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IMPACT OF ENVIRONMENTAL CONTAMINATION WITH MRSA IN CATTLE FARMS

By

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ABSTRACT

Staphylococcus aureus is an important hospital- and community-associated pathogen that can cause a wide variety of infectious diseases. Eighty two (34 %) *Staphylococcus* aureus were isolated from the different samples collected from randomly selected cattle and buffaloes farms in Kalubia governorate. The positive isolates tested against 4 antibiotics (Oxacillin, cefoxitin, amoxicillin clavulanic acid and ampicillin sulbactam) used in the study area, showed twenty one out of 82 (25.6%) to be resistant. PCR was also used to detect the gene *mecA* in 8 (38%) of the 21 MRSA. The highest % of mec A gene was recorded from soil samples, the highest % of positive MRSA S.aureus was recorded from hand swab samples (40%),soil samples(33.3%). The farm environment mainly soil and hand swabs unhygienic management procedures potentiate the survival of *S. aureus* (MRSA- *S. aureus*) and expression of mec A gene form rectal swab and soil samples.

Keywords:

Staphylococcus aureus, MRSA, Antibiotic resistance, *mec A.*

INTRODUCTION

Human and animal health is adversely affected by polluted environment. Livestock are exposed to the challenge of potential pathogens in their sheds, pens, parlours and brooders. The infective agents voided by diseased animals serve as source of infection to healthy stock. Domesticated animals are normally prone to disease. Microorganisms may interfere in animal health and food product obtained from them. Some of the pathogenic organisms, which may be transmitted to man and animals through sewage and farm refuge/surplus as *staphylococcus* aureus **(Singh *et al,* 2012).** *Staphylococcus aureus* is a gram-positive bacterium commonly found on the skin or mucous membranes of both humans and animals. It is associated with many diseases, from less serious skin problems to very serious infections such as bacteremia or pneumonia. *Staphylococcus aureus* is one of the most extensively studied bacteria of genus Staphylococci. *S. aureus* is both commensal and pathogen. *S. aureus* affects skin, soft tissues, bloodstream and lower respiratory tract. It also causes severe deep-seated infections like

endocarditis **(Schito,2006)**. *S. aureus* has been reported as most commonly isolated highly contagious pathogen recovered from bovine raw milk and infected mammary glands **(Tenhagen *et al.,* 2006; Haveri, 2008)**.Traumatized sites such as abrasions on teats, legs and navel, typically infected by *S. aureus*, are regarded as secondary sources of *S. aureus* causing mastitis. Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a significant public health problem in both human and veterinary medicine. Strains of *S. aureus* resistant to β-lactam antibiotics are known as Methicillin-resistant *Staphylococcus* aureus (MRSA). Overuse of antibiotics has been ascribed for MRSA emergence. MRSA in cattle first reported in 1972, MRSA got zoonotic importance when scientists suggested the possibility of cattle serving as reservoirs for human MRSA infection. Problems arise during the treatment, in case methicillin-resistant *S. aureus* (MRSA) strains occur **(Naimi *et al.* 2003)**. Methicillin resistance is caused by the acquisition of the *mecA* gene. This gene encodes an alternative penicillin-binding protein, called PBP2A, which has a low affinity for beta-lactam antibiotics **(Vanderhaeghen *et al.*,2010)**. The *mecA* gene is part of a large mobile genetic element called Staphylococcal Cassette Chromosome mec (SCCmec). MRSA are often multidrug resistant. Those microorganisms have been reported to resist most of the commonly used antibiotics like aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones **(Lee, 2003)**. Despite all the measures taken in livestock breeding, the incidence of *S. aureus* persists in the environment, in milk, on the animal's body, and also in humans **(Golding *et al.* 2010; Harrison *et al.* 2014).** Methicillin-resistant *S. aureus* strains were detected in humans and in animals, including those that are intended for the production of food **(Bardoň *et al.* 2012;Zutic *et al.* 2012; Kolář *et al*2008)**. The incidence of MRSA has been reported in cattle, horses, small ruminants, camels, poultry **(Stastkova *et al.* 2009; Köck *et al.* 2009; Alzohairy 2011; Harrison *et al.* 2014)**. MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans **(AVMA, 2014; Spoor *et al.,* 2013)**. MRSA colonization in cattle may be an occupational risk to the people in close contact with MRSA infected cattle viz. veterinarians, farmers, milkers and people working at slaughterhouses **(Paterson *et al.*, 2012; Juhasz- Kaszanyitzky, 2007).** The purpose of the cross-sectional study was isolation and identification of *Methicillin-Resistant S. aurues* (MRSA) in **dairy farms of** cattle and buffaloes in some Kalubia governorate villages.

