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Using A *Galleria Mellonella* to Investigate the Pathogenicity of *Brachybacterium epidermidis* Isolated from Conjunctivitis Newborn in Mosul City, Iraq

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ABSTRACT

A total of 100 clinical samples were collected from neonates diagnosed with conjunctivitis at three hospitals in Mosul: Al-Salam Teaching Hospital, Mosul General Hospital, and Ibn Al-Atheer Hospital. Among the bacterial isolates, Brachybacterium epidermidis was identified in 2.27% of the isolates, which isolated for the first time in Mosul/Iraq. This Brachybacterium epidermidis grow on chocolate agar and furthermore molecular identification was performed using the universal 16S rRNA primer and the resulting sequence was compared with reference sequences in the NCBI database using the Basic Local Alignment Search Tool (BLAST), thereby confirming a 100% identity with Brachybacterium epidermidis Marseille-Q2903. In order to assess its pathogenic potential, Galleria mellonella larvae were injected with a standardized bacterial suspension compared with control group showed distinct morphological and histological changes indicating the bacterium's ability to cause disease.

Keywords: Conjunctivitis, *Brachybacterium*, *Galleria mellonella*, newborn, Pathogenicity

INTRODUCTION

Conjunctivitis is a common condition that significantly impacts quality of life due to recurrent irritations and uncomfortable symptoms resistance‹ (Kammrath *et al*., 2021; Cupta *et al*., 2023). Any pathogenic organism capable of reaching the eye can cause inflammation, potentially affecting one or both eyes. Infections range from conjunctivitis, which is limited to the outer layer of the eye and the inner surface of the eyelid, to more serious conditions that affect visual acuity, such as keratoconjunctivitis and endophthalmitis, which can cause blindnes (Taha *et al*. 2024). Viral conjunctivitis accounts for approximately 70% of all cases, and 30% of these are of bacterial origin (Al-Eryani, 2021).

In general, viral infections are more commonly seen in the acute phase of conjunctivitis. Bacterial conjunctivitis is most often caused by *Haemophilus aegyptius, Neisseria gonorrhoeae, Neisseria meningitidis, Corynebacterium xerosis,* and *Acinetobacter calcoaceticus* in newborns. The main cause is bacterial transmission from the mother during childbirth, where these strains are pathogenic to both mother and infant. Common bacteria in neonatal intensive care units include *Staphylococcus aureus, Streptococcus pyogenes,* and *Streptococcus faecalis*, while *Aerobacter* is most common in premature infants. (Voinescu *et al*., 2025). The genus *Brachybacterium* was first discovered and recognized by scientists (Colliens *et al*., 1988). These bacteria are non-motile, facultatively anaerobic or aerobic, Gram-positive, and do not produce spores. Cells of different species of *Brachybacterium* are spherical during the stationary phase and rod-shaped during the exponential phase (Ziganshina *et al*.,2018).

Domain: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Order: Micrococcales
Family: Dermabacteraceae
Genus: *Brachybacterium*
Species: *Brachybacterium epidermidis*

This classification came after taxonomic studies conducted on some *Brachybacterium* was proposed as a new genus by scientists since *Brevibacterium* species did not suit the traits of that genus. There are now 25 species with accurate and recorded names. it can be isolated from various environmental sources, such as seawater, plant roots and branches, rivers, cheese surfaces, coastal sand, and soil. Few studies have reported its isolation from human samples (Wu *et al*., 2023). Species of this genus are ubiquitous and have been previously isolated from various sources, such as dogs, laboratory mice, insects, reptiles, fermented foods, poultry droppings, feces, and environmental samples (Hlaing *et al*., 2019). In 2003*, Brachybacterium muris* was initially identified from a lab mouse's liver (Buczolilts *et al*., 2003), and was subsequently cut off from China's Chaka Salt Lake in 2017 (Gu *et al*., 2017).

Additionally, it has been discovered in human nasal canals and skin (Leung *et al*., 2020). A pleural effusion patient was found to have contracted *Brachybacterium muris* (We *et al*.2023), it has also been isolated from a range of clinical specimens, including abscesses in the brain. (Bavbek *et al*., 1998), cultures of blood (Gómez Garcés *et al*., 2001), peritoneal fluid (Radtke *et al*., 2001) and fatal septicemia (Lee *et al*., 2011). It is regarded as an opportunistic pathogen as a result. (Tak *et al*., 2018). Diagnoses at the species level are helpful in elucidating the pathogenic characteristics of *Brachybacterium* species that have been isolated from the human bloodstream. (Tamai *et al*., 2018). A healthy 67-year-old woman's right hand was found to harbor a novel bacterium species called *B. epidermidis* (strain Marseille-Q2903) as part of a study that used cutting-edge bacterial culturing techniques to investigate the human microbiome. (Boxberger *et al*., 2022).

