. After exposure to sidr honey, MRSA strains showed inhibition of *coa* and *spa* genes, but has no effect on *meca* gene. In addition, scanning electron microscopy was used for detection of the morphological characters of MRSA strains before and after treatment with sidr honey. After exposure of MRSA strains to 30% (w/v) Sidr honey for 48 hours, cells surfaces were observable irregular with the appearance of cell debris. In conclusion, MRSA strains could be isolated from fresh water catfish in Egypt which may be attributed to the contamination of water and fish food. Sider honey showed a significant inhibitory effect on the growth of isolated MRSA strains. Moreover, it could inhibit *spa* and *coa* genes. SEM is a valuable tool revealing the abnormal morphological changes that take place in MRSA strains after exposure to Sidr honey.

Keywords: Catfish- Sidr honey - MRSA- PCR- Electron Microscopy.

INTRODUCTION

Fish meat has a high nutritive value as being rich in vitamins, minerals, proteins, and unsaturated fatty acid, it is also one of the most important food stuff as they may be the cheapest supply of animal protein over the last years (Abdulla, 2003).

The prevalence of multidrug resistant of *Staphylococcus* spp. increased during the last several years. Highly pathogenic *S. aureus* strains which yielding powerful enterotoxins are mainly accountable for food poisoning in man with high morbidity rate (Albuquerque, 2007). The major source of *S. aureus* and MRSA contamination in the aquatic environment are entertainment bathers, unprocessed wastewater and urban drainage (Plano *et al.*, 2011).

The restrained improvement of antimicrobial agents in recent times has compounded the situation (Fischbach and Walsh, 2009) and increased the need to search for alternative medications to substitute antibiotics. The chemical analysis of Sidr honey proved that it contains different antimicrobial substances with potent antibacterial activity against the highly virulent pathogens that are incriminated in wound suppuration. (Maddocks *et al.*, 2012). Honey differs from other desserts because of enzymatic constituents such as invertase, diastase and glucose oxidase. Moreover, honey is effective against untreated infections with traditional drugs. Manuka honey is known to has the ability to prohibit the biofilm occurrence and to destruct the existed *S. aureus* biofilms in vitro (Merckoll *et al.*, 2009). Protein A gene of MRSA strains has been inactivated when cells treated with Manuka honey (Jenkins *et al.*, 2011).

This study was aimed to investigate the prevalence of MRSA in catfish *(Clarias gariepinus*) in Egypt as well as evaluation of the inhibitory effect of sidr honey on virulence genes *(meca, spa* and *coa*) of the isolated MRSA strains. In addition, studying the inhibitory effect of Sidr honey on the morphological characters of MRSA strains by Scanning Electron Microscopy.

MATERIALS AND METHODS

Fish samples

A total of 150 of Catfish *(Clarias gariepinus*) were collected from Bahr Elbaker canal, Sharkia Governorate,

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Egypt. Swab samples were collected under complete aseptic conditions from internal organs (pancreas, liver, kidney and intestine), gills and skin for bacteriological examination.

Honey samples

Sidr honey samples obtained from five different sources from Egypt (E), Libya (L), Yemen (Y), Pakistan (P) and Saudi Arabia (S). Samples were diluted in various dilutions using physiological saline. Honey samples were placed in a dark place at (25°C).

Isolation and identification of S. aureus

The collected samples were inoculated on mannitol salt agar, Baird parker medium and 7% sheep blood agar. All plates were incubated at 37°C for 24-48 hours and examined daily for bacterial growth. The identification of bacterial colonies was based on the morphological characters (using Gram's stain), cultural characters as well as biochemical reactions using methods described by (Quinn *et al*., 2002).

Methicillin antibiotic susceptibility testing

The sensitivity to methicillin was estimated using disc diffusion technique according to the methods described by NCCLS (2007). The susceptibility was determined according to the diameter (mm) of the inhibition zone.

