Plant Archives Vol. 20, Supplement 1, 2020 pp. 2861-2865 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ISOLATION AND MOLECULAR IDENTIFICATION OF *CITROBACTER FREUNDII* FROM DIARRHEAL PATIENT IN BABYLON PROVINCE, IRAQ

Ahmed Zaidan Khlaif Imran [1*], Angham Jasim Mohammed Ali [1] and Hasanain Khaleel Shareef [2]

[1]Al_Furat Al-Awsat Technical University, Kufa, Iraq

[2]College of Science for Women, University of Babylon, Iraq

*Corresponding Author Email: ahmedz.khelaif@gmail.com

Abstract

Citrobacter freundii belonging to the family Enterobacteriaceae, it was suspected to causes diarrhea disease. The study aimed to isolation and identification of *C. freundii* strains isolated from diarrhea patients, based on conventional and Molecular assays. The identification of 70 bacterial isolates which deems it belong to *C. freundii* strains, was challenges faced in identification by conventional methods like biochemical tests, then rely partial sequence of 23S rRNA gene as molecular marker for identification. The results shown high frequent percentage (67.74% *C. freundii* out of 70 of suspected strains isolated from diarrhea stool samples based on PCR assay. The specific primer pair which designed in this study was successfully amplified it target 23S gave PCR product size 189bp. Two PCR product of *C. freundii* (Kufa 1 & 2) were subjected to sequencing analysis and alignment with reference sequences deposed in Genbank database. Phylogeny tree was constructed to the native strains (Kufa 1 & 2) with reference sequence. The results shown that molecular tools more reliable for identification *C. freundii* in diarrhea samples more than cultural and biochemical tests.

Keyword: *Citrobacter freundii*, Diarrhea molecular identification, Sequence analysis.

Introduction

The genus *Citrobacter* is a gram-negative bacillus of the family Enterobacteriaceae (Kurtoglu *et al*., 2011). *Citrobacter* spp. are fundamentally occupants of the intestinal tract of well evolved creatures and different vertebrates. Their detachment from natural sources, for example, water and soil likely is the aftereffect of fecal discharge. *Citrobacter* spp. are not basic specialists of human ailment, and are regularly recouped from stool as colonizing verdure of the gastrointestinal tract. . The reason for selecting this age group was based on an increasing number of diarrhea each year. The World Health Organization (WHO) reported that over 700 million cases of diarrhea occurred in children under 5 years of age. The most common cause of bloody infectious diarrhea the toxin produced by *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., toxin produced *Clostridium* spp., *Vibrio cholerae*, *Yersinia enterocolitica* and *Citrobacter* spp.(Dervisoglu *et al*., 2008; Liu *et al*., 2017).

However, use of the 23S rRNA gene for bacterial community analysis is hampered by the lack of established broad-range bacterial PCR amplification and sequencing primers. (Dana *et al*., 2006). Comparative analysis of phylogenetic marker molecules allows the reconstruction of bacterial phylogenies. In comparison with the phenotypic level of evolution, there is a much higher number of definable characters at the molecular and genotypic level. The underlying genetic information for every definable phenotypic function comprises tens to thousands of evolutionary independent sequence residues. Many of these residues can be changed in a neutral fashion without changing or abolishing the overlying phenotypic function (Ludwig and Schleifer, 1994).

Materials and Methods

This study was carried out at Biology department, University of Babylon from May 2019 to November 2019. Patients group consisting of 70 patients (41 males and 29 females) and control group consisting of 11 healthy participants (8 males and 3 females) who were medical free. Tiny portion of diarrhea sample were drawn from each patient in the polyethylene plastic cups and brought direct to laboratory.

Culturing diarrhea samples were performed on MacConkey Agar (MCA) Which consider as selective and differential medium used for isolate and differentiate enteric coliform bacteria based on their ability to ferment lactose . while gram positive organisms inhibited by bile salts and crystal violet., ferments lactose and produces pink colonies (Hashim and AlKhafaji, 2018).

