Study the pathogenicity of the bacteria associated with 
*Periplanta americana* cockroach on *Gelleria mellonella* L worm larva

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**Abstract.** In this study, the bacterial genera associated with the *Periplaneta americana* cockroach were isolated from the residential areas and nurseries in Baghdad city with concentrations 10⁻², 10⁻⁴, 10⁻⁶ and their effect on the larvae of *Gelleria mellonella* L worm was studied in the laboratory by food spraying method. Conclusion: The results showed that the highest percentage of larvae mortality was at the concentration 10⁻² (33.33%) for both *Acinetobacter lwoffii* and *Enterobacter cloacae* bacteria. While the highest rate of larvae mortality was at the concentration 10⁻⁴ (33.33%) for each of *Pseudomonas oleovorans*, *Acinetobacter baumannii*, *Acinetobacter lwoffii* and *Klebsiella pneumoniae*, whereas the concentration 10⁻⁶ showed the highest mortality rate (50%) of *Pseudomonas oleovorans* and *Klebsiella pneumoniae* bacteria. Results of the study showed predominance of the effect of *Pseudomonas oleovorans* and *Klebsiella pneumonia* on the mortality rate of the larvae of the great *Gelleria mellonell* L. worm as it was 50%, while the effect of *Enterobacter cloacae* and *Pseudomonas oleovorans* yielded the highest mortality rate of 33.33% at each of the concentrations 10⁻², 10⁻⁴, 10⁻⁶ whereas the results showed that the concentration 10⁻² for each of *Proteus mirabilis*, *Escherichia coli*, and *Enterobacter aerogenes* had no effect on larvae mortality.

**Key words:** *Periplaneta americana*, *Gelleria mellonella* (L.) worm, Bacterial isolation and diagnosis

1. **Introduction**

*Periplaneta americana* cockroach belongs to order Dictyoptera, suborder Blattaria and Blattidae family, and it is the oldest order of insects, as its existence dates back more than 300 years, where there are about 3500 - 4000 species distributed around the world, but a small number of them are considered disease transmitters [1]. The *Periplaneta americana* cockroach is the most widespread species where it is found in homes, hospitals, health care centers, nurseries, warm and humid places and places where food waste is found such as kitchens and sewers [2][3]. It is considered the most notorious type of insects because of its existence in the human environment, as it causes contamination to the places in which it is found and contaminates foodstuffs through waste and bacteria that it leaves and causes food poisoning, in addition to the transfer of pathogens from one region to another [4].
Cockroaches are nocturnal living insects and feed randomly on waste and sewage water, so they are effective means of transmitting diseases [5] as they feed on human feces, and thus they transfer the internal protozoa in the environment where the feces are found [6]. The mechanism of random transfer between dirty places and food stores made these insects effective vectors for protozoal parasites [7]. In addition, their outer cuticle represents a store for different types of microorganisms that survive on the outer parts of the cockroach body and are transmitted to the environment through contact with objects [8]. The presence of these insects near food and waste made them causative agents of many diseases such as tuberculosis, cholera, leprosy, typhoid and dysentery [9] [10] [11] [12].

Several studies indicated that cockroaches are responsible for transmission of many communicable diseases because they are home insects found in (bathrooms, kitchens and gardens), where at least 32 bacterial species were isolated from cockroaches in the local environments [12].

2. Materials and methods

In the current study, (50) samples of adult Periplaneta americana cockroaches were collected from different areas in Baghdad including (homes, kitchens, sewers, and bedrooms) and the nurseries in Adhamiya and Sab’ abkar quarters. These places were chosen randomly because their inhabitants did not use pesticides recently in Baghdad for the period from 14 / 09/2020 to 01/11/2020, where insect samples were collected the day before their dissection. The insects were placed after catching them in sterile tubes and transferred immediately to the laboratory.

2.1 Isolation and diagnosing bacteria from Periplaneta americana cockroaches

After the insects were diagnosed as Periplaneta americana cockroaches according to a taxonomy key for insects [13], the cockroaches were dissected after being placed in the refrigerator at 0°C for five minutes to anesthetize and immobilize them. Then the cockroaches were fixed with pins on the petri dish and paraffin wax, and the external parts of the insect such as tentacles, legs and wings were cut with scissors and their digestive systems were isolated and the fat bodies surrounding the internal organs were removed by anatomical needles and fine tweezers and placed in test tubes containing 2 ml of 0.85% normal saline and shaken vigorously for two minutes. The insect's gastrointestinal tract was isolated and placed in a sterile pestle with 2 ml of sterile saline solution for the purpose of soaking, after that it was crushed and a swab was taken from the infusion to be grown on the pre-prepared Brain Heart, blood agar and mackonky agar and incubated at 37 °C for 24 hours. The bacteria were diagnosed after performing morphologic and microscopic examinations according to the references specific for bacterial diagnosis [14] and confirmatory diagnosis was done using the Vitek 2 device. A series of bacterial concentrations (10-2,10-4,10-6) were prepared according to the methods of [15] [16].