G. mellonella larvae, also referred to as the "great wax moth," have become a substitute model organism in infectious illness research in recent years, especially for examining internal bacteria and

assessing the efficacy of medications. Due to the numerous benefits these larvae offer over vertebrate animal models, interest in them is expanding. They can be cultivated at 37°C, which is a temperature that many human pathogens can grow at, and practically speaking, they do not require expensive laboratory equipment. (Asai *et al*., 2023). Also, the immune system of *G. mellonella* is functionally extremely similar to vertebrate systems. It is made up of humoral reactions, which include the release of antimicrobial peptides and defensive enzymes like prophenol oxidase, which uses myelinization to detect infection, as well as blood cells (such leukocytes) that carry out phagocytosis. (Browne *et al*., 2013; Hillyer, 2016).

This model's speed of infection and visual monitoring of illness progression through limited mobility or blackening of the larvae's body are noteworthy features. (Li *et al*., 2018).

Additionally, research has demonstrated that some bacterial strains pathogenicity in *G. mellonella* larvae is frequently comparable to that seen in mammalian models, which makes this model highly helpful for evaluating the early effectiveness of medications prior to doing animal or human trials (Tsai *et al*., 2016; Dinh *et al*., 2021). Moreover, the application of *G. mellonella* is consistent with the worldwide movement to lessen the dependence on vertebrates in scientific research, and the *G. mellonella* model is becoming a more significant and ethical tool for acquiring crucial and trustworthy primary data. (Ménard *et al*.,2021).

MATERIALS AND METHODS

At Mosul General Hospital, Ibn Al-Atheer Hospital, and Al-Salam Teaching Hospital, 100 conjunctival swab samples were taken from newborns with conjunctivitis. The swabs were cultured on blood agar, chocolate agar, MacConkey agar, and mannitol salt agar. They were incubated at 37°C for 24-48 hours using a candle jar and using catalase & oxidase tests were performed to identify the bacterial isolates under study (Bauman, 2018).

Molecular Diagnosis

DNA extraction was performed using the Geneaid extraction kit. To determine the sequence of the isolates under study, universal primers for the *16S rRNA* gene were used, as shown in (Table 1).

These primers were obtained from American company Promega. Each primer was dissolved in deionized water to serve as a stock solution at a final concentration of 100 pmol/µL. and the total PCR reaction is 25 µL as shown in (Table 2). (Al-najim; Alsammak., 2024) (Al-jarjary and Alsammak, 2024)

Table (1): universal primers for determining the sequences *16S rRNA*

Gene	Primer	Sequence	Reference
16S rRNA	27F	5'-AGAGTTTGGATCTGGCTCAG-3'	(Salih *et al*., 2019)
	1492R	5'-CGGTTACCTTGTTACGACTT-3'	

Table (2): PCR reaction Mixture

No.	Component	Size in µL
1.	Master Mix	12.5
2.	Forward primer	1.5
3.	Reverse primer	1.5
4.	Dd H2O	5.5
5.	DNA template	4
	Total size	25

DNA amplification, according to the program shown in (Table 3).

Table (3): PCR conditions for amplification the gene of *16S rRNA*

Steps	Temp.	Time	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	30
Annealing	55 °C	30 sec	30
Extension	72 °C	1 min	30
Final extension	72 °C	3 min	3

One hundred base pair DNA ladder was used to estimate the sizes of PCR products, and it was loaded into the first well of the agarose gel. After the gel was fully prepared, it was submerged in 1x TBE running buffer, and electrophoresis was initiated at 12 volts, gradually increasing to 65 volts for one hour. The gel was examined under ultraviolet (UV) light at a wavelength of 320 nm.