Determination of (MIC) of Sidr honey against MRSA strains

MIC detection was carried out by using broth dilution method acc. to (NCCLS, 2007). lowest concentration which inhibits the bacterial growth is known as the MIC (mg/ml).

Determination of (MBC) of Sidr honey against MRSA strains (NCCLS, 2007)

After the determination of the MIC, the number of bacterial cells was counted in each broth tube that showed no visible turbidity after overnight incubation and was compared with the number of bacterial cells in the initial suspension.

Molecular characterization of MRSA strains before and after exposure to sidr honey

1-Extraction of DNA from MRSA strains by using boiling method as described by Heuvelink *et al.* (1998)

2-Polymerase chain reaction

The extracted DNA samples were mixed (in 50µl reaction volume in a 0.2ml PCR tube) with 1.25 unit of taq DNA polymerase, PCR buffer (50mM Pottassium chloride, 10mM tris -HCl, 1mM $MgCl_2$), dNTPS (200uM of each) (dATP, dGTP, dCTP and dTTP) and 50 picomoles of each primer. Thermal cycling was carried out using (Biometra) thermal cycler (Eid *et al.,* 2016: Elfeil *et al.,* 2016). A positive control was given by the Department of Microbiology, Faculty of Veterinary Medicine, Suez Canal University, Egypt. A negative control reaction with no template DNA was also used. Table (1) illustrated Primers used in PCR assay.

-PCR recycling conditions

(1)- *meca* gene according to McClure *et al.* (2006); 39 times (94°C for 1 min.; 58°C for 1 min.; 72°C for 1 min)
(2)- *coa* gene according to Iyer and Kumosani (2011): 30 times (95°C for 1 min.; 55°C for 1 min. ; 72°C for 2min.)
(3)- *spa* gene according to Wada *et al.* (2010): 30 times (94°C for 1 min. ; 60°C for 1 min. ; 72°C for 1 min.).

3- Screening of PCR products

The amplified PCR products were detected by electrophoresis using a 1% agarose gel and 100-bp DNA ladder (QIAGEN). Gels were examined under UV trans-illuminator (UVP, UK) and then were photographed (Elfeil *et al.,* 2012; Abouelmaatti *et al.,* 2013).

Preparation of cells for electron microscopy

It was as carried out according to the procedures of Milani *et al*. (2007).

RESULTS

Prevalence of MRSA strains isolated from cat fish

Results are shown in Table (2) revealed that the prevalence of *S .aureus* strains isolated from fresh water catfish was (46.66%) (n = 70), out of them 15 strains (21.42%) were identified as MRSA strains.

The MIC and MBC of different honey types against the isolated MRSA strains

The MIC and MBC of different honey types against MRSA strains isolated from catfish are shown in Table (2), where the MIC of different Sidr honey types was 40 (w/v) except the MIC of Sider honey (E: Egypt) was 60 (w/v). On the other hand, the MBC of different Sidr honey types was 50 (w/v) except the MBC of Sider honey (E: Egypt) was 70 (w/v). (W/v: Weight by volume)**.**

Molecular typing of virulence genes of MRSA strains before exposure to Sidr honey

Regarding the molecular typing of virulence genes of MRSA strains isolated from catfish before exposure to Sidr honey, as shown in figs. (1.a, b, c) all the selected MRSA strains showed positive results for *meca*, *coa* and *spa* genes with specific amplicon size of 310 bp, 430 bp and 226 bp, respectively.