The aim of the present research is to gain a better understanding of the impact of cattle production in Egypt on the environment in different rearing systems to acquire foundational

knowledge about the dangerous of this infectious microorganism on cattle and its reflection on the surroundings.

MATERIAL AND METHODS

Sampling:

Animal samples:

A total of 241 samples from cattle and buffaloes including 129 animals' rectal swabs, 82 milk,7 soil, 10 water and 13 human hand swabs were collected from smallholder's, Medium sized and Large sized farms village, Kalubia Governorate, Egypt (Table 1). The study was conducted during the period between August 2016 and September 2017. Rectal swabs were directly collected from animals using sterile swabs moistened in sterile buffered peptone water (BPW) (Oxoid, CM509) then inserted in 10 ml of (BPW) tubes for pre-enrichment at 37◦C for 24 hours. In addition eighty- two cattle and buffaloes' milk samples were aseptically collected and transferred into individual sterile screw capped bottles. Soil samples were also collected from surface to a depth of approximately 3 cm, using 13mm diameter sterile corn borer. Samples were placed into sterile 30 ml plastic bottles and Water samples up to 30ml in volumes were transported in sterile plastic bottles. All samples were subjected to laboratory examination under aseptic conditions in laboratory of Hygiene Department, Faculty of Veterinary Medicine, and Cairo University.

Human samples:

Hand swabs were taken from persons in contact with animals under aseptic condition. They were collected on sterile cotton swabs. The hands (Back and palm) were thoroughly swabbed, including the fingernails and between the fingers and then transferred to its sheath. All samples were carried on ice-box to be transferred with a minimum delay to the laboratory for bacteriological examination.

Isolation and identification procedures

Each sample (rectal swab, hand swab, milk and soil) inoculated in brain-heart infusion broth for pre-enrichment and incubated for 24 hours at 37ºC then all samples cultured onto the surface of Mannitol salt agar medium.

Except water samples by filtering suitable volumes through a gridded 0.45-, um membrane filter **(Sartorius Stedim Biotech)**. The filter was placed on mannitol salt agar. **(Silverton and Anderson, 1961).**

The plates were incubated for 24 - 48 hours at 37ºC before examination of characteristic colonies. *S. aureus* appears as yellow colonies with yellow halo.

Microscopic examination:

Films from the pure suspected colonies were stained by Gram's stain and examined microscopically. *Staphylococcus* is gram-positive cocci **(Cruickshank *et al.*, 1975).**

Dryspot Staphytect Plus is a latex slide agglutination test:

1. The latex reagents were brought to room temperature then mixing the latex reagent by vigorous shaking and expel any latex from the dropper pipette for complete mixing.
2. We dispense 1 drop of test latex onto one of the circles on the reaction card and 1 drop of control latex onto another circle.
3. By a loop, we pick up and smear the equivalent of 5 average-sized suspect Staphylococcal colonies (equivalent to 2–3 mm diameter of growth) onto a circle from a culture media plate then mixing this in the Control Latex reagent and Spread to cover the circle then discard the loop appropriately.
4. By using a separate loop proceed in the same way with the Test Latex.
5. We pick up and rock the card for up to 20 seconds and observing for agglutination under normal lighting conditions. Without using a magnifying glass.
6. When the test is completed dispose of the reaction cards safely into disinfectant.