Pathogenicity test on *Galleria mellonella* larvae

1. Last instar larvae of *G. mellonella* were collected from the College of Agriculture, University of Mosul. Selected larvae weighed between 200 and 250 mg and measured 1.5-2.5 cm in length. Only light-colored larvae without dark spots or pigmentation were used.
2. Larvae were placed in Petri dishes and maintained at 37°C for experimentation.
3. For bacterial inoculum preparation, 5 mL of nutrient broth was used culture, incubated for 18-24 hours. Bacterial Cultures were centrifuged at room temperature at 6000 rpm for 7 minutes.
4. The supernatant was discarded, and the pellet was resuspended in 1 mL of phosphate-buffered saline (PBS). The bacterial suspension was then diluted in PBS to an OD600nm equivalent to approximately 1×10^9 CFU/mL.
5. Using an insulin syringe, 10 µL of the diluted bacterial suspension was injected into the second last left proleg of each larva (three larvae per group) after disinfecting the injection site with 70% ethanol. To confirm that the injection procedure did not itself cause mortality, a control group of three larvae was injected with 10 µL of sterile PBS. All larvae were incubated in the dark at 37°C for 24 to 72 hours.
6. The larvae were observed to change color from light brown to dark brown, then black, and were considered dead when touched (Andrea *et al*., 2019).

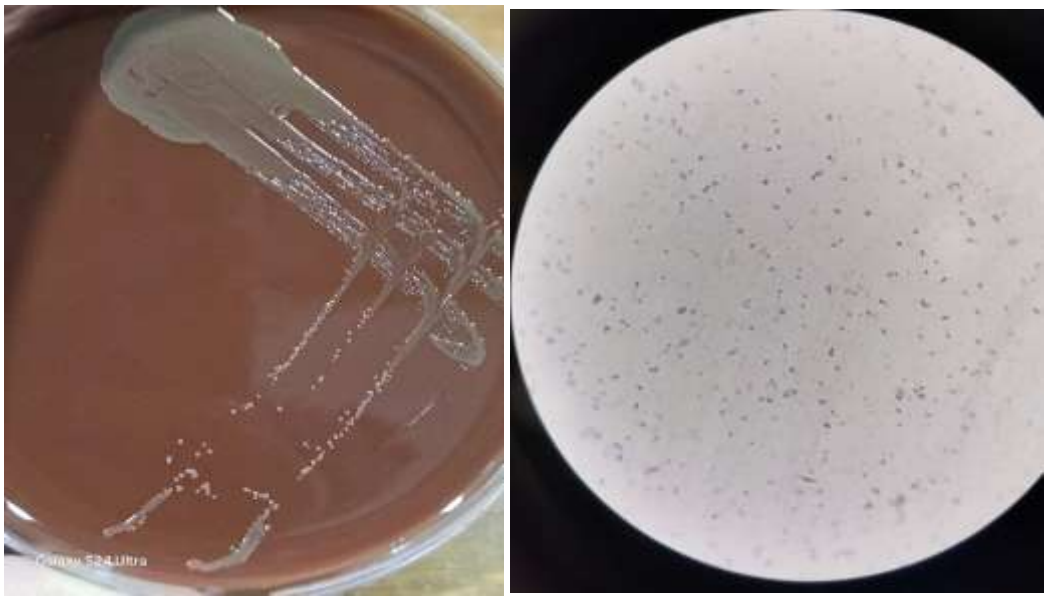
Histological studies:

Dead larvae injected with bacteria and control larvae were preserved in a 10% formalin solution. The preserved larvae were sent to the College of Veterinary Medicine, University of Mosul, for histological examination according to the method (Emery *et al.*, 2019). Before immersing them in wax, the larvae were divided into segments: the head, the midgut, and the posterior (anal) segment, and preserved in 70% ethanol. Samples were dried using series concentrations of ethanol (70%, 80%, and 90%) for one hour for each sample. The samples were then washed three times in 100% ethanol for one hour each to remove any residual traces. The dried samples were washed twice in HistoClear for one hour before being fully immersed in wax. The samples were then resuspended in paraffin wax for one hour. A microtome was used to cut the embedded samples into 6-micrometer-thick slices, which were then fixed on glass slides using egg albumin (1% w/v) and left to dry for 24 hours before staining. Hematoxylin and eosin staining was used to stain the mounted slides.

RESULTS AND DISCUSSION

One *B. epidermidis* isolate was identified from a total of 100 samples. the colonies appeared as small yellow to green, and the cells were spherical under the microscope, Fig. (1: A, B). They were catalase-positive and oxidase-negative. The isolation bacteria are

44% and 2.27% one strain *B. epidermidis* rate was 2.27% as Fig. (2) of the total 100 pathological samples.