DNA Extraction

For 23S analysis, genomic DNA was extracted from bacterial pure colonies of bacteria under interest using the Promega DNA extraction kit (USA) as previously described by Imran (2016). Bacterial 23S were amplified by specific primer pair designed in this study

(F:5-`GAAGAATCCGGACAAACATC`-3' and

R:5`-CCAGGATAGGAAACATCCAG`-3'), The PCR condition was: denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95 °C for 40s, annealing at 58 °C for 1 min and extinction at 72 °C for 40s. Finally, the PCR was extended for an additional 5 min at 72 °C (Labnet Thermal Cycler USA). The results of amplification were analyzed on a 1.2% agarose gel pre-stained with ethidium bromide (Al Kathim, 2019).

Bacterial 23S rDNA Sequence Analysis

23S rDNA genes were amplified using specific primer pair, and PCR products subjected for sequence analysis in Macrogene Lab. (Korea), sequence charts were alignment and identified with the reference sequences deposited in genbank through the NCBI site.

Phylogenetic analysis

To find related species, the sequences obtained in this study were compared to those in genbank database (http://www.ncbi.nlm.nih.gov/blast). The 23S rRNA genes of

related species were used from the genbank database to generate a phylogenetic tree by MEGA6 (Tamura *et al*., 2013), The sequences of reference strains deposited in genbank were retrieved from NCBI website and the tree conducted in MEGA6 software, The evolutionary distances were computed using the UMPG method ,Multiple alignments of sequences were done using (http://www.ebi.ac.uk/Tools/msa/clustalo).

Statistical Analysis

Simple statistical test : Percentage and frequency percentage were applied in calculation the mean values.

Results and Discussion

Isolation and identification of bacteria Age and Gender Distribution of Diarrhea patients with *Citrobacter freundii* :

The results shown that the distribution of 70 isolate of *Citrobacter* out of 200 (35%) undergo diarrhea in Babylon province. The patients numbers and their percentage were calculated after conformation the identification of *C. freundii* based on molecular assays. The Table (1) shown that the age 1-15 years, more frequently infected with *C. freundii* compare with other ages, Table (1), this case (under 1-15 years) may be explained basically on the development of immunity defenses in the infant and childhoods, this explanation agree with the results of Yumuk *et al*. (2008) and Pereira *et al*. (2010), when their study focusing on infantile diarrhea, aggregative *C. freundii* were concomitantly recovered from a severe case of mucous diarrhea. Also, the present results consonant with results of Dervisoglu *et al*. (2008), which suspected *C. freundii* to cause diarrhea and possibly extra intestinal infections including peritonitis.

A 70/200 of suspected *C. freundii* isolate cultured on MacConkey agar shown tentative diagnosis positive ferment lactose. Conventional identification not always considered as clear cut reliable marker for bacterial identification.

On the other hand, the age (16-30) years with low frequent infection with *C. freundii*. The decline of diarrhea infection with young people may be correlated with special sanitations and development of immunity defenses. On the same time, no significant appeared between males and females in their readiness to infected by *C. freundii* (Table 1). The present study observation shown no significant differences between patients attenuated from villages and those from urban. this result was constant with the results of Liu *et al*. (2018) *C. freundii* is a frequent cause of nosocomial infections and a known cause of diarrheal infections.

Table 1: Distribution of Positive Cases with *C. freundii* based on age and gender of patients.

Age interval	Gander		Positive Cases with *C. freundii*		Control With *C. freundii*	
	Male	Female	No.	%	No.	%
1-15	15	10	25	35.7	2	18.1
16-30	9	4	13	18.51	3	27.7
31-45	7	3	10	14.2	2	18.1
46->60	10	12	22	31.4	4	36.3
Total	41	29	70	100%	11	100%

A total of 70 isolates of *Enterobacteriaceae* bacteria (Gram-negative bacillus and oxidase negative) were obtained from the samples which suspected as *Citrobacter freundii* species. These isolates were obtained from stool samples for patient suffer from diarrhea. The phenotypic and the biochemical characters of the presumptive C. *freundii* that had been isolated only.

