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ISOLATION AND IDENTIFICATION OF *S. AUREUS* FROM DIFFERENT SOURCES OF BACTERIAL CONTAMINATION IN LARGE ANIMAL SLAUGHTERHOUSE

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ABSTRACT

A total of 345 swap samples were collected from different sources of suspected bacterial contamination sites in cattle slaughtering hall in El-Monieb slaughter house in Giza governorate. The samples were processed for isolation and identification of coagulase positive *S. aureus*. The results revealed that out of 345 samples, 140 coagulase positive *S. aureus* samples were recorded in prevalence 40.6%. The mean count among the different sources was 4.9 ± 1.5 log10 CFU/ cm^2. The sensitivity testing for 16 different antimicrobials showed that,the140 coagulase positive *S.aureus* samples were multidrug resistance.

The electrophoretic profile of PCR for *mec*A gene was confirming Methicillin Resistant *S. aureus* isolates in 18 samples (13%). It was concluded that they are more than source of Staphylococcal contamination before, during and after slaughtering process could act as a public health risk and play a potential role in food safety issue.

INTRODUCTION

Staphylococci are ubiquitous in the environment. Natural populations are associated with skin, skin glands and mucous membranes of warm-blooded animals. They have been isolated from animal products such as meat, milk and cheese, and other sources such as soil, sand, seawater, fresh water, dust and air. Some Staphylococcus species are known to be frequently encountered in severe infections. Historically, only the *S. aureus* was considered to be pathogenic. *S. aureus*, a species which produces a variety of enzymes and toxins, is the best known and frequently implicated in the etiology of a series of infections and intoxications in animals and humans, **(Harris *et al.*,2002).** The spreading of methicillin resistant *S. aureus* (MRSA) outside hospitals has been encountered recently as a major problem. This has been observed, for example, in veterinary staff and farmers. An increasing number of reports showed that food producing animals could be carriers of methicillin resistant *S. aureus*. It was

observed that animal strains could spread to humans when physical contact is established. In farms where antimicrobials are used, both as therapeutic and feed additives, the risk of spreading is much higher. More than 4% of antimicrobial resistance in human is thought to be associated with animal sources, **(Bergenstrahle, 2015).** The present investigation aims to study the bacterial isolates of *S. aureus* in suspected different sources of contamination in the large animal slaughterhouse by isolation, counting, characterization and identification of the microorganism which might be taken in consideration as a public health and food safety issue in the line of one health approach initiative.

MATERIAL AND METHODS

Collection of swab samples: The samples were collected and processed according to **(Sudhakar *et al.,* 2009)** as follows: Animal surface and hide (muffle area, under tail, back, fore limb and hind limb) before and after slaughtering process, Tools (Hooks and both sides of skinning and evisceration knives) before and immediately after operation, operators' hands (palms and knuckles of both hands) and cloths from back area (loading site) before and after operation, gall bladder, duodenum, ground, wall and washing water. An area of 10 cm^2 of the different surfaces were sampled by sterile cotton swabs (3 cm long and 1 cm in diameter) on sticks. Each cotton swab was moistened with 1 ml of 0.1% peptone water prior to its use. The swabs were rubbed on sites continuously for 30 seconds and transferred to a sterile screw-capped test tube containing 1 ml of sterile maintenance medium peptone water. Ten ml of washing water was also collected in sterile screw-capped test tube. A complete gall bladder and part of duodenum were transferred to sterile caped container. All samples were brought to the laboratory in the ice box containing ice and processed immediately.