Determination of susceptibility of *S. aureus* to antibacterial agents, which were:

4 antibacterial disks were used as shown in (Table 2). The disk diffusion technique was adapted according to **(Finegold and Martin 1982).** Subcultures from the isolates were prepared and the test was applied as follows.

Preparation inoculum: of standardized

Four or five colonies of similar morphology were transferred using a sterile loop to a tube containing 5 ml of brain heart infusion broth.The broth was incubated at 37ºC for 18-24 hours. The turbidity of the inoculated broth was then adjusted to match a McFarland 0.5 barium sulfate standard tube.

Inoculation of the tested plates:

A sterile cotton swab was dipped into the standardized bacterial suspension. The excess fluid was removed by rotating the swab with firm pressure against the inside of the tube above the fluid level. The swab was then used to streak the dried surface of a Mueller-Hinton plate in three different planes by rotating the plate approximately 60 degree each time to ensure an

even distribution of the inoculation. The plates were allowed to remain on a flat and level surface undisturbed for 3 to 5 minutes to allow the adsorption of excess moisture then the disks were applied.

Placement of disks:

With a fine-pointed forceps, the disks were placed on the inoculated plate and pressed firmly into the agar to ensure complete contact with the agar. The disks were distributed evenly in a manner such as to be no closer than 15 mm from the edge of the petridish and so that no two disks were closer than 24 mm from center to center. The plates were inverted and incubated at 37ºC for 24 - 48 hours.

Reading of the results:

After incubation, the degree of sensitivity was determined by measuring the easily visible and clear zone of inhibition of growth produced by diffusion of the antibacterial agent from the disks into the surrounding medium.

DNA extraction:

DNA was extracted from the bacterial colonies by boiling method. Bacterial strains were grown in brain-heart infusion broth at 37 ºC overnight. Organisms from 1.5ml growth were pelleted by centrifugation at 1200xg for 10 min. The bacterial pellet was resuspended in 150µl of sterile distilled water. The bacteria were lysed by boiling for 10 min in a water bath. The lysate was centrifuged again as before and the supernatant was used as template for polymerase chain reaction.

Genotyping of isolated *Staphylococcus aureus* strains (Asfour and Darwish 2011):

DNA samples were amplified in a total of 25µl of the following reaction mixture: 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix. For mec A, gene the PCR program was initiated with incubation at 94°C for 4 min followed by 35 cycle of 94°C for 1 min, annealing was done at 55°C for 1 min whereas, DNA extension at 72°C for 1 min. Final extension was done at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel

For mec A gene the primer used: (de Neeling *et al.* 1998).

