Sidr Honey Inhibitory Effect on Virulence Genes of MRSA Strains From Animal and Human Origin

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Abstract

In order to evaluate the effect of sidr honey on the presence of ***meca***, ***coa*** and ***spa*** genes as well as its antibacterial activities against MRSA strains. A total of (200) clinically mastitic milk samples and (170) samples (45 wounds, 30 sputum, 25 blood, 30 aspirates, 20 urine and 20 cerebrospinal fluids) were collected from human patients at hospitals in Sharkia Governorate. Five different sidr honey samples from {Egypt (E), Libya (L) ,Yemen (Y), Pakistan (P) and Saudi Arabia (S) } were used in this study . After the bacteriological examination of the collected samples, the percentage of *S.aureus* was 31% in mastitic cases and 52.9% in human patients, while the percentage of MRSA strains was found to be 22.58% and 44.4%, respectively. ***Meca, coa*** and ***spa*** genes were detected by PCR in MRSA strains before and after the exposure to sidr honey. The absence of **(*meca, coa*** and **s*pa***) gnes in MRSA strains after exposure to sidr honey was noticed. Briefly, sidr honey has an inhibitory effect on (mec, coa and spa) genes of MRSA.

Introduction

S. aureus is considered to be one of the most frequently prevailing food-borne pathogen worldwide. MRSA was first reported in 1961, two years after the introduction of methicillin for treatment of penicillin-resistant ***S. aureus*** infections ***(Enright et al, 2002).*** MRSA is emerging as a zoonotic and veterinary bacterial pathogen of public health importance.Despite the low occurrence of ***S. aureus*** and MRSA in companion animals, there is concern they may serve as a source of infection or re-infection for humans. In most cases, establishing the direction of transmission of MRSA between animals, humans, and the environment is not possible. Identical MRSA strains have been isolated from pets and their infected human caretakers, and animals participating as therapy dogs at assisted living facilities were shown to acquire MRSA during visitations to these settings but did not remain

persistently colonized ***(Ferreira, 2011).***

Sidr honey is made from bees who only feed on the nectar of the Sidr tree. The floral source of honey plays an important role on its biological properties ***(Molan, 2002).*** Recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, gram-positives and gram negatives. Honey has been known to possess antimicrobial properties, as well as wound- healing activity ***(Amnah, 2013).*** Wounds infected with MRSA have also been cleared of infection and healed by application of honey including a leg ulcer ***(Natarajan et al, 2001),*** cavity wounds ***(Dunford et al, 2000)*** and surgical wounds ***(Betts and Molan, 2001).*** The antibacterial activity of different honeys was studied by many authors ***(Kwakman et al, 2010 and Ahmed and Fyrouz, 2012).*** This study was planned to investigate the inhibitory effect of sidr honey on virulence genes of MRSA strains isolated from cases of clinical mastitis and human patients.

Material and Methods

Sampling:

A total of 370 samples were collected. Out of them 200 quarter milk samples were collected from clinically mastitic cows at different farms in Sharkia Governorate and 170 samples (45 wounds, 30 sputum, 25 blood, 30 aspirates, 20 urine and 20 cerebrospinal fluids) were collected from human patients at hospitals in Sharkia Governorate.

Isolation and identification of *S.aureus*: Samples were cultivated 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. Bacterial colonies were identified morphologically using Gram's stain as well as biochemically using methods described by ***(Quinn et al, 2002).***

Detection of MRSA strains using disc diffusion method:

The susceptibility to methicillin antibiotic was tested according to the procedures of ***NCCLS (2007)*** using discs diffusion technique. The susceptibility of the ***S. aureus*** strains was determined according to the size of inhibition zone.

Honey samples:

Five Sidr Honey samples were used in this study collected from local market of Egypt (E), Saudi Arabia (S) , Yemen (Y) , Libya (L) and Pakistan (P) . All honeys were kept at (23-25°C) in dark glass containers**.** Honey samples were diluted by physiological saline to different dilutions and non-diluted honey.

Gentic amplification of MRSA *meca*, *coa* and *spa*.genes:

1-Extraction of DNA from ***S.aureus*** strains by boiling method according to ***(Van et al, 1989).***

2-Polymerase chain reaction: DNA samples were tested [in 50 µl.