Preparation of samples: One ml from each swab was separately transferred to a test tube containing nine ml of sterile peptone water (0.1%) to provide the original dilution (10^{-1}). From which further ten-fold serial dilutions were prepared up to (10^{-7}), **(Sudhakar *et al.,* 2009).** For duodenum and gall bladder, the organs were sliced into small pieces under complete aseptic condition then; 10 gm of the sliced tissues were added to 90 ml 0.1% peptone water to prepare a stock solution, **(Sudhakar *et al.,* 2009).**

Isolation and counting of *Staphylococcal* species on Baird-Parker agar media:

Baird-Parker agar plates (Oxoid CM 0275) with egg yolk emulsion were prepared according to the manufacturer. One hundred µl from each of the previously prepared swabs were

aseptically spread onto the surface of the plates then, the mentioned plates were incubated at 37˚ C for 48 hours. Typical colonies (black, shiny, smooth, convex, 1-1.5 mm with narrow white margin surrounded by zone of clearing 2-5 mm) were counted and recorded as presumptive Staphylococcal count **(ICMSF, 1978).** Counting of Staphylococci was done according to standard procedures for bacterial culture, **(Reynolds, 2005).** Petri dishes containing between 25 and 250 colonies were selected for bacterial enumeration. The number of colonies counted was expressed as colony forming units (CFU/cm^2).

Identification of suspected *S. aureus*:

Mannitol salt agar plates (Oxoid CM 0085) were prepared according to the manufacturer. The presumptive Staphylococcal colonies were picked up from Baird-Parker agar plates for sub-culturing on Mannitol Salt Agar plate. The plates were incubated for 36 hours at 35ºC. Presumptive coagulase positive Staphylococci produce colonies with bright yellow zones whilst coagulase negative Staphylococci are surrounded by a red or purple zone, **(Cain *et al.,* 2015).** Sheep blood agar plates were prepared by preparation of Nutrient agar (Oxoid CM 0003) according to the manufacturer then enriching with up to 10 % sheep blood then, sterilizing by autoclaving at 121ºC for 15 minutes. The plates were streaked aseptically with presumptive Staphylococcal colonies then incubated for 24 hours at 37ºC to determine the grown colonies surrounded by a zone of beta-hemolysis, **(Cain *et al.,* 2015).** Microscopical examination was done by preparation of glass films from the pure culture of the isolated organisms, stained with Gram stain. The slide was examined under microscope at 1000 x oil immersion to observe Gram-positive cocci in clusters like bunch of grapes, **(Cain *et al.,* 2015).** Catalase test was applied using a sterile loop by transferring a small amount of colony growth on the surface of a clean, dry glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added on the glass slide to observe the evolution of oxygen bubbles, **(Cain *et al.,* 2015).**

Coagulase test, **(Tortora *et al.,*2013).** The tube test was done using citrated rabbit plasma that has been inoculated with a staphylococcal colony (i.e., Gram-positive cocci which are catalase positive).The tube is then incubated at 37º C for 1.5 hours to observe clotting indicating suspension of *S. aureus*.

Determination of antimicrobial sensitivity for Coagulase Positive *S.aureus* (CLSI, 2017):

Mueller Hinton Agar medium (Oxoid) and Antibacterial discs (Oxoid) were used. The types of antimicrobials were selected as follows: AMC: amoxicillin/clavulanic acid (30 µg), AK: amikacin (30 µg), AMC: ampicillin/sulbactam (30 µg), FOX: cefoxitin (30 µg), VA:

vancomycin (30 µg), AM: ampicillin (10 µg), GM: gentamicin (10 µg), CIP: ciprofloxacin (5 µg), RF: rifampicin (5 µg), E: erythromycin (15 µg), AZM: aztreonam (15 µg), CN: clindamycin (10 µg), CTX: cefotaxime (30 µg), CP: cefepime (5 µg), CZ: ceftazidime (5 µg) STX: trimethoprim/ sulfamethoxazole (1.25 / 23.75 µg) and E-test strips for oxacillin and vancomycin. McFarland nephelometer barium sulphate standard (0.5) was used for the determination of the approximate number of bacteria, in which McFarland 0.5 turbidity equals 1.5 X 10^8 organisms per ml. The sensitivity was determined by measuring the diameter of a visible and clear zone of inhibition produced by diffusion of the antibacterial agent from the discs into the surrounding medium. Interpretation according to **(CLSI, 2017).**

PCR identification of *S. aureus mec*A gene primers:

