HTTPS://DOI.ORG/ **10.5281/zenodo.23211636**



Purposeful scientific research as a pillar of sustainable development



Honey bees (*Apis mellifera L.*) as mechanical vectors of Gram-negative bacteria: a field and laboratory study in different environments in Iraq

Hamad Mohammed Jassim[1], Ahmed Ali Issa[2], Marwa Hassan [2]

[1] *Department of Life Sciences, Faculty of Sciences, University of Kirkuk, Kirkuk,Iraq*

[2]*Department of Life Sciences, Faculty of Sciences, University of Tikrit, Salahaddin, Iraq*

hamad.m.jasim@uokirkuk.edu.iq

ABSTRACT

This study aimed to isolate and diagnose Gram-negative bacteria from the wings and hind legs of honeybee workers (*Apis mellifera L.*) collected from different environments in the provinces of Kirkuk and Salah al-Din in Iraq, where 70 isolates were isolated from 76 samples, in addition to evaluating their susceptibility to certain antibiotics. Honeybee samples were collected from apiaries, farms, and home gardens, then cultured on selective and differential media. Isolates were performed on selective and differential media (MacConkey, EMB, Nutrient agar). The isolates were identified by morphological methods and biochemical tests (Gram, Oxidase, Catalase, TSI, Indole, MR-VP, Citrate, Urease, H2S, carbohydrate fermentation) and confirmatory diagnostic methods. Antibiotic sensitivity testing (Kirby–Bauer) was also performed on Mueller-Hinton agar medium.

The results showed the isolation of several Gram-negative bacteria, including *E. coli*, *Acinetobacter spp.*, and *Citrobacter koseri.* and *Klebsiella oxytoca*, with variation in distribution depending on the collection sites. Antibiotic susceptibility tests also showed that many isolates exhibited variation in test results between resistance and sensitivity to a group of commonly used antibiotics. These results suggest that honeybees may act as mechanical vectors for environmental bacteria and some potential pathogens, which is influenced by their foraging behavior and contact with the surrounding environment. The study highlights the importance of monitoring the spread of antibiotic resistance in microbial communities associated with pollinators.

Keywords: microbial diversity, bacteria, honey bees

1. Introduction

Insects have close relationships with microbes that colonize their bodies, both in the digestive tract and on external surfaces such as wings and legs. These microbes perform various roles, such as contributing to

digestion or providing protection against pathogens. Interest in studying these relationships has increased in recent years due to their important role in insect health and immunity [1].

Studies indicate that the composition of microbial communities in insects is influenced by several factors, including food type, physiological functions, and interaction with the environment. The insect body, including its external surfaces, is a suitable habitat for a wide variety of microbes, including both beneficial and pathogenic bacteria [2].

Honey bees (*Apis mellifera*) have been found to have complex microbiota that are not yet fully understood, comprising various species of bacteria. The most prominent of these groups are Gram-negative bacteria such as *Achromobacter*, *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, and *Pseudomonas* [3].

2. Materials and methods

2.1 Sample collection

Seventy-six samples of adult honeybee workers (*Apis mellifera L.*) were collected from different environments in the provinces of Kirkuk and Salah al-Din, including beehives in orchards, farms, and home gardens, during the collection period from April to July 2025. After collection, the workers were collected and placed in special queen cages to safely transport a limited number of samples to the laboratory. The bees were handled under sterile conditions, with the wings and hind legs carefully separated, and the samples were then placed in sterile tubes containing a saline peptone solution (0.1%) with Tween 80 (0.1% w/v) added to ensure the stabilization of bacterial cells and their safe transport to the laboratory. After transfer, the samples were incubated for 24 hours at 37°C under aerobic conditions to facilitate the isolation of Gram-negative bacteria from the external parts of the bees.

2.2 Isolation and diagnosis

Samples were collected from the wings and legs of honeybee workers and cultured on agar plates suitable for isolating Gram-negative bacteria, with the addition of 0.01% cycloheximide to prevent fungal growth. After distributing the samples on the agar surface, the plates were incubated at 37°C for 24 hours under aerobic conditions. Colonies with distinctive morphological characteristics in terms of color, shape, size, and surface texture were selected and purified on new plates to obtain pure isolates suitable for morphological and biochemical analyses. The isolates were diagnosed based on their macroscopic and microscopic characteristics, in addition to basic biochemical tests such as catalase, oxidase, IMViC, and TSI. To confirm bacterial identity, the BioMérieux API 20E system was used according to the attached instructions, which accurately confirmed the diagnosis of the target bacterial species.

2.3 Antibiotic sensitivity testing:

Antibiotic sensitivity testing of bacterial isolates was performed using the Kirby–Bauer method according to CLSI guidelines (2024) to assess the response of bacteria isolated from pooled samples on agar plates to specific antibiotics, adhering to standard conditions in terms of antibiotic concentration, medium thickness, and incubation period to ensure accurate and reliable results.

