How to Cite:
Hasan, H. J., Abdulwahid, M. T., & Ayyez, H. N. (2023). Molecular detection and phylogenetic analysis of Citrobacter freundii isolates from broilers in AL- Diwaniyah province of Iraq. *International Journal of Health Sciences*, *6*(S9), 4736–4752. https://doi.org/10.53730/ijhs.v6nS9.14026

Molecular detection and phylogenetic analysis of *Citrobacter freundii* isolates from broilers in AL- Diwaniyah province of Iraq

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***Abstract*---**Because of the medicinal and economic importance of *Citrobacter freundii*, as well as the fact that there are only a few local studies on it, the purpose of this study was to isolate and identify this important bacterial species from chicken using differential media with comparable biochemical properties. There is a severe concern about the presence of extended-spectrum beta-lactamase (ESBL) creating bacteria in animal husbandry, which could be transmitted to humans. *C. freundii* was detected through the use of specific associated genes that enable this bacterium to resist the effects of antibiotics, making it highly resistant. For detection of this bacteria and recognition of distinct types of resistance genes, polymerase chain reaction was used. Further confirmation, purified PCR product was subjected to sequencing. It showed 99.80% homology with Switzerland isolates available in the NCBI database. Forty two isolates of *C. freundii* were isolated from (450) fecal sample collected from different region of AL-Diwaniyah province. The results shown that molecular tools more reliable for identification *C. freundii* in fecal samples more than cultural and biochemical tests. *C. freundii* haven a large number of antimicrobial resistance-associated genes that allow it to resist the effects of antibiotics, making it highly resistant to them.

Keywords*---Citrobacter freundii*, Biochemical reaction, Beta-lactamase genes and Chicken.

Introduction

Poultry is the world's second most extensively consumed meat, accounting for about 36% of poultry meat output (Abdulwahid, 2015). Poultry meats produced for food include chicken, turkey, and quail. As compared to other species meats, chicken meat is regarded a nutritious food because of its high protein content and low fat level, as well as a higher amount of polyunsaturated fatty acids (Montalvo-Puente *et al.*, 2018). Raw meat, particularly poultry meat, continues to be a source of pathogenic microorganism infection in humans. Because fresh meat is perfect for bacterial growth, it is easily contaminated with bacteria (Auriema *et al.*, 2019). Contamination of poultry meat with foodborne pathogens is a serious public health concern since it can cause illness and even death when improperly handled. Meat products have been related to a number of high-profile outbreaks of foodborne diseases (Aminharati *et al.*, 2019). *Citrobacter* species are facultative, anaerobic, gram negative bacilli or rods (Hashim and AlKhafaji, 2018), motile with flagella that can ferment mannitol and produce H2S, and can use citrate as their carbon source (Thompson *et al.*, 2018). Within the *Enterobacteriaceae* family They're commonly found in animals' and humans' intestines, as well as in water, soil and food. Previously thought to be low-virulence environmental contaminants or colonizers, they are now found to cause a wide range of infections involving the urinary tract (Raphae and Riley, 2017). The liver, biliary tract, peritoneum, intestines, bone, respiratory tract, endocardium, wounds, soft tissue, meninges, and bloodstream are among the organs that can be affected (Liu *et al.*, 2018). Comparative analysis of reconstruction of bacterial phylogenies is possible thanks to phylogenetic marker molecules. The molecular and genotypic levels of evolution have a far higher number of defined features than the phenotypic level. Tens to thousands of evolutionary independent sequence residues make up the underlying genetic information for every specified phenotypic function. Many of these residues can be altered in a non-destructive manner without affecting or eliminating the phenotypic function. This species' significance stems from its link to major nosocomial infections and a high level of resistance to popular antimicrobial drugs used to treat a variety of illnesses (Nayar *et al.*, 2014). Antimicrobial resistance develops as a result of chromosomal -lactamase enzyme overproduction (Mahmood and Atyah, 2021). *Citrobacter freundii* pathogenicity is determined by virulence factors (Tajeddin *et al.*, 2020). The aims of present study isolation of *Citrobacter Freundii* from fasces of live broiler chickens collected from different regions in Al-Diwaniyah province Iraq using conventional bacteriological methods and biochemical tests and confirmation of its isolates by using PCR technique and nucleotide sequences for phylogenetic analysis to detect genetic diversity of local isolates compared with global strains also were screening the beta-lactamase genes.

Materials and Methods

The current study started in 2020 by isolating and identification the bacteria, specimens (cloacal samples) were collected from 450 chicken randomly from local live broiler chickens were collected from three regions in Al - Diwaniyah province there were samples put in sterile tubes, and the samples were sent to the laboratories of the University of AL-Qadisiyah / College of Veterinary Medicine/ Laboratory of the Veterinary Public Health Department. A total of samples were

subjected to bacteriological examinations to give typical reactions of *Citrobacter* genus. (Quinn *et al.*, 2011). Using standard morphological and biochemical testing, the *Citrobacter* isolates were identified to the species level (MacFaddin, 2000). The isolates' identity was confirmed by molecular detection using specific primers for detection bacterium and for detection some antibiotic resistance genes (yang *et al* ., 2008) , as following table (1):

Table (1) Primers for detection and antimicrobial resistance genes sequenced used in PCR