The isolates were sent to animal health research institute for complete confirmed identification. DNA Extraction was applied by harvesting the Pure and young cultures from agar plates. Genomic DNA was extracted using (thermo scientific DNA purification kit) for all samples. According to **(Wielders *et al.,* 2002),** the *mec*A gene was amplified with the primers F: 5-GTT GTA GTT GTC GGG TTT GG-3 (upstream) and R: 5-CTT CCA CAT ACC ATC TTC TTT AAC-3 (downstream) specific for the *mec*A gene with Size of PCR 310 bp. The PCR amplification using 2x PCR master mix solution, negative controls were performed according to the following thermal profile: 30 cycles consisting of 1 minute at 95ºC, 1 min at an annealing temperature at 62ºC and extinction at 72ºC for 10 minutes. The PCR product was visualized on a 1.5% agarose gel using ethidium bromide and a UV trans-illuminator.

Statistical analysis of results' data:

The data of the study were compiled in excel database, and organized for statistical analysis. The analysis was done using IBM SPSS version 21, a computer-based statistical software package, **(Coakes, 2005).** A statistical approach was used for comparing between means which was One Way ANOVA. The data was represented by mean of the samples collected per each suspected source of contamination.

RESULTS

The results analysis revealed that out of 345 samples from different sources of sampling sites in the slaughter house, 140 coagulase positive *S. aureus* samples were recorded in prevalence 40.6%. The mean count among the mentioned sources was 4.9 ± 1.5 log10 CFU/ cm^2.

According to (Table 1-3), it is noticed that, the floor was the highest site had coagulase positive *S. aureus* samples with percentage 93.3% from the collected samples from the floor sites and prevalence 4.1% from the grand total collected samples.

The highest coagulase positive *S. aureus* load was also recorded in floor sites with mean count 6.6 ± 1.2 log10 CFU/ cm^2.

The minimum numbers of samples with positive isolates were found at back of animal before slaughtering, hind limb of animal before slaughtering, pharyngeal region after slaughtering and butchers' clothes before slaughtering in prevalence 0.9%.

While, the lowest mean count of coagulase positive *S. aureus* was 3 ± 0.4 log10 CFU/ cm^2 in pharyngeal region after slaughtering.

There was no detection of *S. aureus* in water samples, duodenum or gall bladder.

By classifying the different sampling sites according to the related category and time of sampling (before or after slaughtering) (Table 3), the sampling sites were categorized as: Sampling sites from animals before slaughtering, sampling sites from animals after slaughtering, butchers (hands and clothes) and tools before slaughtering, butchers (hands and clothes) and tools after slaughtering and finally, sample sites from the slaughterhouse environment (floor and wall). According to the samples category, the results showed that, the butchers (hands and clothes) and tools after slaughtering has the highest number of positive samples (46 positive samples) representing prevalence 13.3% from the total collected sample, followed by slaughterhouse environment and sampling sites from animals after slaughtering (27 positive samples per each category) representing 7.8% then, butchers and tools before slaughtering (22 positive samples) representing 6.4% and finally Sampling sites from animals before slaughtering (18 positive samples) in prevalence 5.2%.

The slaughterhouse environment (floor and wall) had the highest mean count for coagulase positive *S. aureus* 6.41 ± 1.23 log10 CFU/ cm^2, while The butchers (hands and clothes) and tools after slaughtering is the lowest category had coagulase positive *S. aureus* mean count (mean count: 4.36 ± 1.33 log10 CFU/ cm2).

Analysis of variance between the different sources of contamination in the slaughterhouse reveled a significant difference ($P<0.01$) in mean *S. aureus* count with F value 4.65 interpreting that there is a continuous cross contamination between the mentioned sources of contamination in the slaughterhouse.

Table (1): (#) Number and Percentage of coagulase positive *S. aureus* samples isolated from different sampling sites in the slaughterhouse.