Reaction volume in a 0.2 ml PCR tube , containing PCR buffer] (50 mM Kcl , 10 mM tris - Hcl , 1mM $Mgcl_2$) each dNTPS (Deoxy nucleotide Triphosphate) 200 uM each (dATP , dGTP , dCTP and dTTP) , [Two primer pairs each at 50 picomol / reaction] and 0.5 of taq DNA polymerase . Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done.

(1)- *meca* gene according to ***McClure et al (2006):* 39cycles (94 ^{o}C for 1 min.; 58 ^{o}C for 1 min. ; 72 ^{o}C for 1 min)**

(2)- *coa* gene according to ***Iyer and Kumosani (2011):* 30 cycles (95 ^{o}C for 1 min.; 55 ^{o}C for 1 min. ; 72 ^{o}C for 2min.)**

(3)- *spa* gene according to ***Wada et al (2010):* 30 cycles (94 ^{o}C for 1 min. ; 60 ^{o}C for 1 min. ; 72 ^{o}C for 1 min.)**

3-Screening of PCR products: ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

Table (1): *The following were the primer sequences used in the study*

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)
***meca*-FP**	***meca***	**GTA GAA ATG ACT GAA CGT CCG ATA A**	**310bp**
***meca*—RP**		**CCA ATT CCA CAT TGT TTC GGT CTA A**	
Coagulase-FP	***Coa***	**ATA GAG ATG CTG GTA CAG G**	**Four different types of bands may be detected 630 bp 350 bp 430 bp 570 bp**
Coagulase-RP		**GCT TCC GAT TGT TCG ATG C**	
spaF5	***Spa***	**TCA ACA AAG AAC AAC AAA ATG C**	**226 bp**
spaR8		**GCT TTC GGT GCT TGA GAT TC**	

Results

Out of 200 milk samples examined from diagnostic clinical mastitis cows, 62 (31%) pure cultures of ***S. aureus*** isolates were obtained and (22.58%) methicillin resistant ***S. aureus*** while examined 170 human samples were collected from specimens 90 (52.94%) pure cultures of ***S. aureus*** were obtained and (44.4%) methicillin resistant ***S. aureus.*** In order to study the inhibitory effect of different sidr honey in virulence genes (***meca*, *coa*** and ***spa***) in MRSA, 7 MRSA strains were selected (3 from milk

and 4 from human). The selected strains were exposed to 5 different types of sidr honey samples.

Amplification of *meca*, *coa* and *spa* genes of MRSA strains before and after exposure to sidr honey:

Accordingly the 310 bp PCR product of the meca gene was identified in all MRSA strains (100%) before exposure to sidr honey (Photo.1).

After exposure to 30% sidr honey 4 (57%) MRSA strains showed inhibitory effect for ***meca*** gene fragment with sidr honey from (Saudi Arabia and Yemen) Photo2.

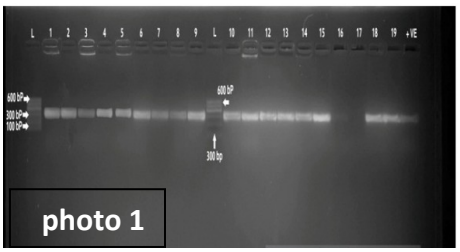
Accordingly, 600 bp PCR product of the ***coa*** gene was identified in all MRSA strains, except trains in lane 6 showed DNA fragment on 570 bp before exposure to sidr honey (Photo 3). Otherwise, after exposure to 30% sidr honey 6(85%) isolates showed inhibitory effect for ***coa*** gene fragment with sidr honey (Saudi Arabia, Yemen, Pakistan, and Libya) Photo (3-5) inhibited by honey samples.

MRSA strains were confirmed to have protein A through the amplification of the ***spa*** gene *(*226 bp). Accordingly most of the DNA fragment of 226 bp was amplified from all MRSA strains (Photo 6**)** .

On the other hand, MRSA strains exposure to sidr honey inhibited ***spa*** gene for two MRSA strains (lane 27 and 33) { Photo. 7 and 8}. The inhibited MRSA strains exposed to 30% sidr honey from Yemen and Saudi Arabia.