Primers	Sequence		**Annealing temp.**	Amplicon	Reference
AspC gene	F	GTTTCGTGCCGATGAACGTC	50◦C	594bp	**NCBI**
	R	AAACCCTGGTAAGCGAAGTC			
BlaCTX-M gene	F	**AACCGTCACGCTGTTGTTAG**	**57◦C**	766bp	**Newier *et al.*, (2013)**
	R	**TTGAGGCTGGGTGAAGTAAG**			
BlaCTE-M gene	F	**CAGCGGTAAGATCCTTGAGA**	**57◦C**	643bp	**Newier *et al.*, (2013)**
	R	**ACTCCCCGTCGTGTAGATAA**			
BlaShv gene	F	**GGCCGCGTAGGCATGATAGA**	**60◦C**	714bp	**Newier *et al.*, (2013)**
	R	**CCCGGCGATTTGCTGATTTC**			
Pse-1 gene	F	**CGCTTCCCGTTAACAAGTAC**	**56◦C**	420bp	**Bacci *et al.*, (2012)**
	R	**CTGGTTCATTTCAGATAGCG**			

Results

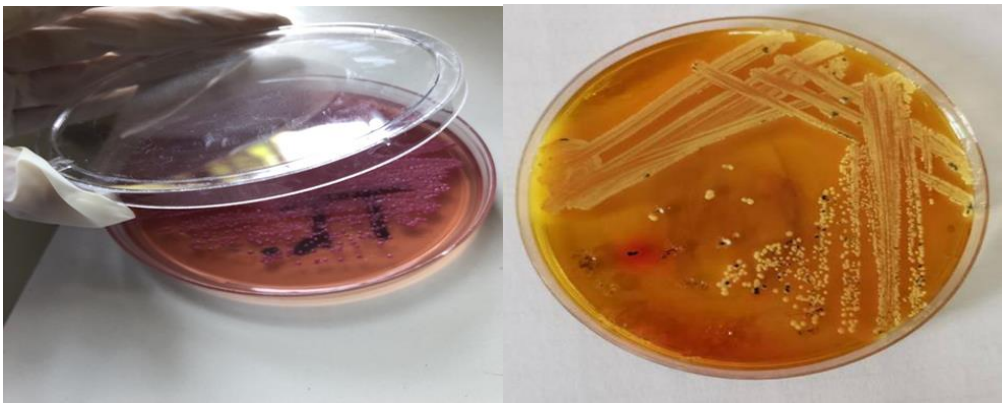
Bacterial isolation and identification

The results appeared the highest percentage of contamination occur with the *C. freundii* at rate 12% in AL- Sunniya farms ,in addition the results showed that the low percentage of contamination with the *Citrobacter freundii* at rate 6% in AL-Shafeyia farms as listed in table (2) , but No significant difference at ($p \leq 0.05$) between farms. The difference in percentage can be caused by variations in hygiene practices among chicken farmers. All samples were cultured on MacConkey agar at typical incubation conditions for initial isolation. According to culture characteristics the results indicated on MacConkey agar the growth of *C. freundii* bacteria were showed small pink colored colonies (lactose fermenter). The suspected colonies were cultured on Xylose lysine deoxycholate (XLD) agar, the growth of *C. freundii* bacteria appeared in the form of yellow colored colonies that are smooth flat and round after a 24hours incubation period at 37°C,and these bacterial isolation were also confirmed on chromogenic agar and showed as metallic blue colored colonies. The colonies of *C. freundii* appeared on Salmonella shigella agar pale colored and flattened colonies with black center after incubation at 37°C for 24 hours due to their ability to produces H2S .In nutrient agar the growth of *C. freundii* bacteria appeared as smooth convex opaque gray color with shine surface and entire margin, while on Eosin Methylene Blue agar the growth of *C. freundii* bacteria appeared brown colored colonies as shown in figure (1).

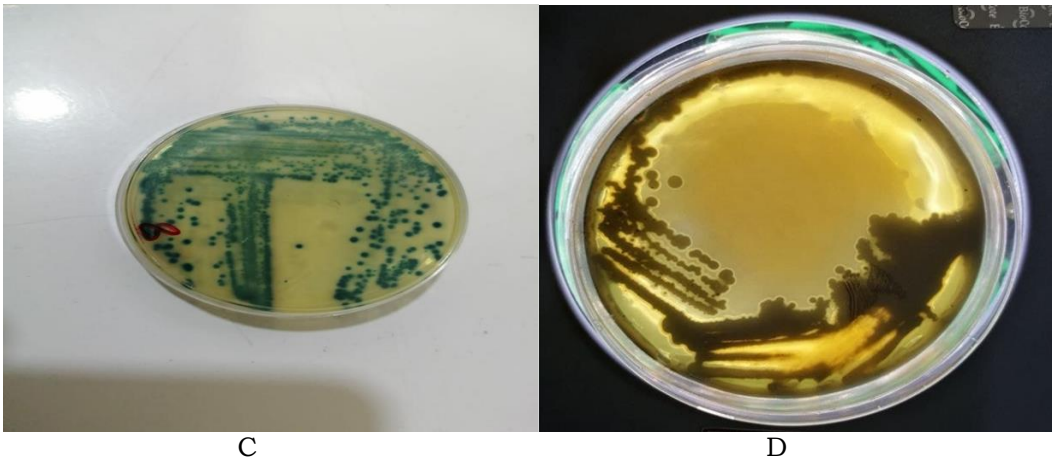
Table (2) Shows number and percentage of bacterial isolation

