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Classical and molecular identification of *Staphylococcus aureus* isolated from infestation cattle wounds with myiasis in Basrah governorate, Iraq

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Abstract

The present study was carried out to describe some epidemiological facts of myiasis infestations in cattle; therefore, Staphylococcus aureus was isolated and identified from various infested sites with maggots from February to September 2019. It involved three districts (Shatt-Alarab, Al-Qurnah, and Al-Dyr) north of Basrah Governorate/Iraq. A total of 54 herds/owners were visited, with 150 cattle were found to be infested with maggots and diagnosed from different sites of the animal body. The result was indicated that 31% (95%Cl, 26.9-35.4) of examined animals were infested with myiasis, and there were no significant differences detected between sex and ages of the animal groups under study. Staphylococcus aureus was diagnosed using classical methods as morphological characteristics, physiological (coagulase tube method), biochemical tests, and growth on selective medium as Mannitol Salt Agar (MSA) at a percentage of 32% (48/150). Polymerase chain reaction (PCR) was performed to amplify the nuc gene in this isolated species, indicating the presence of nuc size (423) bp compared with a ladder used. The study clearly states that myiasis is a severe threat to cattle populations and that veterinary and agriculture authorities must recall control measures. These measures should be forged to include using a trapping/catch system, applying effective treatment, spraying pesticides, and sterilizing male flies with radiation to inhibit producing offspring.

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Introduction

Myiasis is a cutaneous parasite infestation caused by fly maggots that is prevalent in tropical and subtropical regions. The fly has an extreme aggressive behaviour towards all warmblood animals, particularly in livestock. The larvae require nutrition to develop. Hence, they feed on animal tissues, causing severe skin damage and devastating lesion that combines necrosis and haemorrhage. The most common distinctive genera are associated with myiasis infestation in animals include the new world screwworm (NWS) (*Cochliomyia hominivorax*) and old-world screwworm fly (OWSF) (*Chrysomya bezziana*) (1). The ambient humidity and temperature are significant determinants in the increase of fly population in enzootic areas. The geographical distributions of the screwworm fly are positively related to warmer climates, with a favourable annual mean temperature of OWSF being assessed between 28-30ºC (2). Interestingly, the screwworm flies can migrate over 100 kilometres, causing substantial damage to animal herds, particularly in developing countries where sanitary conditions and lack of control programs are more common (3). The screwworm fly is a serious pest that feeds on untreated wounds, pus, and other hormonal secretions. Most of these wounds are associated with the time of branding, dehorning, castrating, earmarking, or shearing wools that are accidentally

perpetuated by animal owners (4). Each gravid female fly lays many eggs (up to 400) whereabouts skin patch or wound site. The pooled eggs hatch to first larvae after 12-20 hours and crawl to feed on the living flesh (4,5). Due to their propensity in digging tunnels within animal tissues using their mouthparts, the larvae leave large lesions or deep cavities. Subsequently, the larvae evolve to different morphological phases, and after a few days, they emerge to pupate in the ground and turn into adult flies (6). The development from a latent pupa to a mature fly takes nearly 14 days (7). The infestation with myiasis has significant health detrimental impacts on the animal industry, causing distress, reduced milk yield, and body weight loss (8). Treatments for myiasis include local use of ether or chloroform, followed by anti-parasitic drugs such as Ivermectin (9). Maintaining sanitation in farmyards and spraying pesticides, on the other hand, can effectively eliminate fly populations and, as a result, lower their occurrences. Geographical distribution of screwworm flies and other important Diptera were reported in many countries of the eastern and western hemispheres (10). The infestation with myiasis also was reported in Arabian Peninsula, included Oman, Kuwait, Saudi Arabia, and Iraq. From 1988 until 1990, more than 12,000 confirmed cases of screwworm fly myiasis caused by *C. hominivorax* were recorded in Libya and other parts of North Africa, with estimated cost losses on the livestock industry reaching US$30 million per year (11). Similarly, in Iraq, there were high incidence cases associated with myiasis in animals from the beginning of 1996, and the FAO estimated the economic loss to the livestock industry as a result of the OWSF invasion at US$ 8,555,000 (12). Since then, myiasis has expanded endemically over many Iraqi pastoral areas, attributing to favourable climatic conditions for fly propagation as well as due to inconsistent veterinary intervention (13).