Table (2): *Number and percentage of S. aureus strains isolated from clinical mastitic milk samples and human patients*

Samples	No. of examined samples	No. of *S.aureus* strains	% of *S.aureus* strains	No. of MRSA	% OF MRSA
Quarter milk samples	200	62	31	14	22.58
Human patients	170	90	52.9	40	44.4
Total	370	152	41.1	54	35.5



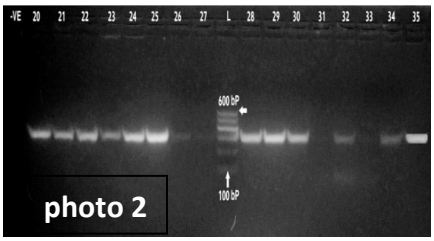
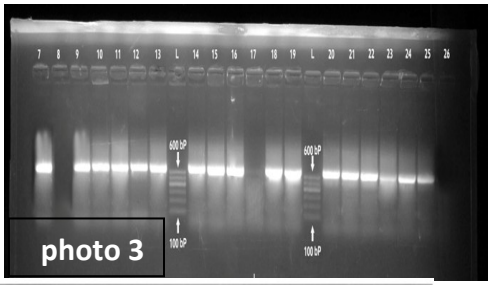
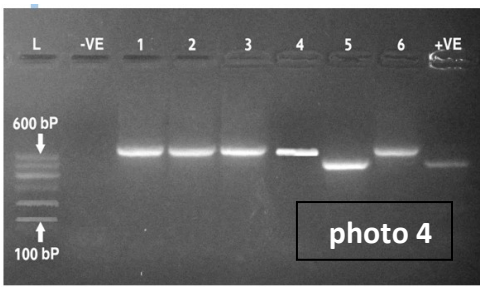
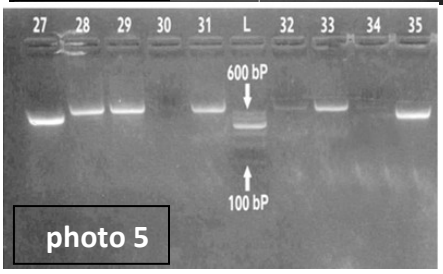


photo 1- 2 .Effect of different sidr honey with different concentration on *meca* gene in MRSA isolates .

Agarose gel electrophoresis showing representative PCR products after meca genes amplification. The lane (L): 100 bp DNA ladder . Lane 1- 7: untreated MRSA isolates .Lane 8-35 show PCR products of the *meca* genes of different strain exposed to sidr honey.

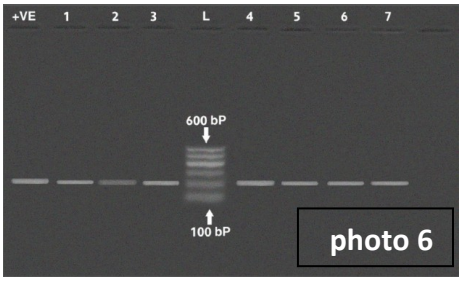


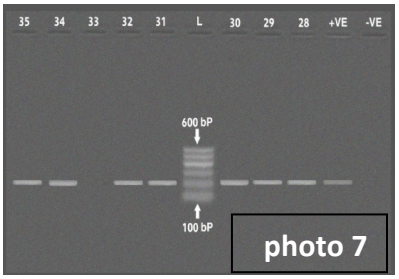




Photos 3- 5 .Effect of different sider honey with different concentration on *coa* gene in MRSA strains .

*Agarose gel electrophoresis showing representative PCR products after coa genes amplification. The lane (L): 100 bp DNA ladder. Lane 1- 7: untreated MRSA strains .Lane 8-35 show PCR products of the **coa** genes of different strains exposed to different sidr honey and honey concentration.*





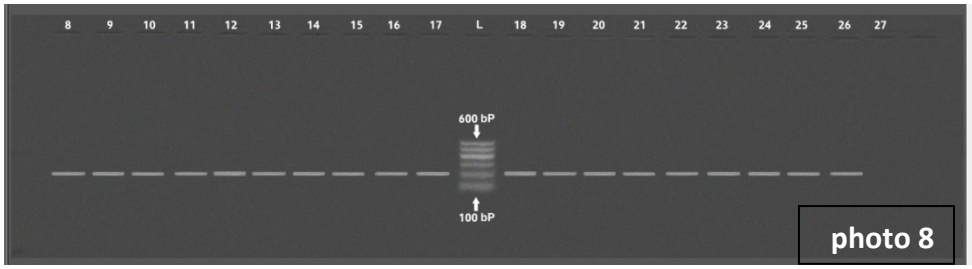


Photo 6- 8 .Effect of different sidr honey with different concentration on *spa* gene in MRSA isolates .