F: CTTCCACATACCATCTTCTTTAAC

R: GTTGTAGTTGTCGGGTTTGG

RESULTS AND DISCUSSION

In Egypt, nearly 70% of the livestock animals are reared by small farmers, where few cattle and buffalo along with small ruminant animals are kept together in their houses to produce milk and other dairy products **(Aidaros, 2005)**. In current study, the total percent of *Staph aureus* isolated was 34% (82/241). These findings was agreeable with **Khan *et al* (2016)** who found that water used at dairy farms did not contain the organism **(Jakee *et al.,* 2008)** found that, the highest isolation rate was observed in human samples (36 %) where staphylococci were responsible for over 80 percent of the suppurative diseases encountered in medical practice. They added that they cause most suppurative infections of the skin but may also invade and produce severe infections, **Christiane *et al.* (2009)** who stated individuals who have contact with animal are more likely to be colonized with *S. aureus.* While such result disagreed with **Khan *et al* 2016** who reported that, the prevalence of *S. aureus* in hand's sample was 30 %. **Kluytmans and Struelens (2009)** found 20% of healthy human acts as carriers for *S. aureus* species, 30% acts as intermittent carriers while 50% were never infected with *S. aureus* species, **Sakwinska *et al.,* (2011)** reported 38% prevalence of *S. aureus* in farm workers. Various researchers identified *S. aureus* in the hand swabs of dairy workers **(Rasha, 2008 and Fadel and Ismail, 2009)**; their isolation rate was 64% and 60% respectively, Moreover, **Abdel-All *et al.* (2010)** identified *S. aureus* with the prevalence of 44.1% in the skin swabs of dairy workers in Aswan, Egypt. The reported high prevalence of *S. aureus* among dairy handlers highlights the great role played by the handlers as a source of milk contamination and considered as a good indicator for inadequate hygiene. So, these results may be attributed to improper hygiene and poor farm management practices contributed to the presence of *S. aureus* especially in those from the smallholder private farms. The overall prevalence of *S. aureus* in the examined fecal swabs was 31%. These findings did not agree with those of **Kang (2013)** with higher percentage (61.5) that isolated *Staph. aureus* from 8 out of 13 of cattle feces. Regarding the occurrence of *S. aureus* in the examined milk samples 29 out of 82 with the percentage 35% .Such result agreed with **Adwan *et al.,* (2005), D'Amico and Donnelly, (2010), Vyletèlova *et al.,* (2011) and Aqib *et al.*,(2017)** with percentages 36.9%, 30%, 35%, 38% respectively, While **Erskine *et al.,* 2002, Moon *et al.,* 2007 and Huber *et al.*,2010** isolated lowest prevalence from milk 0.6%, 5.6% and 1.4% respectively. On the contrary, higher percentages of *S. aureus* infection identified from

the examined milk samples at the following 64, 75, 65.6 and 68% **(Aisha *et al.,* 2002; Elici *et al.,* 2004; Rasha, 2008 and Oliveria *et al*., 2011)**. High frequency of *S. aureus* found in the examined raw milk could be attributed to the presence of keratinized cells in the teat canal of healthy cattle's which act as a reservoir for *S. aureus*, similar finding was previously reported by other researchers **(Zadoks *et al.,* 2002)**. Several other factors as poor animal management, improper cleaning of milk utensils, dirty udder, teat and unclean milkers hands could else be a potential causes **(Bonfoh *et al.,* 2003)**. Given that *S. aureus* is the main leading cause of clinical and subclinical mastitis, contamination of milk by *S. aureus* may come from the animals directly or from the surrounding unhygienic environment **(Jorgensen *et al.,* 2005).** Occurrence of MRSA from positive samples by applying antibiotic diffusion disc: (Table 4) showed that, the highest percent of positive MRSA - *S. aureus* was recorded in hand swab samples (40%), while the water samples didn't reveal the organism. Additionally, MRSA-*S.aureus* isolated from rectal, milk and soil samples with 20, 27.5 and 33.3 % respectively. From total samples of 82, the total percent of MRSA isolated was (25.6%). These findings was closely to **(Spohr *et al.,* 2011)** who isolated MRSA with prevalence rate is 16.7% in Germany while **(Kumar *et al.,* 2011)** isolated MRSA in India with prevalence 13.1% .A low prevalence of MRSA in bovine milk reported by **Huber *et al.*(2010)** with prevalence 1.4% (2 out of 142 *S. aureus* isolates) in Switzerland, 0.6% in Michigan **(Erskine *et al.,*2002)**,1.8% in Wisconsin **(Makovec and Ruegg, 2003)**,0.4% in Hungary **(Juhasz-Kaszanyitzky, 2007)** similar to **Paterson *et al*. (2012)** who found 7 MRSA isolates out of 1500 bulk milk tank samples in UK, and 4% in Minnesota **(Haran *et al.,* 2012)** has been reported, prevalence rate reported by **(Lim *et al*., 2013)** was 6.3% in Korea. Higher prevalence dectected in Egypt by **El-Jakee *et al.,* 2011** who identified five out of 9 (55.6%) *S. aureus* isolated from bovine were MRSA, Also **Suleiman *et al,* 2012** who reported that, the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) from subclinical mastitic milk in Nigeria was 30.9% (105 out 339 quarters of 85 cows) as well **Pu *et al.* (2014)** reported 47.6% prevalence in China. **Aqib *et al.*, (2017)** reported 34% prevalence of MRSA in overall (900) milk samples in Pakistan, while no MRSA isolated by **Peles *et al.,* (2007)** in Hungary these results may be attributed as antibiotics including oxacillin and methicillin were randomly used as a dry-cow treatment; this practice may contribute to the increasing incidence of MRSA strains in cows associated with mastitis, and also high resistance to these antibiotics due to selective pressure. While penicillin has been used, overtime and is the antibiotic of choice for drying-off.