Farms	No. of samples	No. of positive	percentage %
AL- Sunniya	150	18	12
AL-Shafeyia	150	9	6
AL- Daghara	150	15	10
X^2 3.30 P value 0.191(NS)			

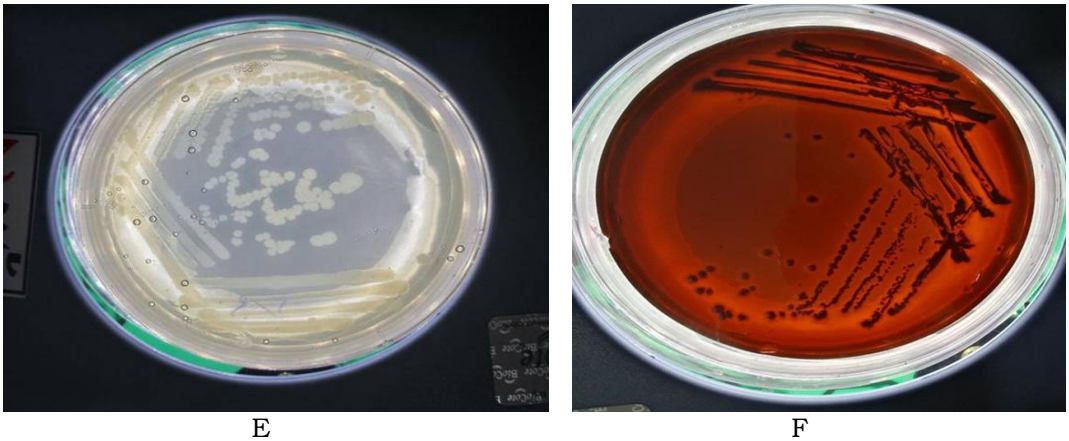
NS :No significant difference at ($p \leq 0.05$).



A B



C D



E F

Figure (1) *Citrobacter freundii* was cultured in a variety of selective and differential media

A. A. MacConkey agar with small pink (Lactose fermenter) colonies.
B. Yellow colonies on XLD agar.
C. Metallic blue colonies on Chromogenic agar.

D. On S.S. agar Pale colonies with black center.
E. Convex opaque gray color colonies on nutrient agar.
F. Brown colonies on EMB

Results of conventional biochemical test

Specific biochemical tests were used for the detection genus of bacteria, methods analytical profile index (API20 E) test to identify positive growth of *C. freundii* .Accordingly the profile of color changes was 3644772 which is characteristic of *C. freundii* bacteria.

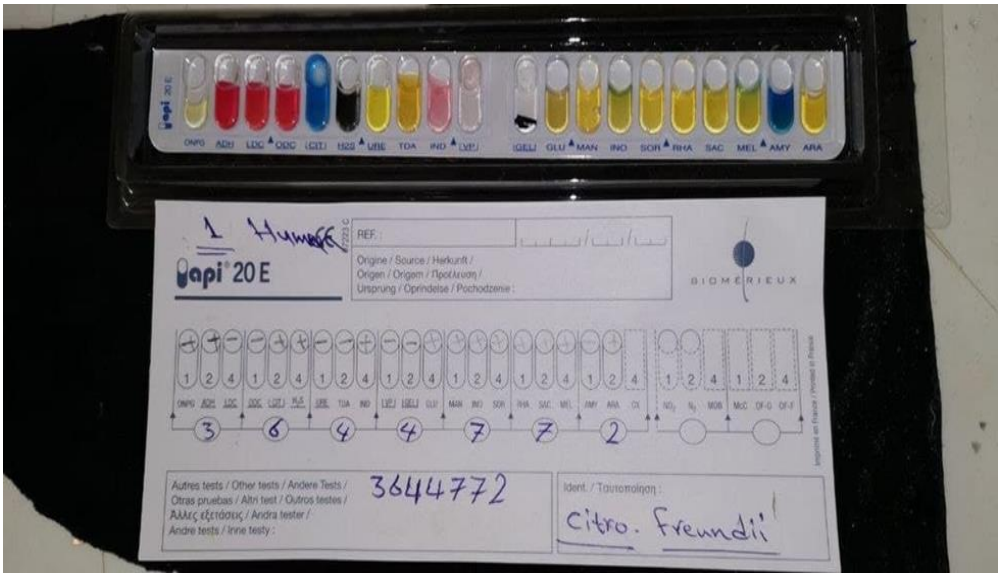
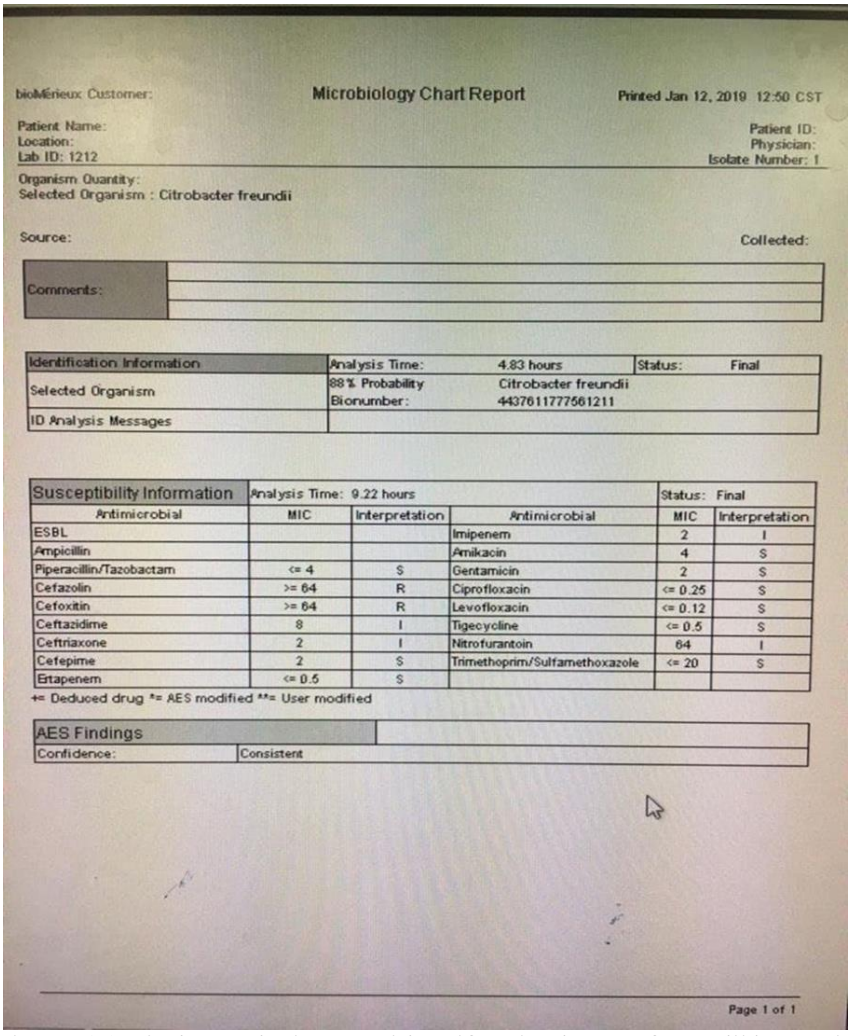