A **B**

Fig. 1: *Brachybacterium epidermidis* in A chocolate ager and B the cells under microscopic at 100x.

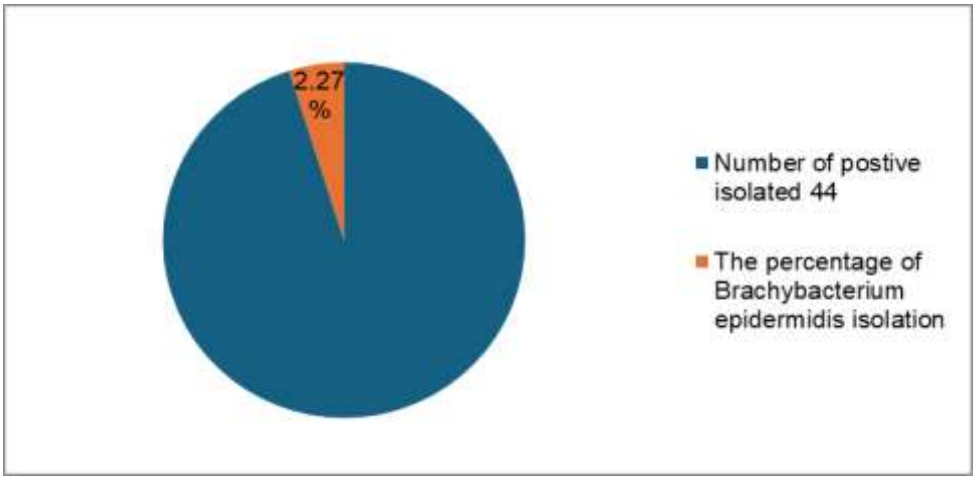


Fig. 2: The percentage of *Brachybacterium epidermidis* isolation

The universal primer *16S rRNA* was used for molecular identification of the isolate, as bands appeared in comparison with the ladder, as shown in Fig. (3)

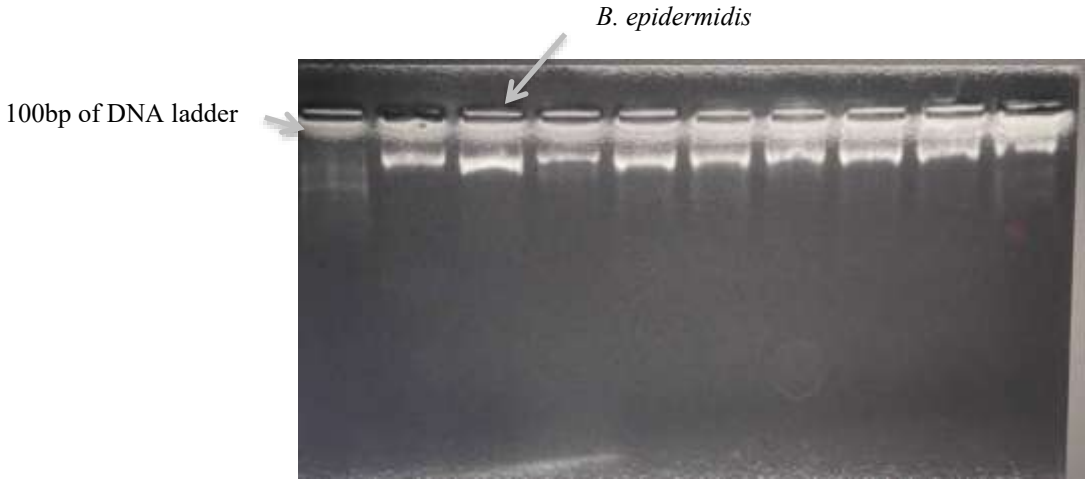


Fig. 3: Amplification of the *16S rRNA* gene

The obtained sequence of the isolate was compared with reference sequences using the Basic Local Alignment Search Tool (BLAST) available on the NCBI database. The results confirmed that the isolate belongs to *B. epidermidis*, showing a similarity range of 96-100% with the reference strain. Following the injection of *B. epidermidis* into *G. mellonella larvae,* phenotypic changes were observed. The larvae changed in color from light brown to dark brown and eventually to black within 24 hours. In contrast, the control larvae remained active and maintained their normal coloration, as shown in Fig. (4)

Fig. 4: The difference between infected *Galleria mellonella* larvae and the control larvae.

Histological results on *G. mellonella* larvae revealed the effects of infection with *B. epidermidis* bacteria, demonstrating clear changes in infected larvae compared to control larvae.