Methicillin resistance requires the presence of *mecA* gene. This mecA gene is chromosomally located on a mobile genetic element called the staphylococcal cassette chromosome (SCC). Further *mecA* gene encodes for penicillin-binding protein 2a (PBP-2a) and responsible for synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2) a 78-kDa protein. Expression of PBP-2a is controlled by mecR1 and mecI regulator genes located upstream of *mecA* gene. A mutation in the mec regulators leads to expression of *mecA* gene (PBP-2a). A PBP2a substitute for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents **(Bhanderi and Jhala 2011)**.

The results of our study showed that, the highest percent of *mec A* was recorded in soil samples (100 %), while water samples not detected (0%). Additionally, *mec A* isolated from rectal, milk and hand swab samples with 62.5, 12.5 and 25.5 % respectively. Therefore, from 21 MRSA samples we detected only eight samples carrying *mecA* gene this result differed from **ElJakee *et al.*, 2011** who found All MRSA isolates were *mecA* gene positive by PCR. Conclusively, there is a high occurrence of *Staphylococcus aureus* in cattle and farm environment. The high occurrence of *Staphylococcus aureus* in the faeces may contaminate environment as well may cross-contaminate other animals in the farms. Farm management practice, environmental factors and workers personal hygiene plays an important role in microbial contaminations. MRSA was prevalent in farm environment and working personal in randomly selected farms in Kalubia governorate.The incidence of MRSA was recorded higher in farm workers in comparison to farm environment. This study may serve as a template to investigate the role of cattle in contamination of farm and surrounding environment.

Table (1): Types of farms from which the samples have been collected.

Type	No. of farms	Location
Small holder farm (up to 10 animals)	**8 farms**	**In small villages in Kalubia governorate.**
Medium sized farms (up to 40 animals)	**4 farms**	**In small villages in Kalubia governorate.**
Large sized farms (40 up to 400 animals)	**2 farms**	**One from small villages in Kalubia governorate, and another from Cairo governorate**

Table (2): Zone diameter interpretation, standard of different antibacterial agents used.

Antimicrobial Agents	Code	Disc potency µg/disc	Zone diameter (mm)		
			S	I	R
Oxacillin	**OX**	**1**	**-**	**-**	**-**
Cefoxitin	**FOX**	**30**	**≥22**	**-**	**≤ 21**
Amoxycillin+clavulanic acid	**AMC**	**30**	**≥29**	**-**	**≤28**
Ampicillin+ sulbactam	**SAM**	**20**	**≥29**	**-**	**≤28**

(National Committees for Clinical Laboratory Standards 2016).

Table (3): Occurrence of *Staphylococus aureus* in different samples in farm.

Samples	Total no. of samples	Positive *S. aureus*	Percentage
Rectal	**129**	**40**	**(31%)**
Milk	**82**	**29**	**(35.3%)**
Soil	**7**	**3**	**(42.8%)**
Water	**10**	**0**	**(0%)**
Hand swab	**13**	**10**	**(76.9%)**
Total	**241**	**82**	**(34 %)**
P- value		**0.002***	

*** using Fisher's Exact Test; P<0.05 (P=0.002) indicated that human working in farms are considered a potential risk of *Staphylococus aureus*.**

Table (4): Occurrence of MRSA from positive samples by applying antibiotic diffusion disc.