2.2 Confirmation of bacterial pathogenicity on G. mellonella (L.) worm.

Frames of infected beeswax were collected for different ages for the G. mellonella.L moth. The Gelleria mallonella moth was grown after obtaining the larvae from infected wax collected from different apiaries in Baghdad, Rashidiya area. The larvae were placed in a special container for breeding containing empty black wax (honeycomb) to feed on it and the insects were placed in appropriate conditions. At the same time, the preparation of treatment of food containing black beeswax which was previously used in bee and pollen grains was performed. They were transported to the laboratory and monitored until virginity and for the purpose of obtaining pure permanent cultures. New virgins were placed in Petri dishes and transferred to wooden breeding cages with dimensions of 30 x 30 x 30 cm. The base of the cage was made of wood and its front sides were made of cloth. The virgins were monitored until the adults emerged and stimulated to lay eggs.

The different larval stages of the Gelleria mallonella worm and their adults were collected from the beekeeping farms in Rashidiya. The larvae were collected by tweezers and placed in special cold-
sterilized plastic containers provided with pieces of dark wax for the purpose of larvae feeding [17] [18].

The isolates were activated by growing them on sterile brain heart infusion agar (BHIA) for 24 hours at 37°C, then they were grown on sterile liquid Nutrient broth for 24 hours at 37°C. A series of dilutions up to 10^-6 were prepared, and the experiment was carried out. Then, (162) larva were used and distributed over (9) dishes, each containing (6) larvae. The wax discs with 3x3 cm in length and pollen grains for each concentration were taken and the hexagonal cells were flooded with the three concentrations used in the treatment, and then one pollen-containing disc was put in each larva-containing dish, and the edges and covers of the dishes were lubricated with Vaseline to prevent the escape of the larvae to the top and to ensure that the larvae were fed on the food treated with bacteria. The dishes were covered tightly with covers that contain holes for ventilation and tied with a rubber band, then the mortality rates were recorded after (24, 48 and 72) hours of the treatment.

3. Results and discussion

In our study, (90) bacterial isolates from (50) Periplaneta americana cockroaches were isolated. Diagnostic tests mainly revealed strains belonging to Gram-negative (-ve) enterobacteria, which included 9 different bacterial genera from both the insect’s outer surface and the digestive system for Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, in addition to genera of Acinetobacter baumannii, Acinetobacter lwoffii, Pseudomonas oleovorans, Enterobacter aerogenes, Kluyvera cryocrescens, Pseudomonas aerogenes. The most prevalent recorded species were Escherichia coli (20%), followed by Klebsiella (20%), Enterobacter cloacae (8%), Acinetobacter lwoffii (4%), Acinetobacter baumannii (4%), Pseudomonas oleovorans (8%), Pseudomonas aerogenes (8%), Proteus mirabilis (8%) and Kluyvera cryocrescens (4%).

Table 1. Distribution and isolation rate of bacterial genera isolated from home Periplaneta americana cockroaches.

| Bacterial genera               | Bacterial isolate numbers | Isolation rate from homes (%) |
|-------------------------------|---------------------------|-------------------------------|
| Acinetobacter lwoffii         | -                         | 0.0                           |
| Pseudomonas oleovorans        | 4                         | 8                             |
| Acinetobacter baumannii       | -                         | 0.0                           |
| Klebsiella pneumoniae         | 10                        | 20                            |
| Kluyvera cryocrescens         | 2                         | 4                             |
| Proteus mirabilis             | 4                         | 8                             |
| Enterobacter aerogenes        | 4                         | 8                             |
| Enterobacter cloacae          | 4                         | 8                             |
| Escherichia coli              | 10                        | 20                            |
| aerogenes Pseudomonas         | 4                         | 8                             |
| Serratia fonticola            | 3                         | 6                             |
| Total                         | 45                        | 90                            |
Table 2. Distribution and isolation rate of bacterial genera isolated from nursery Periplaneta americana cockroaches.