The seventy isolates are oxidase negative and Gram-negative rods matching to the schemes of biochemical reactions provided in Bergey's Manual of Systematic Bacteriology for the identification of genus or species level (Frederiksen, 2004). In addition, they were found to be did not produce indole, urease and tryptophan, and formed glucose, mannitol, produces red pigment and it is Voges-Proskauer positive and ferment lactose ,Hemolytic test showed that all isolates β hemolytic positive. Das and Dash, 2015: Liu *et al*., 2017.

Colonies appeared as pink colonies (Lactose fermenters) while the non-lactose ferment bacteria show brown colonies accordant to key color of manufacture company. In order to differentiated between *Citrobacter* and other bacteria based on MacConkey agar. Bai *et al*., 2012.

Laboratories working in microbiological diagnosis have shown that *C. freundii* is very versatile in its colony morphology, as well as in its biochemical, antigenic and pathogenic behaviors. this phenotypic versatility has made *C. freundii* difficult to identify by conventional methods and it is frequently confused with both *S. enterica* and *E. coli* (Delgado *et al*., 2013).

Primer pair position on 23SrRNA Bacterial gene:

This study was successfully designed specific primer pair based on *C. freundii* with accession No. LR134118.1, and determined it location on the sequence genome of was at 23SrRNA gene of *C. freundii*, the length of 23SrRNA gene about 3kbp, the primer pair located at the first quarter of 23S gene. The primer pair were bounded with it target in plus/minus, the Forward primer (letters labeled by red color) located on the begging of whole sequence (189bp) while Reverse primer (reverse-complementary letters labeled by blue color) binder at the end of sequence, Figure (1) shown detail bioinformatics information about the composition of rRNA gene and amplicon sequence and flanking region of the primer pair at 23S.