Infected wounds with one or more bacteria species might influence flies to become more aggressive in their infestation cycle. A variety of opportunistic micro-organisms can invade untreated wounds, causing tissue damage and releasing toxins, with *Staphylococcus aureus* and *Streptococcus pyogenes* accounting for most infections (14). These pathogenic micro-organisms have a large extent on animal health and maybe ended-fatal hosts if left untreated. Various proteolytic enzymes derived from bacteria were identified as a significant source for attraction flies (15). The enzymes together produce volatile odours which thus attract gravid flies and facilitate oviposition (16). Therefore, the present study's objective was to describe some epidemiological aspects of myiasis infestation in cattle, identify *Staphylococcus aureus* from different infested sites with maggot's species, and detect the *nuc* gene using the PCR technique in its.

Materials and methods

Ethical approve

Ethical approval was obtained from the scientific committee in the college of Veterinary Medicine/University of Basrah, before beginning of the study.

Area of study and sampling

The fieldwork was performed in the Basrah province, located in the south of Iraq. The province has scorching weather during the summer season, with a mean temperature of 37.4°C and a maximum temperature of 45°C. The lowest mean summer temperature was 29.2°C. Basrah is famous for its oil industry and resources, but it has also been recognized as a rural area with diverse palm trees and many animal species. The data from veterinary hospital records show a total of 79982 species of sheep, 6725 species of goats, 55151 species of cattle, and 54467 species of buffalo in the rural and peri-urban areas (Personal communication, data from the Basrah Veterinary Hospital).

Collectively, 54 owners were visited to inspect their animals if they were infested with (myiasis) maggots. A total of 478 cattle was closely examined on the myiasis infestation. The selected owners originated from three districts in the north of Basrah, including Shatt-Alarab, Al-Qurnah, and Al-Dyr, based on-farm visits by the local veterinarian practicians. The owners were visited weekly commenced from the period of February to September in 2019. As a non-probability technique, convenient sampling was chosen as the best appropriate method to select diseased animals. This investigation was done when the owners whose animals were infested with myiasis contacted the veterinarians and called for treatment of myiasis condition. Other owners in the same area guided us to the next owners, all animals inclusively infested with myiasis. From these districts, however, 28 villages were randomly involved in the present study. The owners were asked for verbal consent to access their animals and targeted samples. The sex and ages of the infected animals were documented. The animal ages were determined based on the dentition method.

Protocol of identification of *Staphylococcus aureus*

A sterile swab was used to isolate and identify *Staphylococcus aureus* to collect superficial and deep-infested lesions. Each isolated specimen was preserved in a sterilized broth agar and immediately transferred to the laboratory of veterinary college/University of Basrah. The specimens were incubated aerobically at 37°C for 24 h for growth. All isolates were identified based on their morphology, cultural characteristics, and biochemical properties to differentiate "*Staphylococcus aureus*." Firstly, the isolates of 150 were subjected to growth on mannitol salt agar (MSA) to differentiate between species presumptively. Secondly, the tube coagulase test (TCT) with adding human plasma was performed to an additionally confirmatory

diagnostic test for the targeted bacteria. Other biochemical tests were done using oxidase, coagulase, catalase, Triple sugar Iron (TSI), urease, and indole (17). Finally, the molecular biological Assay by PCR was employed to identify the presence of gene *nuc* in strains of *Staphylococcus aureus*.