Figure (2) API20 E test to identify *Citrobacter freundii* bacteria (profil: 3644772)



bioMérieux Customer: **Microbiology Chart Report** Printed Jan 12, 2019 12:50 CST

Patient Name:
Location:
Lab ID: 1212

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Citrobacter freundii

Source: Collected:

Comments:

Identification Information	Analysis Time:	4.83 hours	Status:	Final
Selected Organism	88% Probability Bionumber:	Citrobacter freundii 4437611777561211		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 9.22 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL			Imipenem	2	I
Ampicillin			Amikacin	4	S
Piperacillin/Tazobactam	<= 4	S	Gentamicin	2	S
Cefazolin	>= 64	R	Ciprofloxacin	<= 0.25	S
Cefoxitin	>= 64	R	Levofloxacin	<= 0.12	S
Ceftazidime	8	I	Tigecycline	<= 0.5	S
Ceftriaxone	2	I	Nitrofurantoin	64	I
Cefepime	2	S	Trimethoprim/Sulfamethoxazole	<= 20	S
Ertapenem	<= 0.5	S			

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Page 1 of 1

Figure (3) Vitek2 technique to identify *Citrobacter freundii* bacteria

Molecular identification

To confirm Citrobacter species identification, aspartate transaminase gene amplification was performed using a PCR technique and 1.5 percent agarose gel electrophoresis was used to detect the positive result, as shown in Figure (4). Six isolates were tested by molecular method (PCR technique by using *AspC* gene primers) to confirm the identification of these isolates after the step of genomic DNA extraction. The use of PCR to identify the *C. freundii* isolates are shown in figure (4).Where, the Lane (M): DNA marker (1500-100bp) and the Lane (1-6) were showed positive PCR amplification of *AspC* gene in *C. freundii* isolate at 594bp PCR product size.

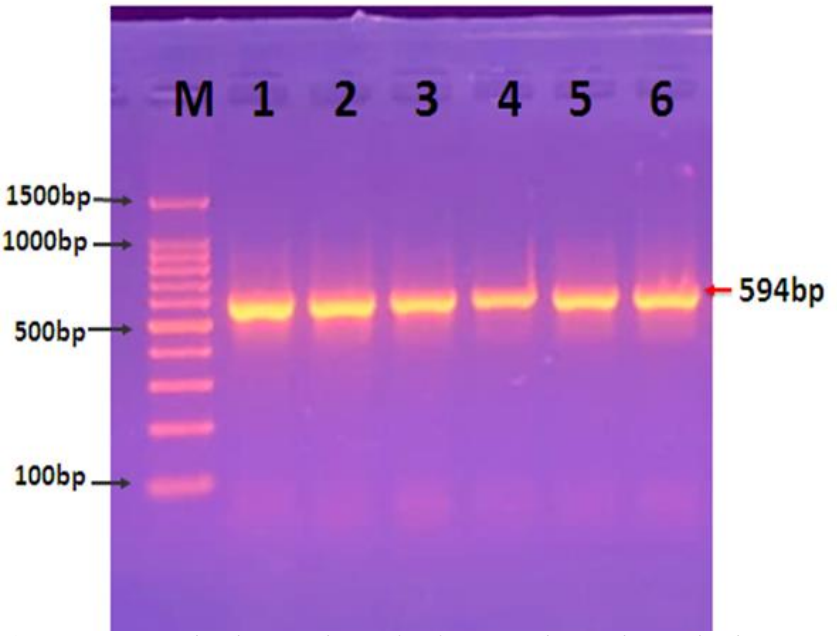


Figure (4) Agarose gel electrophoresis image that showed the PCR product analysis of diagnosis aspartate transaminase gene *Citrobacter freundii* isolates. Where, the Lane (M): DNA marker (100-1500bp) and the Lane (1-6) were showed positive PCR amplification of *AspC* gene in *Citrobacter freundii* isolate at 594bp PCR product size.