Samples	Positive *S. aureus*	Positive MRSA	Percentage
Rectal	**40**	**8**	**(20%)**
Milk	**29**	**8**	**(27.5%)**
Soil	**3**	**1**	**(33.3%)**
Water	**0**	**0**	**(0%)**
Hand swab	**10**	**4**	**(40%)**
Total	**82**	**21**	**(25.6%)**
P- value		**0.492**	

Using Fisher's Exact Test; P>0.05 (P=0.0.492) indicated that occurrence of MRSA in positive *Staphylococus aureus* isolates is not dependent on the source of sample.

Table (5): Presence of *mec a* gene from MRSA positive samples.

Samples	Positive MRSA	PCR	Percentage
Rectal	**8**	**5**	**62.5%**
Milk	**8**	**1**	**12.5%**
Soil	**1**	**1**	**100%**
Water	**0**	**0**	**0%**
Hand swab	**4**	**1**	**25%**
Total	**21**	**8**	**38%**
P- value		**0.093**	

Using Fisher's Exact Test; P>0.05 (P=0.0.093) indicated that presence of mec A gene from MRSA positive samples is not dependent on the source of sample.

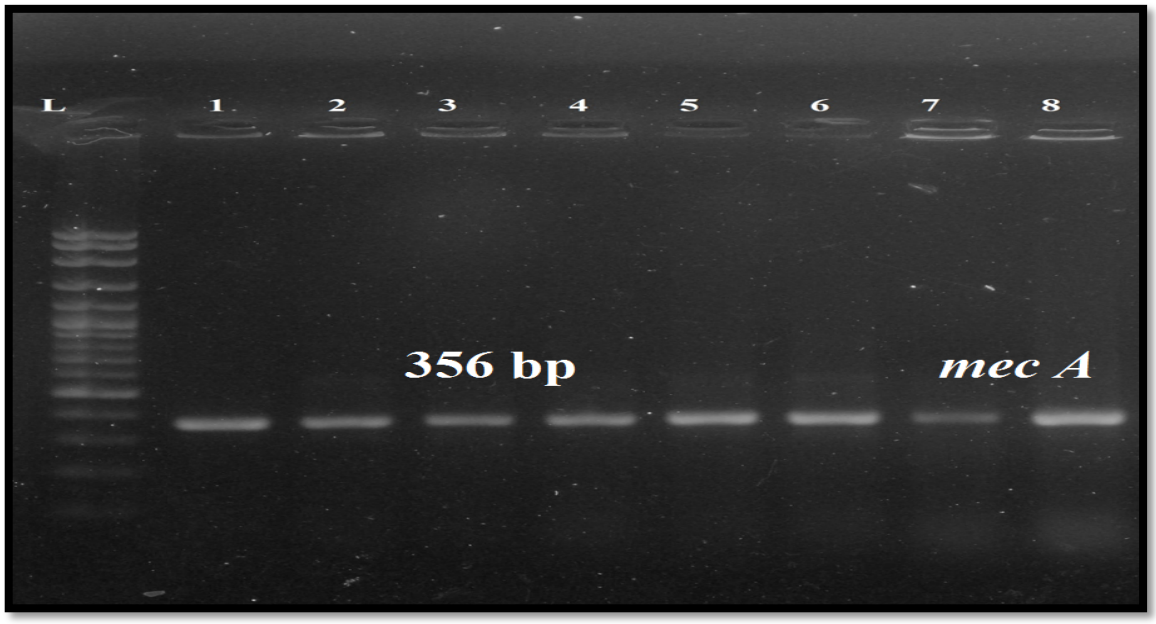

Fig. (1): Agarose (1.5%) gel electrophoresis of *mec A Staphylococcus aureus* PCR products. L = DNA Ladder (100 bp molecular weight standard).

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