| Bacterial genera       | Bacterial isolate numbers | Isolation rate from nurseries (%) |
|------------------------|---------------------------|----------------------------------|
| Acinetobacter lwoffii  | 2                         | 4                                |
| Pseudomonas oleovorans| 4                         | 8                                |
| Acinetobacter baumannii| 2                         | 4                                |
| Klebsiella pneumoniae  | 10                        | 20                               |
| Kluyvera cryocrescens  | 2                         | 4                                |
| Proteus mirabilis      | 4                         | 8                                |
| Enterobacter aerogenes | 4                         | 8                                |
| Enterobacter cloacae   | 4                         | 8                                |
| Escherichia coli       | 10                        | 20                               |
| Serratia fonticola     | 3                         | 6                                |
| **Total**              | **45**                    | **90**                           |

The results of this study showed a wide distribution of pathogenic bacteria pathogens carried on the body and intestine of the Periplaneta americana cockroach, which were chosen from different areas in Baghdad. This finding is similar to the results reported by a study conducted by [19].

The presence of the cockroach in the environment poses a great threat due to its ability to carry and transmit many pathogenic bacterial species [20]. It was shown that Periplaneta americana cockroach can carry and spread pathogenic bacteria, especially members of the Enterobacteriaceae and Pseudomonas family through body parts, faeces and other deposits [20]. In France, nine bacterial species were isolated from the Periplaneta americana cockroach five of which were pathogenic to humans and animals such as E. coli and Klebsiella pneumonia [7],[13]. The results in the study showed that the isolated bacterial species were close to those isolated in the study performed by [21]. The predominance of both E. coli and Klebsiella isolated from the nurseries and houses coincides with the studies of [22] and [23] where the statistical analysis showed no significant differences between bacterial species and the areas of sample collection.

3.1 Confirmation of bacterial pathogenicity on the larva of Galleria mellonella L moth.

Nine different species of bacteria isolated from the Periplaneta americana cockroach were placed on Galleria mellonella L moth at three different concentrations (10^-2, 10^-4 and 10^-6) for all bacterial species, and after 7 days follow-up, the results of table (3) showed the presence of death and carbonization of the treated larvae, as the highest mortality rate (33.33%) was recorded at the concentration 10^-2 for Acinetobacter lwoffii and Enterobacter cloacae, which caused a change of the color of the dead larvae to brown as shown in the photo No. (1)

![Photo 1](image-url)  
**Photo 1.** Effect of bacterial concentrations on the larva of Galleria mellonella L moth.
A- Larva discoloration and blackening after treatment with $10^{-2}$ concentration of *Acinobacter lwoffi* bacteria

B- Larva discoloration after treatment with $10^{-2}$ concentration of *Acinobacter lwoffi* bacteria

No larva emerged after treatment with 10-2 concentration of *Acinobacter lwoffi* bacteria

While the mortality rate was (16.66%) at the concentration 10-2 for *Pseudomonas oleovorans*, Klebsiella pneumonia, and *Kluyvera cryocrescens*, and the mortality rate was 0% when treated with *Acinetobacter baumannii*, Proteus mirabilis, *Enterobacter aerogenes* and *Escherichia coli*. Table (4) showed that the highest larval mortality rate (33.3%) was at the concentration of 10-4 for each of *Pseudomonas oleovorans*, *Acinetobacter baumannii*, *Acinetobacter Iwoffii*, *Enterobacter cloacae* and Klebsiella pneumonia as they caused larval carbonization. Each of *Kluyvera cryocacteris*, *Escherichia coli*, *Enterobacter aerogenes* and *Proteus mirabilis* recorded (16.66%) mortality rate, while the highest mortality rate (50%) occurred at 10-6 concentration for *Pseudomonas oleovorans* and Klebsiella pneumonia. And (33.3%) for each of *Acinetobacter baumannii* and *Acinetobacter Iwoffii*, whereas the mortality rate was (16.66%) for each of *Kluyvera cryocrescens*, *Escherichia coli*, *Enterobacter aerogene* and *Proteus mirabilis*, and the mortality rate in the control treatments was (0.0%) as shown in table (5).

These findings agree with the results of the study of [24] in using gram negative bacteria to determine the virulence of each of *Pseudomonas aeroginosa* *Escherichia coli*, Klebsiella pneumonia and *Acinetobacter baumannii* as the infection rate with *Pseudomonas aeroginosa* increased leading to proteolysis of elastase B serine protease IV enzymes which play a role in the deterioration of the lipoprotein APOlip III. Studies demonstrated that *P. aeruginosa* strain is pathogenic to insects and causes major changes in morphology and ability to spread, and ultimately causes apoptosis of granulocytes and plasmocytes. This has been associated with significant changes in the morphology, vitality and spread ability of immunocompetent blood cells [25].