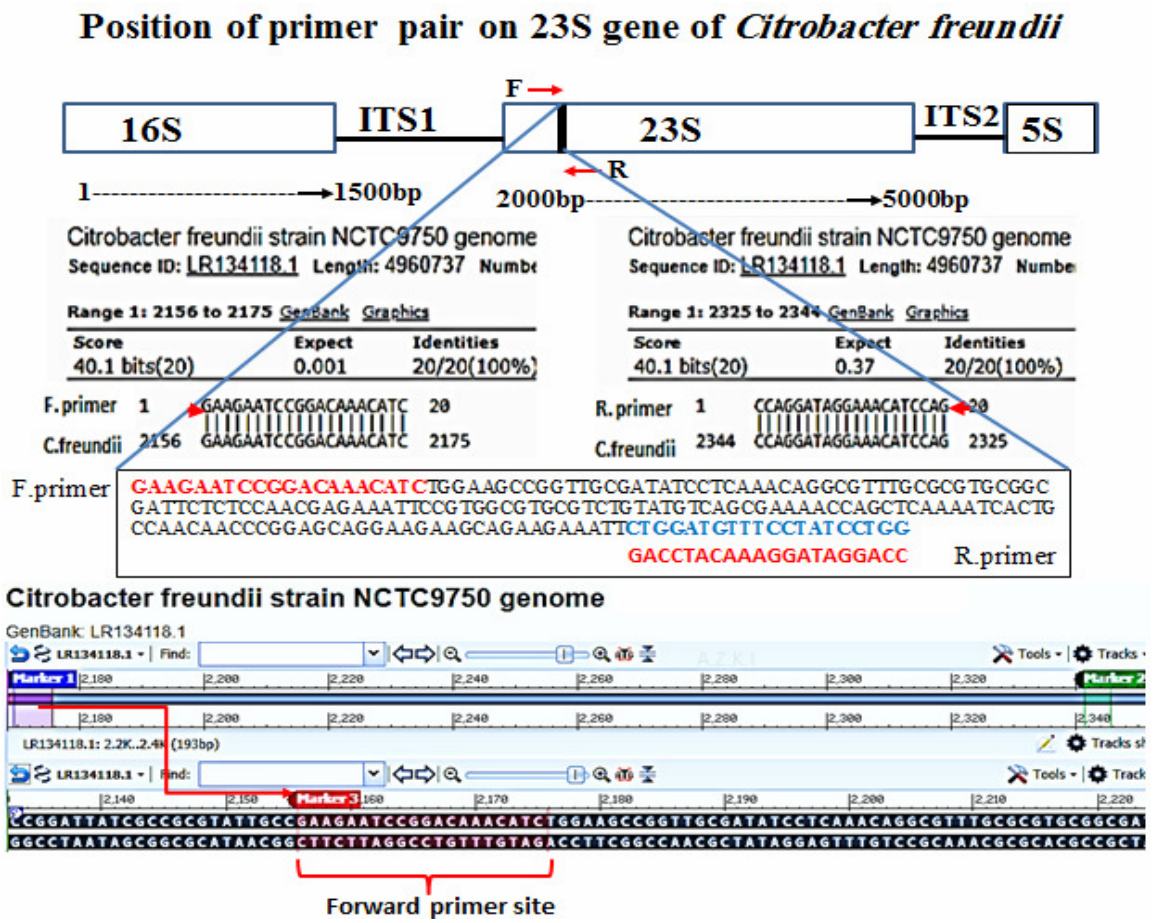

Fig. 1 : Presentation of Location for specific primer pair sequence (F and R) on 23S gene of *C. freundii* chromosome to the reference strain *C. freundii* Accession No.LR134118.1 Marker 1 and 2 the boundaries of amplicon started from 2175 -2344,Marker3 pointed to location and sequence of forward primer.

The specific primer pair was successfully identified 67.74% *C. freundii* out of 70 as DNA of suspected bacterial isolates designated it's *C. freundii* based on MCA and biochemical tests from all other closed related colonies grown on MacConkey agar by shown PCR amplicon 189 bp for 21 isolates of *C. freundii* (Figure 2).