Molecular assay using PCR technique

Genomic DNA of suspected *Staphylococcus aureus* strains was extracted using a DNA isolation kit (Geneaid, USA). PCR-amplification of the *nuc* gene was used to detect and confirm *Staphylococcus aureus* from the isolates positive in the tube coagulase. The PCR protocol was based on reactions contained 20 p moles each of the *nuc* forward and reversed primers (5´- GCT TGC TAT GAT TGT GGT AGC C 3', 5´- TCT CTA GCA AGT CCC TTT TCC A 3', respectively, synthesized by Bioneer, Korea. The PCR program consisted of initial denaturation at 94°C, 5 min, followed by 37 cycles each consisting of denaturation at 94°C, 1 min; primer annealing at 55°C, 0.5 min, and extension at 72°C, 1 min; followed by a final extension at 72°C, 7 minutes.

Statistical analyses

The data were stored in an excel sheet and analyzed using Statistical Package for the Social Sciences (SPSS) software (version 23). A chi-square test was used to determine an association between the infested animals with myiasis and animal factors (sex and age), considering a p. value at the level of significance if it was ≤ 0.05. The percentages and 95% Confidence intervals (Cl) of animals with myiasis were calculated. The incidence cases of myiasis recorded are visualized as a radar chart in the result section.

Results

The examined cattle were 478 cattle; 150 were infested with maggots, as shown by Figure 1, and the prevalence was calculated as 31% (95%Cl, 26.9-35.4). There were no significant differences between characterization allocated animal groups infested with maggots, as seen in Table 1. Regarding the sites of infestation, feet 26% (39/150) and vaginal regions 16% (24/150) were the most frequent infested sites with maggots (Table 2).

Staphylococcus aureus on MSA was identified at 32% (48/150) as in Figure 2. The culture media appeared shining yellow. There was a significant difference for the presence of *Staphylococcus aureus* isolated from different infested sites, with P< 0.05 (Table 2). The TCT detected 25% (37/150) of *Staphylococcus aureus* as a pathogenic strain, based on forming a clot. The PCR-amplification of the *nuc* gene (423bp) confirmed all 37 positive specimens that detected by TCT as *Staphylococcus aureus* strains (Figure 3). Figure 4 depicts the monthly incidence of myiasis cases among cattle; however, the months of April and July were found to have the highest peak of incidence, with maggot infestation estimated as 0.8 and 0.9, respectively.

Table 1: Number and percentage of cattle characterisation groups infested with myiasis

Group	No. Examined	No. Infested (%)	95%Cl	P
Sex				
Males	90	38 (30)	22.2, 38.7	0.6
Females	238	112 (32)	27.1, 37.2	
Age				
<1	22	8 (27)	12.5, 46.2	
1-2	60	28 (34)	24.2, 44.9	0.8
>2	246	114 (32)	27.2, 37.1	

Figure 1: Cattle infested with maggots.

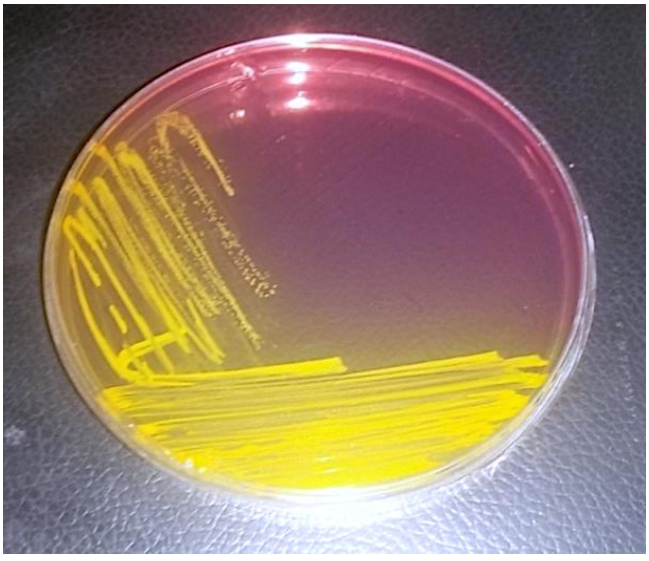

Figure 2: *Staphylococcus aureus* colonies on MSA.