Source of Sampling	# Samples	# Positive	Percentage
Abdomen after slaughter	15	5	33.3%
Back after slaughter	15	4	26.7%
Back before slaughter	15	3	20%
Floor	15	14	93.3%
Fore limb before slaughter	15	4	26.7%
Hind limb after slaughter	15	10	66.7%
Hind limb before slaughter	15	3	20%
Muffle before slaughter	15	4	26.7%
Neck after slaughter	15	5	33.3%
Pharyngeal region	15	3	20%
Hand after slaughter	15	11	73.3%
Hand before slaughter	15	9	60%
clothes after slaughter	15	11	73.3%
clothes before slaughter	15	3	20%
Tool 1 after slaughter	15	12	80%
Tool 1 before slaughter	15	6	40%
Tool 2 after slaughter	15	12	80%
Tool 2 before slaughter	15	4	26.7%
Under tail before slaughter	15	4	26.7%
Wall	15	13	86.7%
Water sample	15	0	0%
Duodenum	15	0	0%
Gall bladder	15	0	0%
Total	345	140	40.6%

Table (2): Mean counts (log10 CFU/ cm^2 ± Standard Deviation), 95% confidence interval of mean and % prevalence of coagulase positive *S. aureus* in the different sources of sampling in the slaughterhouse.

Source of Sampling	Mean log10 CFU/ cm^2	Std. Deviation	Prevalence
Abdomen after slaughter	**4.278**	**1.544**	**1.5%**
Back after slaughter	**5.612**	**0.137**	**1.2%**
Back before slaughter	**4.996**	**0.429**	**0.9%**
Floor	**6.610**	**1.167**	**4.1%**
Fore limb before slaughter	**5.275**	**0.591**	**1.2%**
Hind limb after slaughter	**4.826**	**1.004**	**2.9%**
Hind limb before slaughter	**5.473**	**0.074**	**0.9%**
Muffle before slaughter	**4.510**	**1.093**	**1.2%**
Neck after slaughter	**3.823**	**1.292**	**1.5%**
Pharyngeal region	**2.957**	**0.424**	**0.9%**
Hand after slaughter	**4.411**	**1.038**	**3.2%**
Hand before slaughter	**3.738**	**1.381**	**2.6%**
clothes after slaughter	**4.317**	**1.226**	**3.2%**
clothes before slaughter	**4.144**	**1.528**	**0.9%**
Tool 1 after slaughter	**4.407**	**1.504**	**3.5%**
Tool 1 before slaughter	**5.362**	**0.277**	**1.8%**
Tool 2 after slaughter	**4.325**	**1.610**	**3.5%**
Tool 2 before slaughter	**5.887**	**0.406**	**1.2%**
Under tail before slaughter	**6.163**	**0.395**	**1.2%**
Wall	**6.195**	**1.309**	**3.8%**
Total	**4.935**	**1.454**	**40.6%**

Table (3): Total Mean counts (log10 CFU/ $cm^2 \pm$ Standard Deviation), 95% confidence interval of mean and % prevalence of coagulase positive *S. aureus* in the different sources before and after slaughtering process.

Source of Sampling	Mean log10 CFU/ cm^2	Std. Deviation	Prevalence
Slaughterhouse environment	**6.41**	**1.23**	**7.8%**
Sampling sites from animals before	**5.29**	**0.82**	**5.2%**
Butchers and tools before slaughtering	**4.63**	**1.35**	**6.4%**
Sampling sites from animals after	**4.45**	**1.26**	**7.8%**
Butchers and tools after slaughtering	**4.36**	**1.33**	**13.3%**
Total	**4.935**	**1.454**	**40.6%**

The results of antimicrobial sensitivity revealed that, the 140 coagulase positive *S. aureus* samples determined as multidrug resistance against the 16 antimicrobials tested for the sensitivity, (Table 4).

Out of 16 different antimicrobials, all the 140 coagulase positive *S. aureus* samples (100%) showed sensitivity for vancomycin (30 µg) and only 118 samples from the total coagulase positive *S. aureus* samples (84.3%) showed sensitivity for cefoxitin (30 µg). Cefotaxime (30 µg) is considered the most antimicrobial showed intermediate action (82 samples) followed by Cefepime (5 µg) (74 samples were intermediate) while, the other antimicrobials were resisted by the most of the coagulase positive *S. aureus* samples.