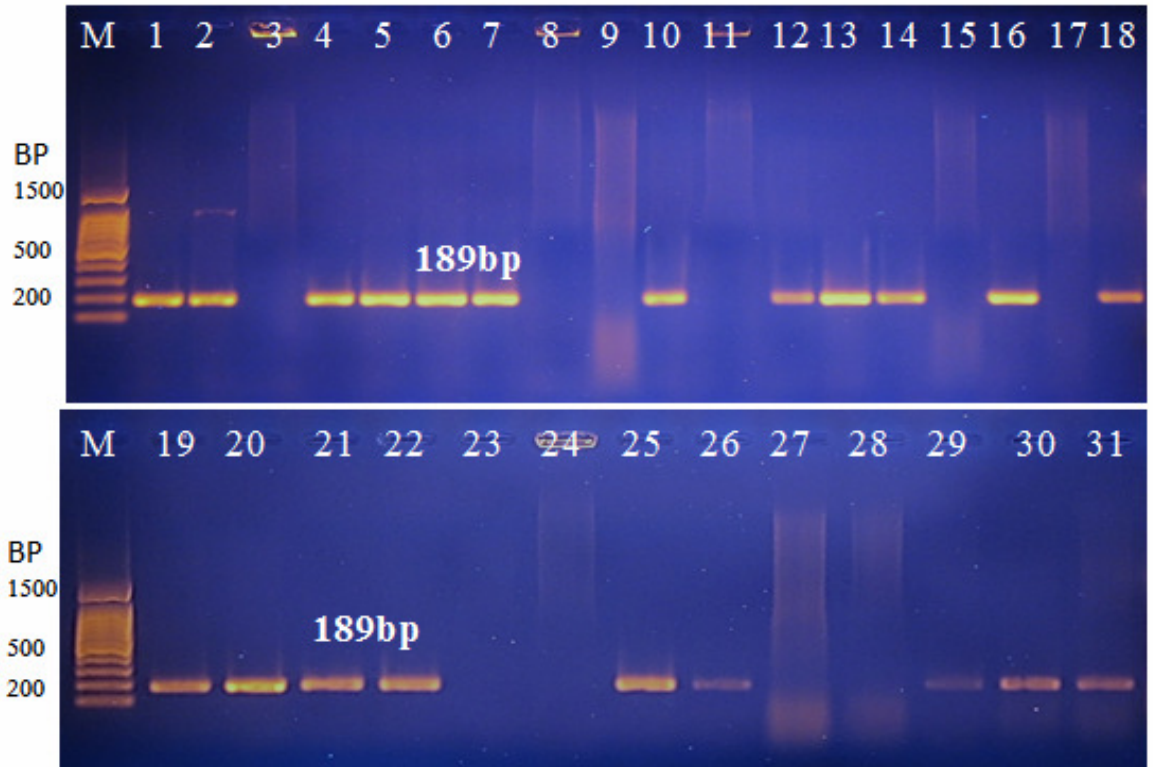

Fig. 2 : Gel electrophoresis profile of PCR product for 31 bacterial isolates of Enterobacteriaceae amplified by specific primer pair for *Citrobacter freundii*, bacterial isolates shown positive *C. freundii* 189bp, M=molecular marker 100bp for each step,1-31 bacterial isolates.

The molecular diagnostic of *C. freundii* by using specific primer design in this study 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).

Analysis partial sequence of 23S rRNA:

The specific primer pair (F and R) was succeeded to amplify it target sequence with flanking region in the 23S rRNA gene (Figure 3), in order to accurately and conformed the identify percentage of *C. freundii*. The sequence charts of two *C. freundii* Kufa1 & 2 were Compared with reference strain of *C. freundii* those deposited in genbank the results shown high similarity ranged between 99.35% to 100% match with those sequence of *C. freundii* strains with accession numbers CP037734.1, CP036435.1, LR134118.1, CP033744.1, CP033742.1 and LS992175.1 GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the results of the NCBI report(shotgun of report shown in Figure 3).

Citrobacter freundii Kufa 1 Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Citrobacter sp. CF971 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP041051.1
Citrobacter freundii strain R47 chromosome R47, complete sequence	289	289	95%	3e-74	100.00%	CP040698.1
Citrobacter freundii strain CAV1857 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP037734.1
Citrobacter freundii complex strain ABFQG, complete genome	289	289	95%	3e-74	100.00%	CP036435.1
Citrobacter freundii strain NCTC9750 genome assembly, chromosome: 1	289	289	95%	3e-74	100.00%	LR134118.1
Citrobacter freundii strain FDAARGOS_549 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP033744.1
Citrobacter freundii strain FDAARGOS_550 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP033742.1
Citrobacter freundii strain HM38 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP024672.1
Citrobacter freundii strain UMH19 chromosome, complete genome	289	289	95%	3e-74	100.00%	CP024673.1
Citrobacter freundii Kufa 2 Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Citrobacter sp. CF971 chromosome, complete genome	279	279	93%	2e-71	99.35%	CP041051.1
Citrobacter freundii strain R47 chromosome R47, complete sequence	279	279	93%	2e-71	99.35%	CP040698.1
Citrobacter freundii strain CAV1857 chromosome, complete genome	279	279	93%	2e-71	99.35%	CP037734.1
Citrobacter freundii complex strain ABFQG, complete genome	279	279	93%	2e-71	99.35%	CP036435.1
Citrobacter freundii strain NCTC9750 genome assembly, chromosome: 1	279	279	93%	2e-71	99.35%	LR134118.1
Citrobacter freundii strain FDAARGOS_549 chromosome, complete genome	279	279	93%	2e-71	99.35%	CP033744.1
Citrobacter freundii strain FDAARGOS_550 chromosome, complete genome	279	279	93%	2e-71	99.35%	CP033742.1

Fig. 3 : Description Blast Report of sequences alignment of *Citrobacter freundii* Kufa 1 and 2 isolate sequences with many reference sequence of *C. freundii* deposited in genbank with accession numbers, Identity 100% and 99.35% for each.