Histological sections of uninfected larvae showed the integrity of various tissues, such as the skin, skeletal muscles, silk glands, adipose tissue, respiratory system, and gastrointestinal tract, indicating absence of pathological alterations and the absence of any signs of inflammation or necrosis Fig. (5), Fig. (6) and Fig. (7).

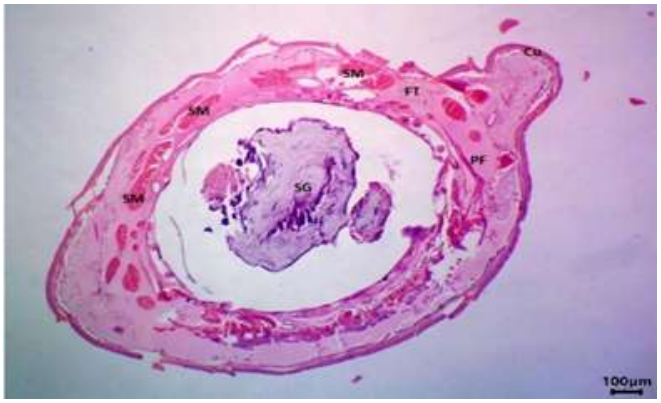


Fig. 5: Transverse histological section of the Galleria mellonella larvae of the control group shows the intact histology of the cuticle (Cu), skeletal muscles (SM), silk glands (SG) and fat tissue (FT). Hematoxylin & Eosin stain, 40X, Scale bar=100µm.

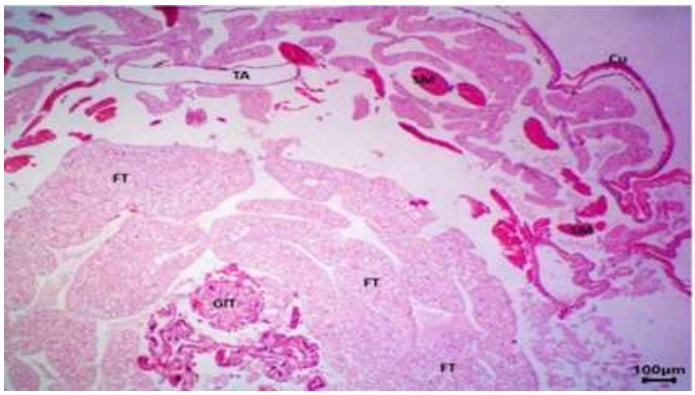


Figure 6: Transverse histological section of the Galleria mellonella larvae of the control group shows the intact histology of the cuticle (Cu), skeletal muscles (SM), tracheal apparatus (TA), gastrointestinal tract (GIT) and fat tissue (FT). Hematoxylin & Eosin stain, 40X, Scale bar=100µm.

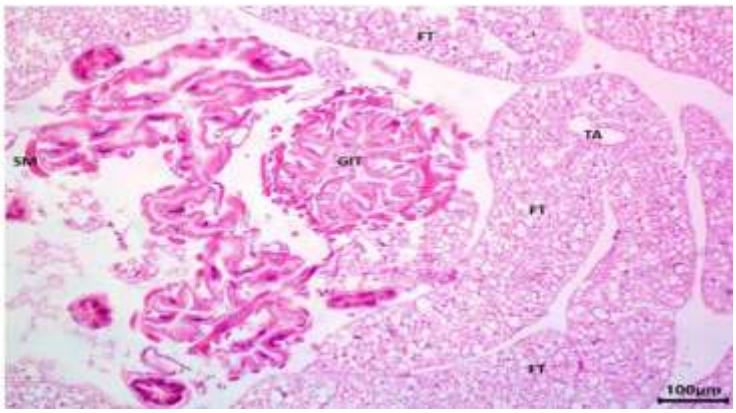


Figure 7: Transverse histological section of the Galleria mellonella larvae of the control group shows the intact histology of the cuticle (Cu), skeletal muscles (SM), tracheal apparatus (TA), gastrointestinal tract (GIT) and fat tissue (FT). Hematoxylin & Eosin stain, 100X, Scale bar=100µm.

In contrast, larvae infected with *B. epidermidis* exhibited multiple pathological histological signs, including:

1. Disruption and rupture of the cuticle, which may indicate degradation of the larval protective barrier due to enzymatic activity or bacterial toxicity.