*Agarose gel electrophoresis showing representative PCR products after spa genes amplification. The lane (L): 100 bp DNA ladder . Lane 1- 7: untreated MRSA strains .Lane8-35 show PCR products of the **spa** genes of different isolates exposed to different sidr honey and honey concentration.*

Discussion

Recently there is a growing interest in exploring natural antimicrobial as honey bee**.** Our study examined the inhibitory effect of (5) sidr honey samples against of MRSA virulence genes.

PCR amplification of (3) different genes (***meca*****,** ***spa*** and ***coa*****)** in (7) MRSA strains was applied before and after exposure to (5) different types of sidr honey.

meca gene is responsible for the methiciliin resistance and confirmed the presence of MRSA in isolated strains .PCR amplification of ***meca*** gene for untreated strains(1-7) was positive (DNA fragment at 310 bp in Photo 1).our results are with agreement with those obtained by ***Langlois et al (1984)***. As shown in Photos 1 -2, sidr honey had an inhibitory effect on ***meca*** gene of (4) MRSA strains (17, 27, 31 and 33), this may lead to easy treatment of MRSA strains.

In our study, ***coa*** gene was detected in all untreated strains. The length of DNA fragment of ***coa*** gene is 600pb (Photo 3).our results agree with those obtained by ***(Akineden et al, 2001)*** . After exposure of MRSA strains to sidr honey, 6 (85%) strains (8,17,26,30,32 and 34) showed un detected ***coa*** gene as shown in (Photos 5 and 6), this agrees with results obtained by (***Cooper et al, 1999)***. Also Protein A was detected through the amplification of the ***spa*** gene ***(***226 bp). Accordingly most of the DNA fragment of 226 bp was amplified from all MRSA strains **(**Photo 6**)** . On the other hand, after exposure of MRSA strains to sidr honey, ***spa*** gene inhibited in (2) MRSA strains (lane 27 and 33) { Photo. 7 and 8}. ***Jenkins (2011)*** detected 16-fold decrease in the expression of UspA by treatment of MRSA and ***E.coli*** with manuka honey in honey-treated cells compared with control cells and UspA expression was confirmed by quantitative PCR.

Undetected genes (***meca, coa*** and ***spa***) in MRSA by PCR after exposure to different types of honey may be due to inhibition of genes or kill the bacteria as mentioned by ***(De, N et al, 2010)*** or may honey also prevent the formation of biofilms and to disrupt established ***Staphylococcal*** biofilms in vitro ***(Alandejani et al, 2009).***

Briefly, sidr honey has an inhibitory effect on meca, coa and spa genes in some MRSA strains that may limits its virulence as well as antibiotic resistance abilities.

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التأثير المثبط لعسل السدر على جينات الضراوة لعترات الميكروب العنقودى الذهبى المقاوم للميثيثيلين والمعزول من الحيوان والإنسان

من اجل دراسة تاثير عسل السدر الجبلي على تواجد جينات (meca ,coa, spa) وكذلك تاثيراته المضادة للبكتيريا على عترات الميكروب العنقودى الذهبى.المقاوم للميثيثلين ، تم تجميع ٢٠٠ عينة لبن من الأبقار المصابة بالتهاب الضرع و ١٧٠ عينة من المرضى بمستشفيات محافظة الشرقية. وتم استخدام ٥ انواع مختلفة من عسل السدر الجبلي في هذه الدراسة (المصري والليبي و اليمني و الباكستاني والسعودي) .بعد الفحص البكتريولوجي للعينات المجمعة وجد أن نسبة الميكروب العنقودى الذهبى المعزول من حالات التهاب الضرع كانت (٣١ %) ونسبته فى المرضى كانت (٥٢,٩ %) ، ووجد أن نسبة الميكروب العنقودى الذهبى المقاوم للميثيثلين المعزول من حالات التهاب الضرع (٢٢,٥٨%) ، ونسبته فى عينات المرضى كانت (٤٤,٤%). تم إجراء إختبار البلمرة المتسلسل لتحديد تواجد جينات (meca ,coa, spa) في عترات الميكروب العنقودي الذهبي المقاوم للميثيثلين قبل وبعد التعرض لعسل السدر. وأظهرت النتائج أن الجينات متواجدة فى جميع العترات قبل المعامله بعسل السدر.وبعد معاملة العترات بعسل السدر وجد أن له تأثير مثبط على هذه الجينات في بعض العترات.باختصار ان عسل السدر له تاثير مثبط على جينات (meca ,coa, spa) الخاصة بالميكروب العنقودي الذهبي المقاوم للميثيثلين.