Table (4): The interpretation pattern of antimicrobial sensitivity testing for coagulase positive *S. aureus* against 16 antimicrobials.

	Resistant	Intermediate	sensitive
Amoxicillin/ Clavulanic acid	**140**	**0**	**0**
Ampicillin	**140**	**0**	**0**
Amikacin	**132**	**8**	**0**
Ampicillin/ Sulbactam	**140**	**0**	**0**
Vancomycin	**0**	**0**	**140**
Aztreonam	**140**	**0**	**0**
Ciprofloxacin	**124**	**16**	**0**
Cefoxitin	**12**	**10**	**118**
Gentamycin	**107**	**33**	**0**
Sulpha/ Trimethoprim	**132**	**8**	**0**
Rifampicin	**124**	**16**	**0**
Clindamycin	**132**	**8**	**0**
Erythromycin	**124**	**16**	**0**
Ceftazidime	**140**	**0**	**0**
Cefotaxime	**58**	**82**	**0**
Cefepime	**66**	**74**	**0**

The electrophoretic profile of PCR for *mec*A gene was confirming Methicillin Resistant *S. aureus* isolates, Fig. (1). 18 samples were confirmed as Methicillin Resistant *S. aureus* (MRSA) in percentage 13% from the total 140 coagulase positive *S. aureus* samples and prevalence 5.2% from the total 345 samples collected from the different sites of sampling in the slaughterhouse, (Table 5).

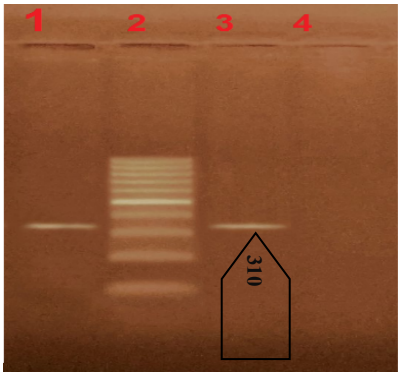

Fig. (1): PCR for *mec*A gene Lane 1 sample Lane 2 marker (NEB 100bp)Lane3control+ve (310 bp) Lane 4 control-ve

Table (5): Numbers and percentage of Methicillin Resistant *Staphylococcus aureus* (MRSA) isolates recovered from different sources in the slaughterhouse.

Source of samples	# of isolated coagulase positive *S. aureus*	# of positive *mec*A gene
Abdomen after slaughter	**5**	**-**
Back after slaughter	**4**	**1**
Back before slaughter	**3**	**-**
Floor	**14**	**2**
Fore limb before slaughter	**4**	**-**
Hind limb after slaughter	**10**	**-**
Hind limb before slaughter	**3**	**-**
Muffle before slaughter	**4**	**2**
Neck after slaughter	**5**	**1**
Pharyngeal region	**3**	**-**
Hand after slaughter	**11**	**2**
Hand before slaughter	**9**	**2**
clothes after slaughter	**11**	**1**
clothes before slaughter	**3**	**1**
Tool 1 after slaughter	**12**	**1**
Tool 1 before slaughter	**6**	**3**
Tool 2 after slaughter	**12**	**1**
Tool 2 before slaughter	**4**	**1**
Under tail before slaughter	**4**	**-**
Wall	**13**	**-**
Total	**140**	**18**

DISCUSSION

The results analysis revealed that there were 140 coagulase positive *S. aureus* samples were recorded in prevalence 40.6%. This is meaning that *S. aureus* has considered probability for contaminating the slaughterhouse environment and infection of their workers. On the other hands, **(Abdalla *et al.*, 2009)** recorded 10.5%, **(Ahmed *et al.*, 2013)** recorded 72% and **(Sudhakar *et al.*, 2009)** recorded 59.3% positive samples from the total collected samples in the slaughterhouse. The mean count among the mentioned sources was 4.9 ± 1.5 log10 CFU/ cm^2. The same result was concluded by **(Kedir, 2013)** (4.9 log10 CFU/ cm^2). The present records are slightly higher than, the records of **(Ahmed *et al.*, 2013)** (2.75 log10 CFU/ cm^2),

(Bergenstrahle, 2015) (3.15 ± 0.185 log10 CFU/ cm^2) and **(Sudhakar *et al.,* 2009)** (3.5 ± 0 .45 log10 CFU/ cm^2), however we might consider that **(Ahmed *et al.,*2013) and (Sudhakar *et al.,* 2009)** were higher in prevalence than the present study.