Table 1. Antibiotics used in the study

Antibiotic	Symbol	Antibiotic concentration
Penicillin	P	G10 UI
clavulanate –Amoxicillin	AMC	10/20 µg
Ceftrazidime	CAZ	30 µg
Ciprofloxacin	CIP	10 µg
Ceftriaxone	CRO	10 µg
Cefotaxime	CTX	10 µg
Gentamycin	CN	10 µg
Levofloxacin	LEV	5 µg
Trimethoprim + Sulfamethoxazole	SXT	23.75/1.25 µg
Amikacin	AK	µg10

3. Results and Discussion

3.1. Results

The results showed that 70 bacterial isolates were obtained from honey bees carrying bacteria after examining the samples collected from the sites mentioned above. These isolates were distributed as follows: *E. coli*, *Klebsiella oxytoca*, *Citrobacter oxytoca*, *Acinetobacter baumannii*, and *Acinetobacter lwoffii*. Table (3) shows that the largest number of isolates were contaminated with *E. coli*, with 32 isolates, representing 45.7% of the isolates and 42.1% of the total number of insects. followed by *Citrobacter koseri* with 20 isolates, accounting for 28.6% of the isolates and 26.3% of the total number of insects, followed by *Acinetobacter lwoffii*, with 12 isolates, representing 17.1% of the isolates and 15.8% of the total number of insects. Then came *Acinetobacter baumannii* bacteria, with 2 isolates, accounting for 5.7% of the isolates and 5.2% of the total number of insects. Finally, *K. oxytoca* came in last place with 2 isolates, representing 2.8% of the isolates and 2.6% of the total number of insects.

Table 3. Types of Gram-negative bacteria and their percentages in honeybee samples from the collection sites

Bacterial type	Number of isolates	Percentage of isolates	Percentage of isolates from total number of insects
E.coli	32	45.7	42.1
K.oxytoca	2	2.8	2.6
Citrobacter koseri	20	28.6	26.3
Acinetobacter lwoffii	12	17.1	15.8
Acinetobacter baumannii	4	5.7	5.2
Total	70	99.9	

3-2 Diagnosis of bacteria

The results of the diagnosis of Gram-negative bacterial isolates were based on a set of diagnostic methods that included macroscopic and microscopic examination. Smears of the colonies were prepared and stained with Gram stain to identify Gram-negative bacteria, documenting the morphological characteristics of the

cells in terms of shape (rod-shaped or spherical) and arrangement (single, pairs, chains, or clusters), as well as the distinctive characteristics of the colonies, such as color, texture, and odor. Microscopic examination was also performed to confirm cellular features, followed by a series of standard biochemical tests for the differential identification of isolates. The results for some samples were supported by the Vitek system as a confirmatory diagnostic tool.

E. coli isolates appeared as short, Gram-negative, small, pink rods on MacConkey medium and green, shiny rods on EMB medium. They were positive for indole, methyl red, and catalase tests, negative for citrate and Voges-Proskauer tests, and capable of fermenting glucose, lactose, and mannitol [4]. *Klebsiella oxytocae* appeared as Gram-negative rods with a mucous consistency, producing pink colonies on MacConkey, negative for indole and methyl red, positive for catalase, Voges-Proskauer, and citrate consumption, and nonmotile due to the presence of a capsule [5, 6]. *Acinetobacter spp.* were characterized as short or spherical rods, nonmotile and nonfermentative for lactose, negative for oxidase and positive for catalase, and classified as glucose-nonfermentative bacteria [7, 8]. *Citrobacter koseri* were motile rods, capable of producing H_2S and fermenting glucose and mannitol, catalase-positive and citrate-positive, with some species indole-positive, while their ability to ferment lactose varied [9, 8].

Table 2. Distinctive characteristics of bacterial isolates used in the study

Type Test	**Type of bacteria**			
	Acinetobacter spp.	***Citrobacter koseri***	***Klebsiella oxytoca***	***E. coli***
(Shape)	Rod	Rod	Rod	Rod
(Gram stain)	-	-	-	-
(Catalase)	+	+	+	+
(Oxidase)	+	-	-	-
(Indol test)	-	+	-	+
(Methyl red)	-	+	+	+
(Voges- proskauer)	+	-	+	-
(Citrate utilization)	+	+	+	-
(Motility)	-	+	-	+
(Fermentation glucose)	A/A	A/A	A/A	A/A
(H_2S)	-	+	-	-
(Lactose)	+	+	+	+
(Mannitol)	+	+	+	+
(Urase Production)	-	-	+	-