Molecular typing of virulence genes of MRSA strains after exposure to Sidr honey in vitro

Regarding the effect of Sider honey on virulence genes of the isolated MRSA strains as shown in figs. (figs. 2a,b,c), the results revealed that *meca* gene is not affected by Sider honey (fig. 2a), otherwise, both *coa* gene and *spa*

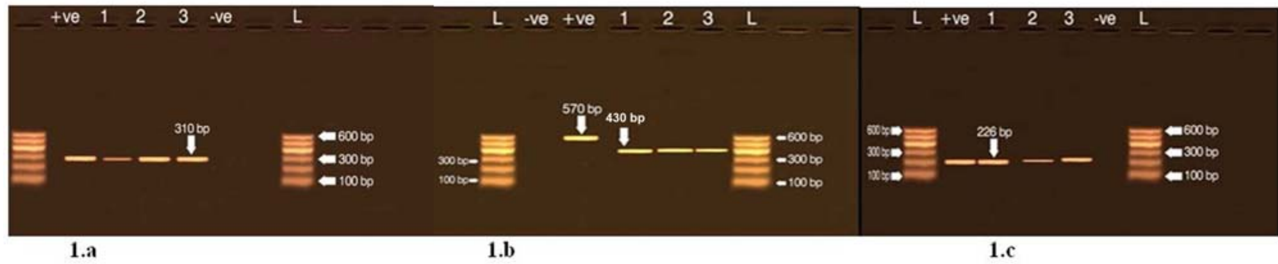

Fig. 1: Electrophoretic pattern of PCR products of MRSA *meca*, *spa* and *coa* genes before exposure to Sidr honey.
a: L: 100bp DNA ladder, +ve: positive control, -ve: control negative, Lanes 1, 2, 3, +ve for *meca* gene (310bp).
b: L: 100bp DNA ladder; +ve : positive control , -ve: control negative, Lanes 1,2,3, +ve for *coa* gene(430 bp).
c: L: 100bp DNA ladder, +ve: positive control , -ve: control negative, Lanes 1, 2, 3, +ve for *spa* gene (226 bp).

Fig. 2: Electrophoretic pattern of PCR products of MRSA *meca, spa* and *coa* genes isolated from catfish after exposure to Sidr honey *in vitro.*
a: L: 100bp DNA ladder, +ve: positive control,-ve: control negative, Lanes 4, 5, 6, 7, 8 +ve for *meca* gene (310 bp).
b: L: 100bp DNA ladder, +ve : positive control , -ve: control negative, Lanes 4,5,6,7,8 -ve for *coa* gene.
c: L: 100bp DNA ladder, +ve: positive control, -ve: control negative, Lanes 4, 5, 6, 7, and 8 -ve for *spa* gene.