Table 2: Number and percentage of identified *Staphylococcus aureus* from different infested sites with myiasis

Sites of infestation	Frequency (%)	Number of positive (%)	Number of negative (%)	P value
Abdomen region	13 (8.7)	5 (38.5)	8 (61.5)	
Feet	39 (26.0)	15 (38.5)	24 (61.5)	
Horns	8 (5.3)	0 (0.0)	8 (100.0)	
Mandibular region	17 (11.3)	11 (64.7)	6 (35.3)	
Rectum	14 (9.3)	0 (0.0)	14 (100.0)	0.001*
Testicle	16 (10.7)	3 (18.8)	13 (81.3)	
Thigh	19 (12.7)	9 (47.4)	10 (52.6)	
Vaginal region	24 (16.0)	5 (20.8)	19 (79.2)	
Total	150 (100.0)	48 (32.0)	102(68.0)	

* Significant as the P ≤ 0.05.

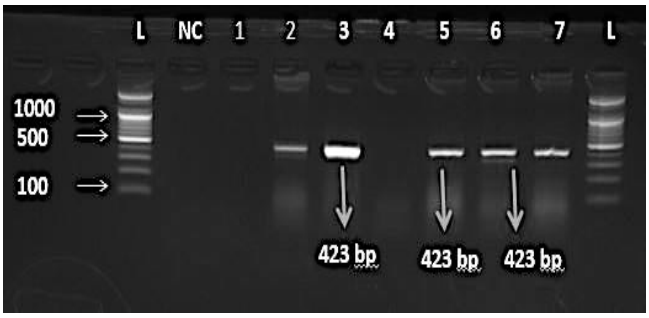

Figure 3: Amplification of *nuc* gene from strains of *Staphylococcus aureus* showed by agarose gel electrophoresis. Lane L: 1 Kb molecular weight DNA ladder, Lane 1-22: the product size (423) bp.

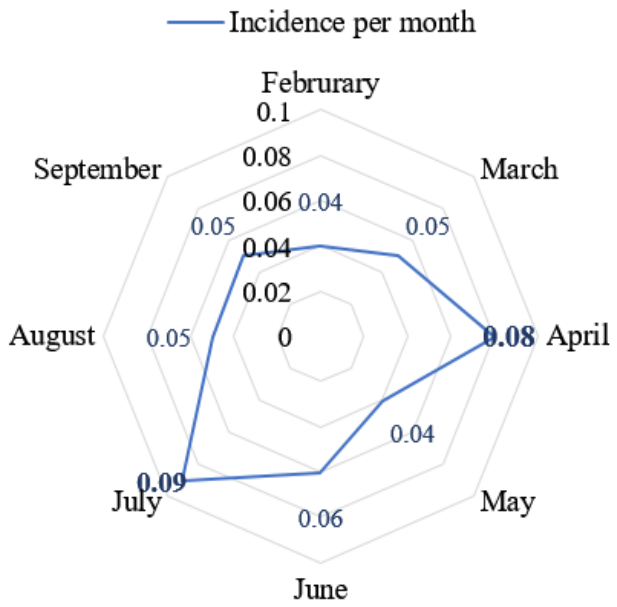

Figure 4: The incidence cases of myiasis/month observation are visualized as Radar Chart.