(+)Positive result, (-) Negative result, (K) Basic, (A) Acidic

3.3. Testing the sensitivity of bacterial isolates to antibiotics

The results in Table (4) show that *E. coli* bacteria exhibited high resistance to traditional β-lactam antibiotics such as penicillin and amoxicillin-clavulanic acid, with no isolates showing sensitivity to these antibiotics (0%). It also showed complete resistance to trimethoprim + sulfamethoxazole at a rate of 0%. Meanwhile, it

showed moderate susceptibility to aminoglycosides, with a positive response to amikacin and cefotaxime (59.3%) at a diameter of 19 mm and gentamicin (62.5%) at a diameter of 20 mm. while relatively higher sensitivity was shown to Ciprofloxacin (87.5%) with a diameter of 28 mm and Ceftriaxone (75%) with a diameter of 24 mm. The isolates also showed good sensitivity (81.2%) to Levofloxacin with a diameter of 22 mm. This pattern of resistance is attributed to E. coli possessing extended-spectrum β-lactamases (ESBLs) in addition to changes in the permeability of the outer membrane, which hinders the entry of the drug into the target site [10, 11].

The results revealed that *Klebsiella oxytoca* isolates were completely resistant to Trimethoprim + Sulfamethoxazole, with a rate of 0%, as no sensitive isolates were recorded. They also showed partial resistance to Amoxicillin–clavulanic acid, with an inhibition diameter of 14 mm at a rate of 50%. , while showing moderate sensitivity to Amikacin, with a positive response (52.3%) at a diameter of 16 mm. Ciprofloxacin showed relatively higher sensitivity (87.5%) at a diameter of 28 mm, and (81.2%) to Levofloxacin with a diameter of 22 mm and (75%) to Cefotaxime and Ceftriaxone with a diameter of 20 mm. The lowest sensitivity was to Gentamycin (60%) with an inhibition diameter of 19 mm. This pattern of sensitivity and resistance is partly attributed to the production of *K. oxytoca* of broad-spectrum β-lactamases (ESBLs), which reduce the effectiveness of some antibiotics such as Amoxicillin–clavulanic acid, in addition to changes in the permeability of the outer membrane of the bacteria, which may hinder the entry of the antibiotic into its site of action [12, 10].

Citrobacter koseri showed complete resistance to both Amoxicillin–clavulanic acid and Ceftrazidime, with no sensitive isolates recorded (0%). Trimethoprim + Sulfamethoxazole recorded an inhibition diameter of (10 mm), indicating complete resistance (0%). In contrast, the isolates showed the highest level of sensitivity to Ciprofloxacin with an inhibition diameter of 33 mm and a rate of 100%, followed by good sensitivity to Gentamycin with a diameter of 24 mm and a rate of 82%, Amikacin with an inhibition diameter of 22 mm and a resistance rate of 75%, and Levofloxacin with an inhibition diameter of 21 mm and a resistance rate of 71%. As for third-generation cephalosporins, Cefotaxime (17 mm) and Ceftriaxone (19 mm) showed sensitivity but with low levels of effectiveness. Studies indicate that the resistance of Citrobacter spp. is often due to the production of AmpC β-lactamase in addition to mutations in the genes responsible for permeability [13].

Acinetobacter lwoffii showed moderate to good sensitivity to Ciprofloxacin and Trimethoprim + Sulfamethoxazole (78.1%) with an inhibition diameter (25 mm), Levofloxacin (75%), Gentamycin (65.6%), and Amikacin (59.3%), with inhibition diameters ( ), while it showed complete resistance to Amoxicillin-clavulanic acid, Ceftrazidime, and Cefotaxime at a rate of (0%). This is consistent with the documented resistance pattern for this species [14].

Finally, *Acinetobacter baumannii* showed almost complete resistance to all antibiotics tested, showing no response to Ceftrazidime, Amoxicillin-clavulanic acid, Cefotaxime, Ceftriaxone Amikacin, or Trimethoprim + Sulfamethoxazole, and showed very low sensitivity to Ciprofloxacin and Levofloxacin with a diameter of 18 mm and a percentage of 52.8% and Gentamycin with a diameter of 15 mm and a percentage of 46.2%. These results are attributed to the nature of this species, which is known to possess multiple resistance mechanisms, including efflux pumps, production of β-lactamases, and modification of binding sites [15]. It should be noted that this species is less virulent than A. lwoffii, but it still carries resistance genes associated with plasmids that can be transferred between bacteria [7].