DNA sequence results

The DNA sequencing method was carried out to identification genetic relationship analysis in aspartate transaminase gene of local *C. freundii* Iraq isolate and NCBI-Genbank country related species isolates. The phylogenetic tree genetic relationship analysis was showed that The local *C. freundii* was showed closed related to NCBI-BLAST *C. freundii* Switzerland isolate (CP022151.1). as showed in figure (5). The homology sequence identity between The local *C. freundii* was displayed closed related to NCBI-BLAST *C. freundii* country isolates were showed genetic homology sequence identity ranged from (98.09-100%). As showed in table (3). Finally, the local *C. freundii* Iraq were submitted into NCBI Genbank and identified by accession numbers (MZ202354.1).

MZ202354.1_C.freundii_Iraq -CTCGGGATCGGTGTATATAAAGATGAGACAGGCAAAACGCCGGTGCTGA
CP022151.1_C.freundii_Switzerl -CTCGGGATCGGTGTATATAAAGATGAGACAGGCAAAACGCCGGTGCTGA
CP055247.1_C.freundii_China -CTCGGGATCGGTGTATATAAAGATGAGACAGGCAAAACGCCGGTGCTGA
CP056256.1_C.freundii_Spain -CTCGGGATCGGTGTATATAAAGATGAGACAGGCAAAACGCCGGTGCTGA
LR134118.1_C.freundii_UK AAGCTGAGCCAGCGTTTGCCA---CTGCTCAAGCGTAGGATCGATACCGG
CP070549.1_C.freundii_Malaysia AAGCTGAGCCAGCGTTTGCCA---CTGCTCAAGCGTAGGATCGATACCGG

```
CP024683.1_C.freundii_USA                AAGCTGAGCCAGCGTTTGCCA---
CTGCTCAAGCGTAGGATCGATACCGG
                          * *  * * ** *  *     ** **  *   ** * * *
MZ202354.1_C.freundii_Iraq
CCAGCGTTAAAAAAGCCGAGCAATATATACTGGAAAATGAAACCACCAAA
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CP055247.1_C.freundii_China
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CP056256.1_C.freundii_Spain
CCAGCGTTAAAAAAGCCGAGCAATATATACTGGAAAATGAAACCACCAAA
LR134118.1_C.freundii_UK                 TTGG-GTTATGACAGC--AGCCGTGGA-
ACAGTACCACATCGCCAGCCTG
CP070549.1_C.freundii_Malaysia           TTGG-GTTATGACAGC--AGCCGTGGA-
ACAGTACCACATCGCCAGCCTG
CP024683.1_C.freundii_USA                TTGG-GTTATGGCAGC--AGCCGTGGA-
ACAGCACCACATCGCCAGCCTG
                        * ****   *** *** *  * ** * *  *   *** *
MZ202354.1_C.freundii_Iraq                          AACTATCTTGGCATTGAC----
GGTATTCCAGAGTTTGGTC-GTTGTACT
CP022151.1_C.freundii_Switzerl                      AACTATCTTGGCATTGAC----
GGTATTCCAGAGTTTGGTC-GTTGTACT
CP055247.1_C.freundii_China                         AACTATCTTGGCATTGAC----
GGTATTCCAGAGTTTGGTC-GTTGTACT
CP056256.1_C.freundii_Spain                         AACTATCTTGGCATTGAC----
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LR134118.1_C.freundii_UK
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CP070549.1_C.freundii_Malaysia
AGCTTCGCTGAGGCTGGCCAGCAGCGCATCAAAGTCTAATGAGTGGTTCG
CP024683.1_C.freundii_USA
AGCTTCGCTGAGGCTGGCCAGCAGCGCATCAAAGTCTAATGAGTGGTTCG
                        * **   **   ** *   *   ** *** *  *  ** ** *
MZ202354.1_C.freundii_Iraq                                              CAGGAAT-
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CP022151.1_C.freundii_Switzerl                                          CAGGAAT-
TACTGTTTGGTAAAACGAGTTCGCTTATCAACGA---TAAAC
CP055247.1_C.freundii_China                                             CAGGAAT-
TACTGTTTGGTAAAACGAGTTCGCTTATCAACGA---TAAAC
CP056256.1_C.freundii_Spain                                             CAGGAAT-
TACTGTTTGGTAAAACGAGTTCGCTTATCAACGA---TAAAC
LR134118.1_C.freundii_UK
CCGCATCGTAATAGTTGTATTCACGAACTTCCAGACCGGCAGAGTTAAAG
CP070549.1_C.freundii_Malaysia
CCGCATCGTAATAGTTGTATTCACGAACTTCCAGACCGGCAGAGTTAAAG
CP024683.1_C.freundii_USA
CTGCATCGTAATAGTTGTATTCACGAACTTCCAGACCTGCAGAGTTAAAG
                        * * *  ** * ***   **** * * * * *   ****
MZ202354.1_C.freundii_Iraq
GCGCACGTACAGCACAGACCCCTGGTGGCACTGGCGCAC-TGCGCGTGGC
```