2. Dense aggregations of hemocytes, representing an early immune response to the infection.
3. A noticeable increase in proteinaceous fluid, which may be associated with enhanced vascular permeability or cellular death.
4. Gastrointestinal tract necrosis and epithelium shrinkage are clear signs of infection-induced cellular damage.
5. Deterioration of the structure of the muscle fibers combined with skeletal muscle necrosis.
6. Melanized nodules, a recognized immunological signature that encapsulates pathogenic pathogens, are seen in *G. mellonella* (Browne *et al*., 2013).

These histological alterations, which include nodule formation, melanin synthesis, and arterial phagocytosis, are a definite sign of *G. mellonella* typical immunological response (Tsai *et al*., 2016). These findings align with those published in research by (Tsai *et al*. 2016). It showed how bacterial infections in *G. mellonella* can seriously harm tissue, particularly when very aggressive bacterial strains are used.

A recent study by Asai *et al*. 2023, supports the use of as a substitute model for researching bacterial virulence and immunological tests since it has a widely recognized immune response that is comparable to that of mammals. The same study was released by Iwański *et al*. 2023, noticed comparable histological alterations, such as pigment nodules, muscle tissue necrosis, and structural degradation, in *Pseudomonas aeruginosa* infections. The mechanism of action of *Shigella* bacteria on *G. mellonella* larvae was studied. The results showed that mortality rates depended on the type of strain and the dose of infection. Melanin pigmentation was also shown to be a common indicator of larval response to stress or infection, as this response causes the larvae to change color from their normal cream color to dark brown or black. Experiments showed that high concentrations of bacteria spread rapidly within the larvae compared to low concentrations, causing a clear change in the larval appearance, indicating the severity of the disease reaction, as a show in (fig from 8 to 12) (Barnoy et al., 2017).

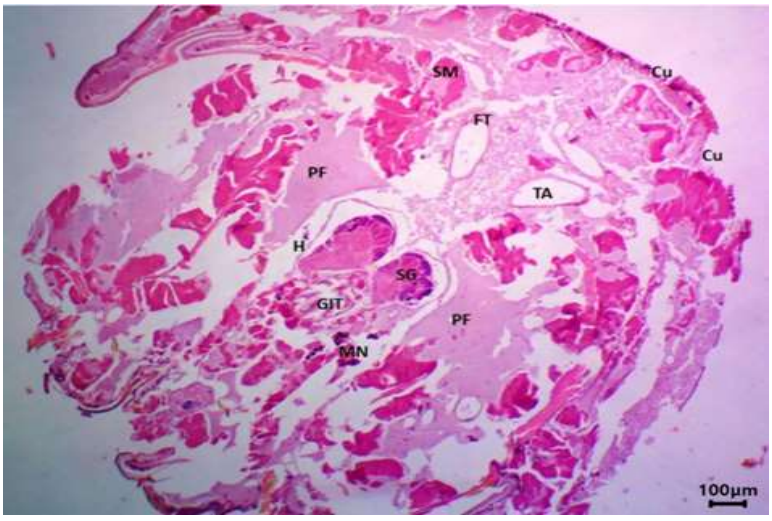


Fig. 8: Transverse histological section of the Galleria mellonella larvae of the bacterial infected group shows the interruption of the cuticle (Cu), presence of silk glands (SG), aggregates of hemocytes (H), increase proteinaceous fluid (PF) necrosis in gastrointestinal tract (GI), necrosis in the skeletal muscles (SM), and presence of melanized nodule (MN). Hematoxylin & Eosin stain, 40X, Scale bar=100µm.