Table 4. Sensitivity of bacteria isolated from the outer surface of honeybee workers to antibiotics

Bacterial isolates	**Antibiotics**									
	R & S	Amoxicillin-clavulanic acid	Ceftrazidime	Ciprofloxacin	Ceftriaxone	Cefotaxime	Gentamycin	Levofloxacin	Trimethoprim + Sulfamethoxazole	Amikacin
E.coli (n=32)	S	-	-	28	24	19	20	22		19
	R	0	-	-	-	-	-	-	0	-
Klebsiella oxytoca (n=2)	S	-	-	25	20	20	19	22	-	-
	R	14	-	-	-	-	-	-	0	16
Citrobacter koseri (n=20)	S	-	-	33	19	-	24	21	-	22
	R	0	0	-	-	17	-	-	10	-
Acinetobacter lwoffii (n=12)	S	-	-	25		-	21	24	25	19
	R	0	0	-	8	-		-	-	-
Acinetobacter baumannii(n=4)	S	-	-	18	-	-	-	18	-	-
	R	0	0	-	0	0	15	-	0	0

Images showing the effect of antibiotic sensitivity tests on the bacteria studied



K. oxytoca



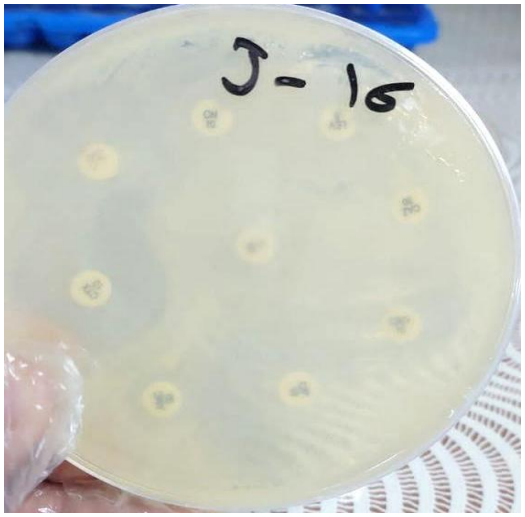
E. coli



A. baumannii



A. lwoffii



C. koseri

References

[1] Engel P, Moran NA. The gut microbiota of insects–diversity in structure and function. *FEMS Microbiol Rev*. 2013;37(5):699-735.

[2] Babendreier D, Joller D, Romeis J, Bigler F, Widmer F. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. *Microb Ecol*. 2007;53(2):269-78. http://dx.doi.org/10.1007/s00248-006-9203-3

[3] Endo A, Salminen S. Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. *Syst Appl Microbiol*. 2013;36(6):444-8. http://dx.doi.org/10.1016/j.syapm.2013.06.002

[4] Mahon CR, Lehman DC, Manuselis G. *Textbook of diagnostic microbiology*. 3rd ed. Philadelphia (PA): Saunders Elsevier; 2007.

[5] Holt JG, Krieg NR, Sneath PH, Sraley JT, Williams ST. *Bergy's Manual of Determinative Bacteriology*. 9th ed. Baltimore (MD): Williams and Wilkins; 1994. p.135-8.

[6] Atlas MR. *Handbook of Media for Environmental Microbiology*. 2nd ed. New York (NY): Taylor & Francis; 2005. p.157-89.

[7] Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: Emergence of a successful pathogen. *Clin Microbiol Rev*. 2008;21(3):538-82.

[8] Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*. 7th ed. Philadelphia (PA): Wolters Kluwer Health; 2017.

[9] Forbes BA, Sahm DF, Weissfeld AS. *Bailey & Scott's Diagnostic Microbiology*. 12th ed. St. Louis (MO): Mosby Elsevier; 2007.

[10] Rawat D, Nair D. Extended-spectrum β-lactamases in Gram-negative bacteria. *J Glob Infect Dis*. 2010;2(3):263-74. http://dx.doi.org/10.4103/0974-777X.68531

[11] Ramatla T, Obi CL, Nchabeleng M, Olukosi Y. Antibiotic resistance mechanisms of *Escherichia coli*: An emerging global health concern. *Front Microbiol*. 2025;16:1523498. http://dx.doi.org/10.3389/fmicb.2025.1523498

[12] Forbes BA, Sahm DF, Weissfeld AS. *Bailey & Scott's Diagnostic Microbiology*. 14th ed. St. Louis (MO): Elsevier; 2020.

[13] Mezzatesta ML, Gona F, Stefani S. *Enterobacter cloacae* complex: Clinical impact and emerging antibiotic resistance. *Future Microbiol*. 2012;7(7):887-902. http://dx.doi.org/10.2217/fmb.12.68

[14] Bonomo RA, Szabo D, Paterson DL. Role of β-lactamases in antibiotic resistance of *Acinetobacter baumannii*. *Clin Infect Dis*. 2006;43(Suppl 2):S29-36. http://dx.doi.org/10.1086/504477

[15] Howard A, O'Donoghue M, Feeney A, Sleator RD. *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence*. 2012;3(3):243-50. http://dx.doi.org/10.4161/viru.19649