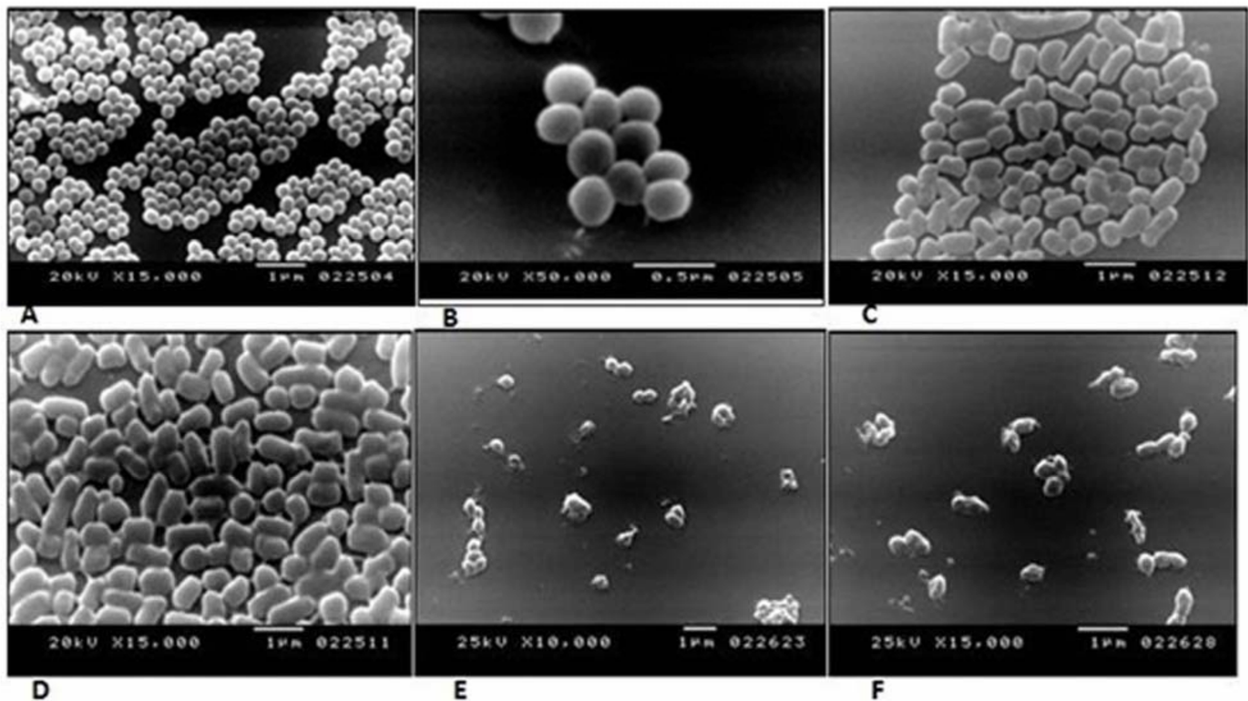

Fig. 3a, b: SEM micrograph of untreated cells of MRSA at x15,000 and x50,000 magnification.
c, d: SEM micrograph of MRSA cells exposed to 30% (w/v) sider honey after 24 hours at x10,000 and x15,000 magnification.
e, f: SEM micrograph of MRSA cells exposed to 30% (w/v) sider honey after 48 hours at x15,000 magnification.

Fig. 3a, b, c, d, e and f: SEM micrograph of untreated and treated cells of MRSA

Table 1: list of primers used for PCR assay:

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)
meca-FP		GTAGAAATGACTGAACGTCCGATAA	310 bp
meca-RP	*meca*	CCAATTCCACATTGTTTCGGTCTAA	
Coagulase-FP	*coa*	ATAGAGATGCTGGTACAGG	Four different bands may be detected 630; 350; 430; 570 bp
Coagulase-RP		GCTTCCGATTGTTCGATG C	
spaF5		TCAACAAAGAACAACAAAATGC	
spaR8	*spa*	GCTTTCGGTGCTTGAGATTC	226 bp

(1)- *meca* gene, according to (McClure *et al.*, 2006) ; 39 times (94°C for 1 min.; 58°C for 1 min.; 72°C for 1 min)
(2)- *coa* gene, according to (Iyer and Kumosani, 2011): 30 times (95°C for 1 min.; 55°C for 1 min.; 72°C for 2min.)
(3)- *spa* gene according to (Wada *et al.*, 2010) : 30 times (94°C for 1 min.; 60°C for 1 min.; 72°C for 1 min.)

Table 2: Number of MRSA strains isolated from catfish and (MIC) and (MBC) of different Sidr honey types:

No. of examined samples	No. of *S.aureus*	% of *S.aureus*	No. of MRSA	% of MRSA	Honey samples	MIC	MBC
150	70	46.66	15	21.42	L	40 (w/v)*	50 (w/v)
					E	60 (w/v)	70 (w/v)
					P	40 (w/v)	50 (w/v)
					S	40 (w/v)	50 (w/v)
					Y	40 (w/v)	50 (w/v)

(W/v)*: Weight to volume

genes were inhibited after the exposure to different types of Sidr honey (fig. 2b, c).

Scanning electron microscopy

SEM micrographs of control cells of MRSA strains incubated with broth only showed regular, smooth surfaces with spherical grape-like clusters (figs. 3a, b). After 24 hours exposure to 30% (w/v) Sidr honey, slightly elongated cells could be observed (figs. 3c, d).

After exposure to 30% (w/v) Sidr honey for 48 hours, cells surfaces were observable irregular with the appearance of cell debris, indicating the occurrence of cell lysis in some bacterial cells (fig. 3.E,F).