The floor was the highest site had coagulase positive *S. aureus* samples and highest mean count. This result is matching with the results of **(Ahmed *et al.,* 2013) and (Sudhakar *et al.,* 2009).** On the other hands, the present study showed that, the average mean count *S. aureus* in swabs from butchers and tools were around 4 log10 CFU/ cm^2 and this is higher than the records of **(Amani *et al.,* 2017)** who recorded 2.75 log10 CFU/ cm^2.

There was no detection of *S. aureus* in water samples, the result that achieved by **(Ntanga, 2013) and (Sudhakar *et al.,* 2009).**

By grouping the different sources of contamination in the slaughterhouse, the butchers (hands and clothes) and tools after slaughtering has the highest number of positive samples, followed by slaughterhouse environment and sampling sites from animals after slaughtering then, butchers and tools before slaughtering and finally Sampling sites from animals before slaughtering. The same conclusion was achieved by **(Amani *et al.,* 2017) and (Branko *et al.,* 2007).** Analysis of variance between the different sources of contamination in the slaughterhouse interpreted that there is a continuous cross contamination between the mentioned sources of contamination in the slaughterhouse which matched with results of **(Sudhakar *et al.,* 2009), (Ahmed *et al.,* 2013), (Bhandare *et al.,*2010), (Ntanga, 2013), (Amani *et al.,* 2017), (Branko *et al.,* 2007) and (Fathy *et al.,* 2016)** and controversy to statistical significance achieved by **(Nicoline *et al.,* 2015).**

The results of antimicrobial sensitivity revealed that, the detected coagulase positive *S. aureus* samples determined as multidrug resistance against the 16 antimicrobials tested for the sensitivity. *S. aureus* samples (84.3%) showed sensitivity for cefoxitin and this is against the results which achieved by **(Nicoline *et al.,* 2015)** who recorded 100 % resistance to oxacillin.

The electrophoretic profile of PCR for *mec*A gene was confirming Methicillin Resistant *S. aureus* (MRSA) isolates in 18 samples in percentage 13% from the total 140 coagulase positive *S. aureus* samples and prevalence 5.2% from the total 345 samples collected from the different sites of sampling in the slaughterhouse indicating significant role of slaughterhouse ground, workers and tools in spreading and contamination of Methicillin Resistant *S. aureus* (MRSA) directly and indirectly. On the other hands, **(Amani *et al.,* 2017)** recorded MRSA in 24% of the examined samples indicating more risk.

CONCLUSION

From the present study, it is concluded that, the presence of multidrug resistant *S. aureus*, in particular MRSA, in different sources in bovine slaughterhouse, has an epidemiological significance and represents a potential threat for the spread of this pathogen in the community through cross contamination of meat and/or contamination of utensils, workers' hands and cloths and environment of the slaughterhouse as well as possibility for directing infection of the works who could play a significant role in public and animal health. The contamination is mainly due to bad hygiene and improper practices in the slaughter house that ensure a continuous contamination. It is needed to study the antigenic relation of MRSA inside slaughterhouse to ensure the main genetic source whether from animal or human origin for better understanding of the main reservoir of the microorganism. A quantitative risk assessment study with genetic epidemiological investigation have to be applied for better understanding of the way and source of spreading of MRSA before introducing animals for slaughtering. It is recommended to follow the proper hygienic measures before, during and after slaughtering process and use the appropriate disinfectants to ensure decreasing the *Staphylococcal* load in the slaughter house environment.

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