Discussion

The bacterial species "*staphylococcus aureus*" remains a widely spread, facultative and important micro-organism in humans and animals due to producing harmful toxins and lytic enzymes. Ideal identification of *staphylococcus aureus* requires sequential laboratory tests in order to differentiate it from other bacterial species. Therefore, three laboratory tests were efficiently utilized to identify the isolates included growth colonies, biochemical, and molecular biology assay. The mannitol salt agar (MSA) and tube coagulase test (TCT) identify *Staphylococcus aureus*. This bacterium can rapidly ferment the mannitol in MSA, while TCT with human or sheep plasma is also used to diagnose *Staphylococcus aureus* strains. Although both tests culminate validating results, further reliable tests like PCR are highly recommended must be preceding by laboratory classical methods. A combination of MSA/DNase and TCT (human plasma) was evaluated to possess a sensitivity of 75% and specificity as 100%, giving a credence in the identification of *Staphylococcus aureus* (18).

In the current research, 150 samples from myiasis infested sources were screened for presumptive *Staphylococcus aureus* using MSA & TCT. The culture media MSA is the most successful selective media for the bacteria growth that differentiates several *Staphylococcus* species, particularly *Staphylococcus aureus*. The test has a sensitivity and specificity of 95% and 79%, respectively (18). The specimens from the current study were also undergone to the TCT as the second consecutive test. The test recognized 25% of strains of *Staphylococcus aureus* as a pathogenic strain based on the forming a clot. *Staphylococcus aureus* can produce the enzyme "coagulase," which basically can turn fibrinogen in the plasma to fibrin. Although the test has 91% sensitivity, it was reported to give some discordant results due to anti-staphylococcus antibodies in the human plasma, and some of the animal Staphylococci have negative clumping factors. Other researchers indicated that the sensitivity of the TCT could be lower than 60% due to using human plasma. A study was done by Kateete *et al*. (18) to compare identification methods

highlighted that the TCT is less sensitive (sensitivity 81%) with sheep plasma as more false-negative were diagnosed in comparing with the human plasma (sensitivity 91%).

All 37 strains from the TCT were detected positive to *Staphylococcus aureus* from the deoxyribonuclease (DNase) test results. We used the PCR to amplify a sequence of the *nuc* gene. This gene is an important pathogenic factor, and the thermostable nuclease (TNase) gene encodes *Staphylococcus aureus* (19). The DNase test for the *Staphylococcus aureus nuc* gene was reported to possess a sensitivity of 75% and specificity of 96% (18). On the other hand, several microbiologists have used different gene encodes to characterize *Staphylococcus aureus* from human and animal samples. For example, they sequence the *mec* gene, a specific gene for methicillin-resistant *Staphylococcus aureus* MRSA (20,21). Nevertheless, both TCT and DNase laboratory techniques in the current work showed the similar results.

Staphylococcus aureus and other bacteria can produce a combination of enzymes compounds that cause connective tissue lysis and decomposition. These enzymes can play a more significant role in attracting flies due to a volatile odour emitted from the contaminated wound. An experimental study was manipulated *in vitro* by Zhu *et al*. (15) identified five volatile compounds, including dimethyl disulfide, dimethyl trisulphide, phenol, P-cresol, and indole collected from five species of bacteria and showed considerable effectiveness in attracting adult flies. Their study, however, concluded that the blend of different mixture compounds could be utilized as a trapping trial for surveillance and early monitoring of myiasis flies. It is essential to treat wounds with antisepsis, maintain a hygienic barnyard, and cleanse animal skin regularly to avoid flies from attracting. These critical practices can help minimize the likelihood of myiasis incidence and the number of flies striking.

The current study reported that 150 cattle in the northern Basrah region were inspected to find out the occurrence of myiasis. Between 2014 and 2017, more than one hundred myiasis cases were recorded in sheep and cattle in the middle and south of Iraq, with *Chrysomya bezziana* identified as the most predominant species (22). The statistical results did not indicate a significant difference between males and females or even between age groups. This finding is contrary to what Islam *et al*. (23) found that among cattle and goats, 67% of myiasis-infested animals were females, whereas just 33% were males. It is necessary to develop a monitoring system to detect screwworm flies using a specific synesthetic attractant blend; it would aid in the identification of species of flies as well as their seasonal activation. Application of pesticides to eliminate traceability of fly niche sources or/and use of sterile insect method to sterilize males' flies should be included in implementing a control program.