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CP022151.1_C.freundii_Switzerl
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CP055247.1_C.freundii_China
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CP056256.1_C.freundii_Spain
GCGCACGTACAGCACAGACCCCTGGCGGCACTGGCGCAC-TGCGCGTGGC
LR134118.1_C.freundii_UK
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CP070549.1_C.freundii_Malaysia
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CP024683.1_C.freundii_USA
ACGCTCTTATGGTTCGGCCAGCTTGGGTTGCTCACCCATACGCGTTTAGC
                    *** * **  *  * * * * ** * *  **  * **   **  * **

MZ202354.1_C.freundii_Iraq
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CP024683.1_C.freundii_USA
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                    *  *  ***** ** *****  *  * ** *  ***  ** *  **

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CP024683.1_C.freundii_USA
CCGCCAGGGGTCTGTGCTGTACGTGCGCGTTTA---TCGTTGATAAGCGA
                    * * **  * * *  *  ** * *** ****    *  * *  *  *
```

Figure (5) Analysis of multiple sequence alignments of aspartate transaminase gene in local *C. freundii* Iraq isolate and NCBI-Genbank country related species isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). This demonstrated the nucleotide alignment similarity. (*) and substitution mutations in aspartate transaminase gene between isolates.

Table (3) The percentage of NCBI-BLAST Homology Sequence identity between local *Citrobacter freundii* Iraq isolate and NCBI-BLAST country isolates

NCBI-Blast isolate No.	Country	Accession number	Homology Sequence Identification Using NCBI-BLAST (%)
1.	Switzerland	CP022151.1	100%
2.	China	CP055247.1	100%
3.	Spain	CP056256.1	100%
4.	UK	LR134118.1	99.61%
5.	Malaysia	CP070549.1	99.02%
6.	USA	CP024683.1	98.24%

The results of PCR for detecting antibiotic resistant genes revealed that all isolates under study harboring these gene as illustrated in following figures (6,7,8 and 9).

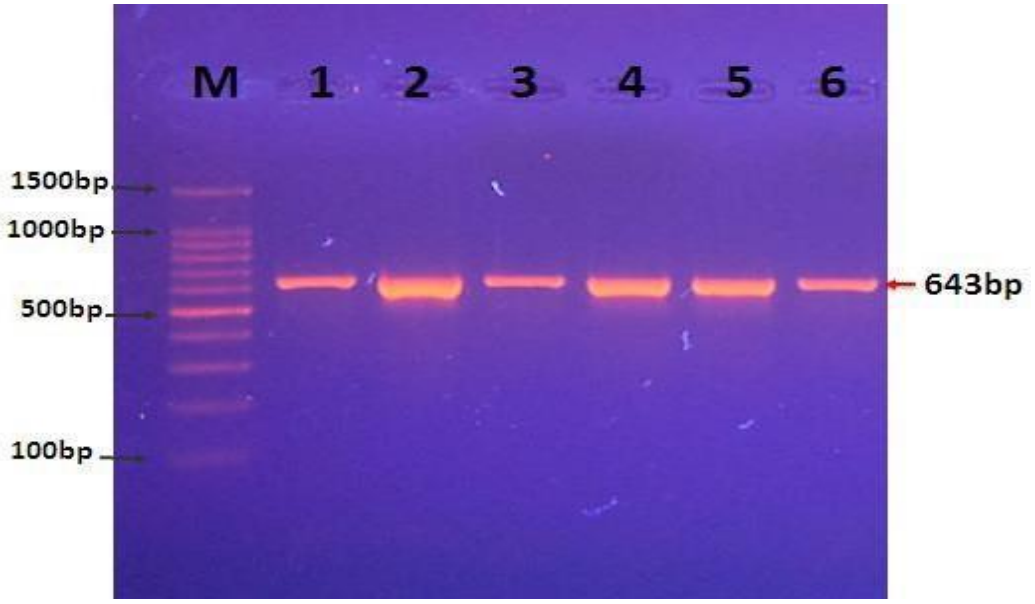

Figure (6) Agarose gel electrophoresis image that showed the PCR product analysis of antibiotic resistance *bla-tem* gene *Citrobacter freundii* isolates. Where, the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1-6) were showed positive PCR amplification of *bla-tem* gene in *Citrobacter freundii* isolatesat 643bp PCR product size.

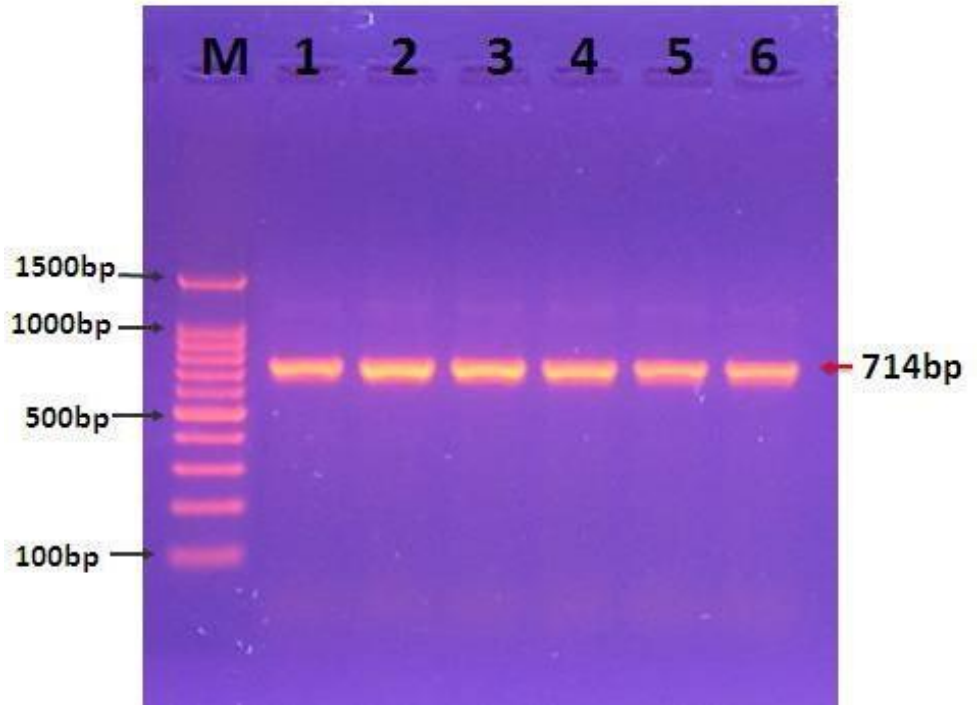

Figure (7) PCR product analysis of antibiotic resistance gene *bla-shv* gene *Citrobacter freundii* isolates on an agarose gel electrophoresis picture. Where, at 714bp PCR product size, the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1-6) demonstrated positive PCR mplification of the *bla-shv* gene in *Citrobacter freundii* isolates. amplification of the *bla-shv* gene