Data in the study of [26] indicated that *E. cloacae* has thermal control of virulence factors or that in *G.mellonella* the larva are more vulnerable to infection at the highest levels of temperatures, but yet the mechanisms of action of *E. cloacae* are not known. Regardless of the differences between pathogenicity of species, it was noted that the most obvious strains of *Acinetobacter* is *A. baumannii* and the infectious syndrome that causes them, followed by *A. Iwoffii*, which is the most distinct clinical type that causes bacteremia, acute respiratory distress syndrome (pneumonitis) and disseminated intravascular coagulation (DIC). The mechanisms of action of this pathogen are still unknown [27].

The *G. mellonella* moth was used in facilitating the pathogenicity examination of the bacteria isolated from the *Periplaneta americana* cockroach, where it has been used to determine the virulence of different species of human pathogens. *G. mellonella* possesses a number of advantages over conventional mammals such as developed cellular defenses, including the production of antimicrobial peptides which is similar to the immunologic response of mammals, as well as blood cells which are the main cell mediated immunity that perform the same functions of neutrophils and macrophages and they are not subject to the ethical limitations of mammals, and they can be easily maintained at 37°C, and they are suitable for studying causative agents of human diseases. The *Mellonella larvae* were considered as an in vivo infection model. It can be observed that the killing rate of *G. mellonella* was dependent on the incubation temperature after infection, as the killing rate increased at 37°C compared to 25-27°C and this result is similar to the study conducted by [27]. *A. Iwoffii* and *E. cloacae* strains caused greater killing, and their pathogenic mechanisms are still unknown, a result which is close to what was reported by [26] and [27]. Data in the study of [26] showed that *E. cloacae* has thermal controlled virulence factors and that the larva of *G. mellonella* moth are less subject to infection at high temperature levels, but yet little is known about mechanism of action of *E. cloacae* pathogen.

One of the most important symptoms appeared on the larva is the lack of movement and carbonization of the infected larvae. The effectiveness of the pathogenic bacteria on insects is often
related to the appropriate application. In case of the products that function throughout ingestion, it was proven that bacterial toxins remain in the environment until they are ingested by insects, and after ingestion of bacteria by the host, they germinate in the midgut and may lead to the entry of toxic substances into the blood, leading to septicemia, or they may produce insecticidal and pathogenic toxins that interact with the epithelial cell receptors in the insect's midgut thus causing imbalance of membrane permeability, degeneration, paralysis and death of epithelial cells, or they produce proteinous toxins such as the Sep proteins (SepA, SepB and SepC), which is a group of insecticidal toxins or the production of some extracellular enzymes such as proteases, lipases, and chitinases that act as pathogens having the ability to suppress the insect's immune cells as seen in Serratia species [28].

Results in table (3) showed that the highest virginity rate (100%) of the larva was when they were treated with P. mirabilis, E. aerogenes, E.coli, and A. baumannii bacteria at 10-2 concentration, while each of P. oleovorans, K. pneumonia and Kluyvera cryocrescens bacteria recorded (83.33%) virginity rate at 10-2 concentration, and that Acinetobacter Iwoffii and E. cloacae recorded the lower virginity rate (66.6%). The concentration 10-4 recorded the highest virginity rate (83.33%) during the treatment with Kluyvera cryocrescens, P.mirabilis, E.aerogenes and E.coli, while this concentration recorded the lowest virginity rate (66.6%) with each of A. Iwoffii, A. baumannii, P. oleovorans and E. cloacae. The concentration 10-6 recorded the highest virginity rate (83.33%) with Kluyvera cryocrescens, Proteus mirabilis, En. Aerogenes and E. coli, and (66.6%) with E.cloaca, K.baumannii and P.oleovorans, while this concentration recorded the lowest virginity rate (50%) with P.oleovorans and Klebsiella pneumonia. These results were almost close to the findings of a study which showed that the strain of P.aerogenes pathogenic to insects caused changes in morphology and ability to spread and eventually apoptosis of granulocytes and plasma cells.

The infection model for a pathogenic Escherichia coli study was published in 2012 and showed that the larvae of G. mellonella could be killed by entero pathogenic Escherichia coli (EPEC) and indicated that EPEC does not remain within the hemolymph but rather is rapidly encapsulated in the pigment nodules by the larvae of G. mellonella. The activation of the humoral arm of the innate immune response to G. mellonella by induction of the enzyme prophenoloxidase that leads to changes in melanin color which is a common feature of many microbial infections of this organism. Worms that appear black (pigment) or do not move were considered dead and worms that were moving without stimulation or not stained were considered alive.