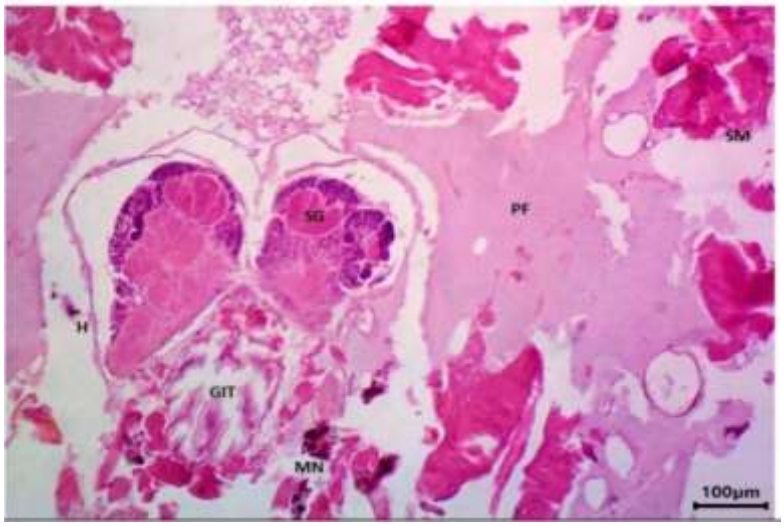


Fig. 9: Transverse histological section of the Galleria mellonella larvae of the bacterial infected group shows the interruption of the cuticle (Cu), presence of silk glands (SG), aggregates of hemocytes (H), increase proteinaceous fluid (PF) necrosis in gastrointestinal tract (GI) with shortened of epithelium (arrows) necrosis in the skeletal muscles (SM), and presence of melanized nodule (MN). Hematoxylin & Eosin stain, 100X, Scale bar=100µm.

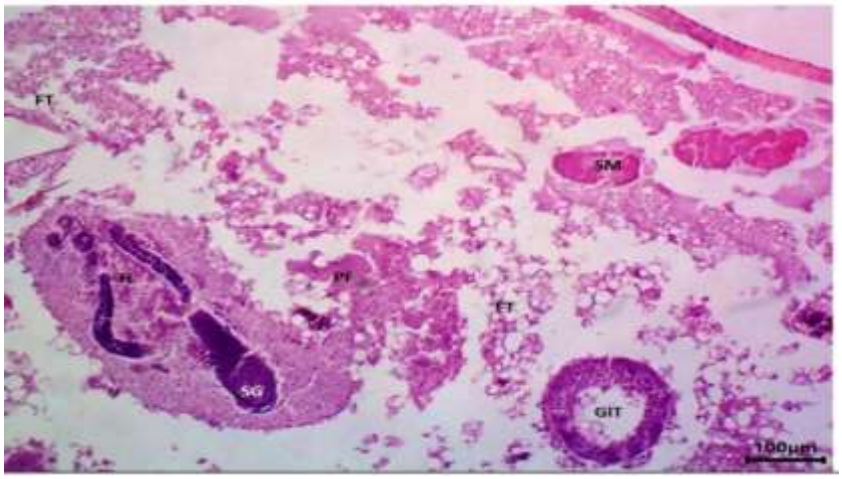


Fig. 10: Transverse histological section of the Galleria mellonella larvae of the bacterial infected group shows the interruption and loss of the fat tissue (FT), presence of silk glands (SG), aggregates of hemocytes (H), increase proteinaceous fluid (PF) necrosis in gastrointestinal tract (GI), necrosis in the skeletal muscles (SM). Hematoxylin & Eosin stain, 40X, Scale bar=100µm.

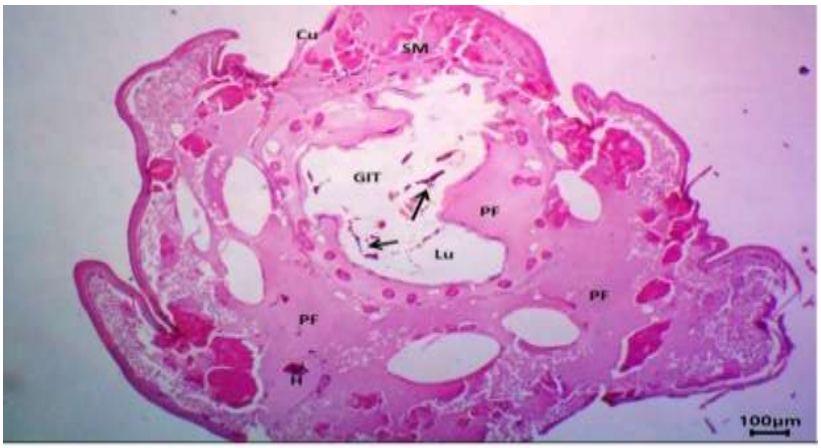


Fig. 11: Transverse histological section of the Galleria mellonella larvae of the bacterial infected group shows the interruption and loss of the cuticle (Cu), aggregates of hemocytes (H), increase proteinaceous fluid (PF) necrosis in gastrointestinal tract (GI) with sloughing of the epithelial cells (arrows) in the lumen (Lu), and necrosis in the skeletal muscles (SM). Hematoxylin & Eosin stain, 40X, Scale bar=100µm.