The present study also determined the temporal distribution of the infestation with myiasis. The estimated prevalence was high (31%), with the most significant incidence of myiasis monitored during April and July. Probably the main reason, from north to south, Iraq has tropical weather and the most extended summer, with temperature increased 2 to 7 times faster than global temperature (24). The temperature shown above is a linearly optimal degree for unleashing screwworm flies, leading them to become overly abundant and potentially causing infestation (25). Additionally, a large number of livestock in the north of Basrah could contribute to ecological changes due to a vast amount of manure produced. As a result, a large quantity of ammonia and carbon dioxide are freely emitted into the atmosphere. Accordingly, our research shows that these significant variables are highly linked to the occurrence of myiasis in cattle in Basrah. Climate change and weather warming may contribute to the emergence of a new exotic pathogen or vector-borne illness that poses a health concern to humans and animals. The technique for trapping sample flies is essential for monitoring fly seasonal activity in the locations where myiasis is often reported.

Conclusion

Myiasis is a severe pest striking livestock in Basrah. *Staphylococcus aureus* was the most common bacteria identified from different sites of infestations using laboratory diagnostic techniques, included MSA, TCT, other biochemical tests, and polymerase chain reaction (PCR). The current study conspicuously stated that the bacterium *Staphylococcus aureus* precedes the infestation with maggots may be due to volatile compounds released from the metabolism of bacteria and can attract flies ovipositional. Myiasis infestation can be ultimately reduced by using a fly trapping/catching system, administering treatment, spraying pesticides, and using a sterilized male fly technique.

Acknowledgments

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Conflicts of interest

All the authors declare that there is no conflict of interest.

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التشخيص التقليدي والجزيئي للمكورات العنقودية القيحية (الذهبية) المعزولة من جروح الأبقار المصابة بيرقات الذباب في محافظة البصرة، العراق

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الخلاصة

أجريت الدراسة الحالية لتوصيف بعض من الجوانب الوبائية لانتشار داء النغف في الأبقار، إذ عزلت وشخصت بكتريا المكورات القيحية من مختلف الجروح للأبقار المصابة بالنغف، للفترة من شهر شباط إلى أيلول لسنة ٢٠١٩ من ثلاث مناطق مختلفة (شط العرب والقرنة والدير) من شمال محافظة البصرة/العراق. تم زيارة ٥٤ قطيعا/مالكا إذ وجد ١٥٠ من المواشي مصابة بيرقات الذبابة الحلزونية في مناطق مختلفة لجسم الحيوان. أوضحت النتائج إلى أن ٣١٪ (٩٥٪ CI، ٢٦,٩-٣٥,٤) من الحيوانات التي تم فحصها مصابة بالنغف، حيث لا توجد فروقات معنوية بين مجاميع المواشي قيد الدراسة من ناحية الجنس والعمر. شخصت المكورات القيحية اعتمادا على الطرق التقليدية كالصفات الشكلية والاختبارات الأيضية (اختبار تجلط البلازما في الأنبوب)، والاختبارات الكيموحيوية والنمو على وسط أكار المانيتول والملح إذ بلغت نسبتها ٣٢% (١٥٠/٤٨). أجريت تقنية تفاعل السلسلة المتبلمرة لتضخيم جين *nuc* في السلالات المعزولة واتضح حجم الجين ٤٢٣ كيلودالتون مقارنة مع الدليل الحجمي المستخدم. أوضحت الدراسة أن يرقات النغف تشكل تهديدا خطيرا على قطعان الماشية لذا يجب اتخاذ تدابير المكافحة من قبل الجهات البيطرية والزراعية بحيث تشمل تلك التدابير كاستخدام نظام الشبك، تطبيق العلاج الفعال، رش المبيدات وتعقيم ذكور الذباب الحلزوني بالإشعاع لمنع إنتاج النسل.