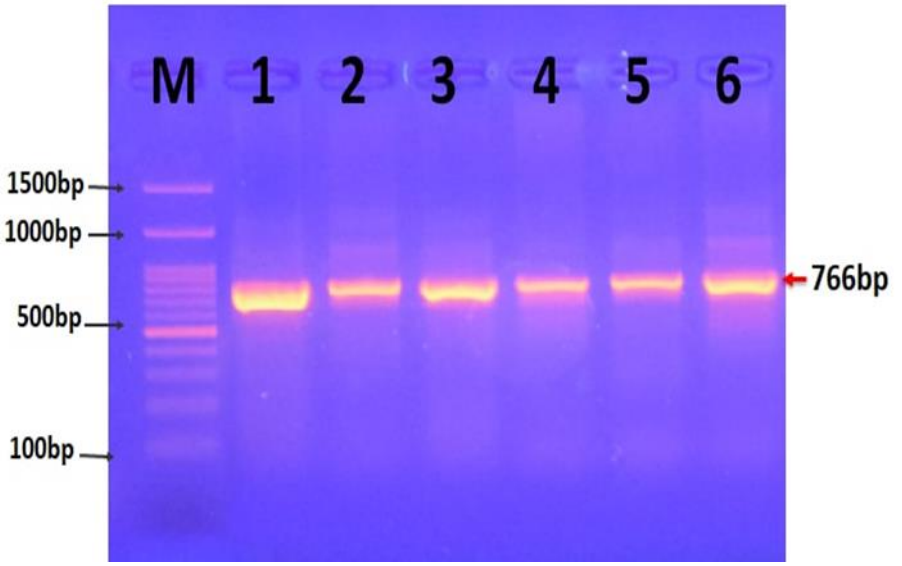

Figure (8) PCR product analysis of antibiotic resistance gene *bla-ctxm* gene *Citrobacter freundii* isolates on an agarose gel electrophoresis picture. Where, at

766bp PCR product size, the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1- 6) demonstrated positive PCR amplification of the *bla-ctxm* gene in *Citrobacter freundii* isolates.

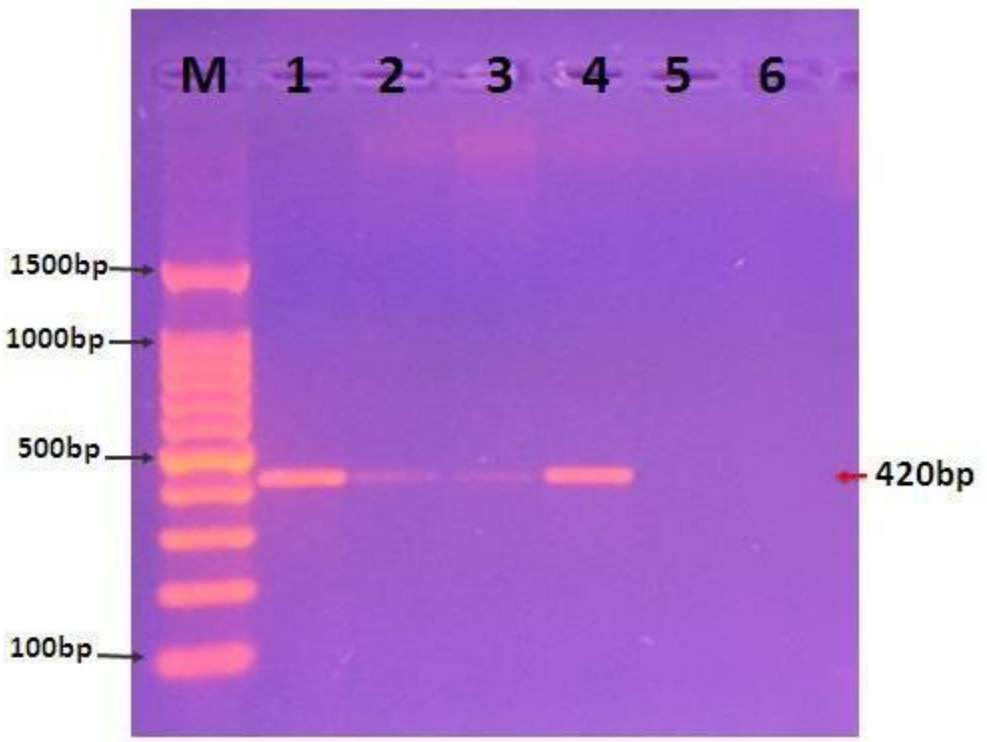

Figure (9) The PCR product analysis of antibiotic resistance gene *pse-1* gene *Citrobacter freundii* isolates was shown on an agarose gel electrophoresis image. Where the Lanes (M): DNA marker ladder (1500-100bp) and (1,4) showed positive PCR amplification of the pse-1 gene, whereas the Lanes (2,3) exhibited a weak band in *Citrobacter freundii* isolates at 420bp PCR product size. The researchers discovered beta-lactamase genes in *Citrobacter freundii* isolates from chicken feces. As a result of the widespread dissemination of chickens and their feces, antibiotic resistance is likely to spread.

Discussion

Poultry represent an important source of meat, eggs and edible offals. Many bacterial agents were found to affect the raising of chickens. The present results revealed this bacterium were isolated from fecal of diseased chickens representing different ages. The results showed (42) bacterial isolated were *Citrobacter freundii* (12%) higher percentage obtained in AL- Sunniya farms, Our results agree with (Hashim and AlKhafaji, 2018).

According to the cultural and biochemical tests, the results of the current study proved it has been diagnosed as *Citrobacter freundii*. The primary identification of *C. freundii* isolates was based on cultural, morphological, and biochemical testing (Castanheira *et al.*, 2017), and the confirmatory identification test was based on

selective Chrome agar media and biochemical assays. AP20E test, these findings are in agreement with (Soud *et al.*, 2018). Regarding XLD agar, our results are similar to that of (Darweesh, 2017) who stated that *C. freundii* bacteria appear as yellow, smooth ,flat and round colonies (Sekhi ,2021). In addition, our results regarding chrome agar are similar to that of (Milhem *et al.*, 2016) in which the growth of *C. freundii* bacteria appears in the form of metallic blue colored colonies. Our results concerning the use of Analytical Profile Index (API20 E) are in agreement with (Al-Daraghi and AL-Behadili, 2020).