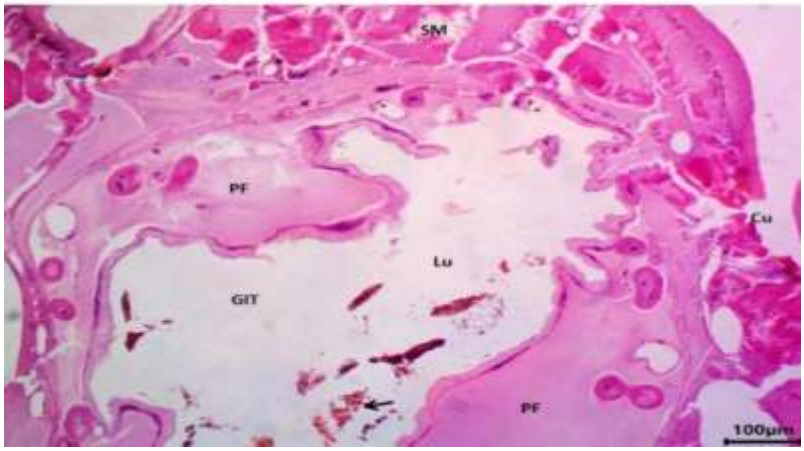


Figure 12: Transverse histological section of the Galleria mellonella larvae of the bacterial infected group shows the interruption and loss of the cuticle (Cu), increase proteinaceous fluid (PF) necrosis in gastrointestinal tract (GI) with sloughing of the epithelial cells (arrows) in the lumen (Lu), and necrosis in the skeletal muscles (SM). Hematoxylin & Eosin stain, 100X, Scale bar=100µm

CONCLUSIONS

B. epidermidis isolated for the first time in Iraq from a case of neonatal conjunctivitis and reported for only the second time worldwide, induced histopathological alterations in the larvae of demonstrated a clear ability to induce significant pathological effects in the tissues of *G. mellonella* larvae.

These results represent an important scientific addition, as they are among the few studies that shed light on the tissue-mediated effects of this bacterium on this model organism. This opens the door for further studies on the mechanisms of bacterial virulence and resistance using alternative vertebrate animal models.

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استخدام يرقة *Galleria mellonella* للتحري عن الضراوة المرضية لبكتريا *Brachybacterium epidermidis* المعزولة من التهاب ملتحمة العين لدى حديثي الولادة في مدينة الموصل

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الملخص

تم جمع 100 عينة سريرية من أطفال حديثي الولادة المصابين بالتهاب ملتحمة العين من ثلاثة مستشفيات في مدينة الموصل: مستشفى السلام التعليمي، مستشفى الموصل العام، ومستشفى ابن الأثير. أظهرت 44% من هذه العينات نمواً على الأوساط الزرعية، وكانت نسبة عزل بكتيريا *Brachybacterium epidermidis* 2.27% وهي المرة الأولى التي يتم فيها عزل هذا النوع البكتيري من حالات التهاب الملتحمة في مدينة الموصل/العراق

واظهرت Brachybacterium *epidermidis* نمواً على وسط أكار الدم المطبوخ(Chocolate agar) ، وتم تشخيصها مظهريًا، ثم أُجري عليها التشخيص الجزيئي باستخدام البادئ العام *16S rRNA* تمت مقارنة نتائج تسلسل القواعد النوكليوتيدية للعزلة مع التسلسلات القياسية المثبتة في National Center for Biotechnology Information (NCBI)باستخدام أداة(BLAST (Basic Local Alignment Search Tool ، وأكدت النتائج أن العزلة تعود إلى *Brachybacterium epidermidis* اذ أظهرت نسبة تشابه 100% مع Marseille-Q2903.

ولغرض تقييم التأثير الحيوي لهذا النوع البكتيري Marseille-Q2903 *Brachybacterium epidermidis*، تم استخدام يرقات *Galleria mellonella*، حيث حُقنت مجموعة من اليرقات بمعلق بكتيري قياسي، في حين حُقنت مجموعة السيطرة بمحلول منظم. أظهرت اليرقات المصابة تغيرات مظهرية ونسيجية واضحة مقارنة بمجموعة السيطرة، مما يشير إلى قدرة البكتيريا المعزولة على إحداث تأثيرات مرضية.

الكلمات الدالة: التهاب المخيخ، *Brachybacterium*، *Galleria mellonella*، حديثي الولادة، الامراضية.