DISCUSSION

S.aureus has been reported as the third main causative agent of food borne disease transmitted by fish and fish products in the European Union (Authority, 2009).

Regarding the results shown in table (2), the percentage of *S.aureus* isolated from the examined catfish was (46.6%) (n=70), out of them 15 strains (21.4%) were identified as MRSA strains. It is the first record of isolation of MRSA strains from catfish in Egypt; these results are in agreement with those obtained by Hammad *et al.* (2012). The fecal pollution of the aquaria is the major source of *S.aureus.* Many studies insured that *S.aureus* could be found in fresh and brackish water fish globally (Abdel-Gawad, 2012).

During the recent years, a great interest has been shown to exploring natural antimicrobial such as honey. The antimicrobial activity of sidr honey has been evaluated using different methods with special reference to the MIC and the MBC. In the present study, the MIC and MBC of sidr honey were determined against MRSA strains indicating that there is no significant difference in sensitivity. Moreover, the different types of sider honey showed an inhibitory activity ranged from 40% to 60% (w/v) depending upon source, geography and place of honey, the present results are supported by those obtained by (Alem, 2013). This difference in the antibacterial activity of honey may be due to the type of honey or the source of the nectars (the flowers from which bees collect nectar to produce the honey). The bactericidal activity of Sidr honey is mainly due to H2O2 content, the elevated osmotic pressure, the low water content and the antibacterial biologically active substances (Molan, 1992).

PCR amplification of 3 virulence genes (*meca*, *spa* and *coa*) in the isolated MRSA strains was applied before and after exposure to 5 different types of sider honey (Yemen, Pakistan, Saudi Arabia, Egypt and Libya).

In this study, all the selected MRSA strains showed positive results with *meca*, *coa* and *spa* genes before exposure to Sidr honey with specific amplicon size at 310 bp, 430 bp and 226 bp, respectively as shown in figs. 1a, b, c. After exposure to Sidr honey, as shown in fig. 2a, there is no effect on *meca* gene (no gene inhibition or no down regulation). On the other hand, as shown in fig.2b, c, all treated strains with sidr honey showed gene inactivation for both *coa* and *spa* genes. The result of *spa* gene is in agreement with those obtained by Faria *et al.*

(2008). Moreover, some genes were inactivated when MRSA exposed to Manuka honey (Jenkins *et al.*, 2011; Jenkins *et al.*, 2013). Undetected genes (*coa* and s*pa*) after exposure to all types of Sidr honey may be due to inactivation of genes or bacterial lysis.

Electron microscopy is an essential reliable tool used to observe the ultra structural details that take place in cells after exposure to particular substances. The characteristics of the bacterial morphology help in explaining of the effect of different agents on bacteria. In the present study, SEM was used to observe the morphological characters of the bacterial cells, before and after exposure to sidr honey. As shown in fig. 3c, d, After 24 hours exposure to 30% (w/v) sidr honey, slightly elongated cells had appeared (losing the normal spherical structure). After exposure to 30% (w/v) Sidr honey for 48 hours, cells' surfaces were became irregular and cell debris had appeared, indicating the occurrence of cell lysis in some bacterial cells (fig. 3e, f). These results are in agreement with those obtained by Ibrahim *et al.* (2013). The abnormal changes including the existence of holes and the cell wall retraction illustrated by Electron Microscope are mainly attributed to cell wall formation disorder. Cell death is mainly occurred due to the cell wall damage (Amnah, 2013).

CONCLUSION

MRSA strains could be isolated from fresh water catfish in Egypt, and this may be attributed to the contamination of water. Sider honey showed a significant inhibitory effect on the growth of MRSA strains. Moreover, it has the ability to inactivate or down regulate *spa* and *coa* genes. SEM is a significant tool revealing the morphological abnormal changes that take place in MRSA strains after exposure to Sidr honey. The potent antibacterial activity of Sider honey, suggesting the usage of honey as a natural agent for treatment of MRSA associated infections.

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