### Table 3. Confirmation of pathogenicity of bacteria on *Galleria mellonella* moth at 10^{-2} concentration.

| No. | Concentration 10^{−2} | Death and carbonization | Virginity without emergence % |
|-----|------------------------|-------------------------|-------------------------------|
| 1   | *Acinetobacter Iwoffii* | 33.33                   | 66.66                         |
| 2   | *Pseudomonas oleovorans* | 16.66                   | 83.33                         |
| 3   | *Acinetobacter baumannii* | 0                       | 100                           |
| 4   | *Klebsiella pneumoniae*  | 16.66                   | 83.33                         |
| 5   | *Kluyvera cryocrescens*  | 16.66                   | 83.33                         |
| 6   | *Proteus mirabilis*      | 0                       | 100                           |
| 7   | *Enterobacter aerogenes* | 0                       | 100                           |
| 8   | *Enterobacter cloacae*   | 33.33                   | 66.66                         |
| 9   | *Escherichia coli*       | 0                       | 100                           |
### Table 4. Confirmation of pathogenicity of bacteria on *Galleria mellonella* moth at $10^{-4}$ concentration.

| No. | Concentration $10^{-4}$ | Death and carbonization | Virginity without emergence % |
|-----|-------------------------|--------------------------|-------------------------------|
| 1   | *Acinetobacter lwoffii*  | 33.33                    | 66.66                         |
| 2   | *Pseudomonas oleovorans*| 33.33                    | 66.66                         |
| 3   | *Acinetobacter baumannii*| 33.33                   | 66.66                         |
| 4   | *Klebsiella pneumoniae*  | 33.33                    | 66.66                         |
| 5   | *Kluyvera cryocrescens*  | 16.66                    | 83.33                         |
| 6   | *Proteus mirabilis*      | 16.66                    | 83.33                         |
| 7   | *Enterobacter aerogenes* | 16.66                    | 83.33                         |
| 8   | *Enterobacter cloacae*   | 33.33                    | 66.66                         |
| 9   | *Escherichia coli*       | 16.66                    | 83.33                         |

### Table 5. Confirmation of pathogenicity of bacteria on *Galleria mellonella* moth at $10^{-6}$ concentration.

| No. | Concentration $10^{-4}$ | Death and carbonization | Virginity without emergence % |
|-----|-------------------------|--------------------------|-------------------------------|
| 1   | *Acinetobacter lwoffii*  | 33.33                    | 66.66                         |
| 2   | *Pseudomonas oleovorans*| 50                       | 50                            |
| 3   | *Acinetobacter baumannii*| 33.33                   | 66.66                         |
| 4   | *Klebsiella pneumoniae*  | 33.33                    | 66.66                         |
| 5   | *Kluyvera cryocrescens*  | 50                       | 50                            |
| 6   | *Proteus mirabilis*      | 16.66                    | 83.33                         |
| 7   | *Enterobacter aerogenes* | 16.66                    | 83.33                         |
| 8   | *Enterobacter cloacae*   | 16.66                    | 83.33                         |
| 9   | *Escherichia coli*       | 16.66                    | 83.33                         |
Photo 2. Effect of bacterial concentrations on *Gelleria mollenella*. L moth.

A- Discoloration and blackening of larva treated with *Acinobacter lwoffii* $10^{-4}$ concentration

B- Discoloration of larva after treatment with *Acinobacter lwoffii* $10^{-4}$ concentration

C- Virginity and non emergence of larva treated with *Acinobacter lwoffii* $10^{-4}$ concentration

Photo 3. Effect of bacterial concentrations on *Gelleria mollenella*. L moth.

A- Discoloration and blackening of larva treated with *Acinobacter lwoffii* $10^{-6}$ concentration

B- Virginity and non emergence of larva treated with *Acinobacter lwoffii* $10^{-6}$ concentration

C- Virginity and emergence of the control treatment

4. Conclusion

Results of the study showed predominance of the effect of Pseudomonas oleovorans and Klebsiella pneumonia on the mortality rate of the larvae of the great *Gelleria mellonell* L. worm as it was 50%, while the effect of Enterobacter cloacae and *Pseudomonas oleovorans* yielded the highest mortality rate of 33.33% at each of the concentrations $10^{-2}, 10^{-4}, 10^{-6}$ whereas the results showed that the concentration $10^{-2}$ for each of *Proteus mirabilis*, *Escherichia coli*, and Enterobacter aerogenes had no effect on larvae mortality.
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