The isolates were confirmed as *C. freundii* using *aspc* gene PCR products were exposed to the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information was used to assess the sequences obtained from direct sequencing (NCBI). Sequencing technique, are in agreement with (Yang *et al.*, 2018). The molecular diagnostic of *C. freundii* by using specific primer was facility direct identification compare with followed cultural, microscopically criteria and biochemical test, the conventional methods were consuming time, costly ,unclear and sometimes shown conflict results. More than one *Enterobacteriaceae* strains grew on of MacConkey agar and have ability to ferment lactose and increase the foggy of identification *C. freundii* strains from other bacteria (Al-Hasnawi, 2014).

Because the poultry environment is continually in contact with humans, a high prevalence of resistant bacterium genes in the environment may raise the danger of transfer to humans. In current study out of 6 isolates, all isolates were positive for (*bla-tem, blaCTX-M* and *bla-SHV*) genes, this result is in agreement with the study by Faife *et al.*,(2020), While this study is not in agreement to the study by Akinbami *et al.* (2018)., It was discovered that 10.4 percent of *C. freundii* isolates tested positive for the bla-tem gene. *Bla-tem* gene was the most prevalent ESBL gene detected followed by *bla-SHV* and *blaCTX-M* genes in clinical isolates (Sedighi *et al.*, 2017 and Olajumoke *et al.*, 2018). Whereas *blaPSE-1* were not detected in all isolates, this result is in agreement with (Elhariri *et al.*, 2020). Mutations in the *bla-tem* and *bla-shv* genes have resulted in extended spectrum beta lactamase enzymes, which have been well documented in the *Enterobacteriaceae* family (Liakopoulos *et al.*, 2016). The high rate of disposal of chicken droppings in the environment, according to these data, is likely to hasten the transmission of antibiotic resistance genes. *C. freundii* contains a large number of antimicrobial resistance-associated genes, allowing it to become more virulent and resistant to most antimicrobials, making it a more harmful bacteria.

Conclusions

C. freundii has number of antimicrobial resistance-associated genes, allowing it to become more virulent and resistant to most antimicrobials, making it a more harmful bacteria. PCR may also provide the sensitivity and specificity needed for the direct detection of *Citrobacter* spp.in broiler chicken specimens.

Ethical approval

In Iraq, the Research Ethical Committee oversees scientific research with ethical permission from the ministries of the environment, health, higher education, and scientific research.

Acknowledgments
We gratefully thank University of Al-Qadisiyah Unit of Zoonotic Research, College of Veterinary Medicine, for supporting and agree to performed this work in it Genetic Lab.

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