Phylogeny Tree based on pairwise sequence analysis

The two sequence of Kufa isolates of *C. freundii* were multiple alignment by MEGA 6 software with close related bacterial sequences to construct the phylogeny tree to provided two aims ,First to confirmed the specify of primer pair designed for *C. freundii* nor other *Citrobacter spp*. neither closed related Enterobacteriaceae bacteria like *E. coli*. second, the result shown the *C. freundii* Kufa stain 1 and 2 came with reference strains of *C. freundii* with accession numbers CP033744.1,CP033742.1 and CP024677.1 in the one cluster, while others *Citrobacter* spp became in different clusters so with the *E. coli*, Figure(4).

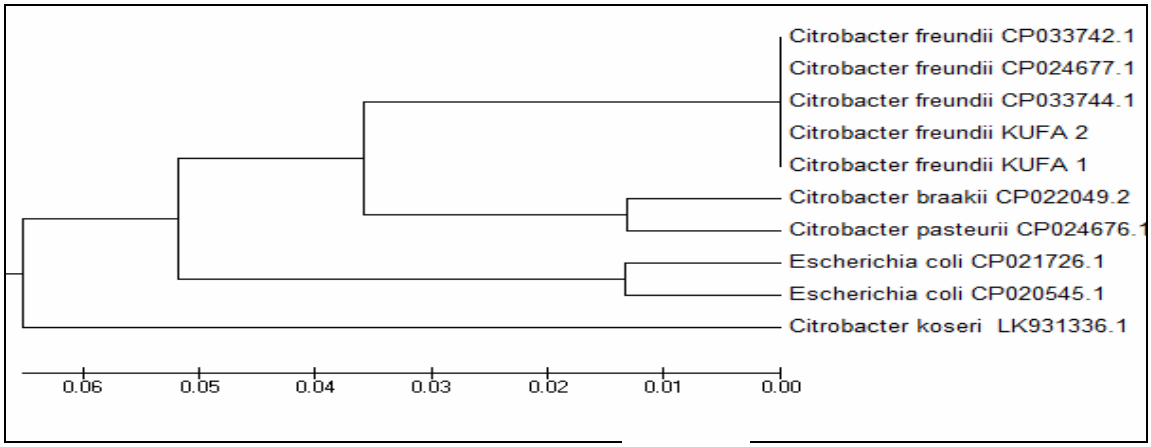

Fig. 4 : Construction of Phylogeny tree for two isolates of *C. freundii* Kufa 1 and 2 with other closed related bacterial strains with their accession numbers based on Mega6 software.

This study was conducted to conviction *C. freundii* as causes of diarrhea cases based on it high frequency isolation and identification based on molecular assy. this study recorded a new results correlated with high frequency of *C. freundii* with diarrhea cases under interest compare with previous studies in Iraq. These results consonant with results of Al-Jubori *et al*. (2012); Al-Hissnawy (2012) when she found this bacteria have acquired specific virulence traits that enable them to cause diarrhea in human, unfortunately, like isolates of *C. freundii* was recorded in the previous studies performed in Iraq, Al-Hissnawy *et al*., 2012,isolated only 11 isolates of *C. freundii* with percent 2.60% were isolated from 282 clinical stool samples.

Ethical approval

All authors hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of Interests

The authors did not declare any conflict of interest.

Acknowledgments

We gratefully thank University of Babylon, Science college for girls for supporting and agree to performed this work in it Genetic Lab. and Marjan hospital staff for supporting and supplying the clinical samples for this study.

References

Abd. Al-Kahdum, S.A.; Imran, Z.K. and Khdhier, H.M. (2019). Molecular Typing of *Malassezia* species By RFLP-PCR and Evaluate Antifungal Activities of Some Plant Extracts. *Plant Archives*, 19 (Suppl.1): 217-221.

Al-Hasnawi, A. (2014).Comparison of biochemical tests, Api system, Vitek 2 system and PCR of the enteropathogenic bacteria isolated from children with persistent diarrhea. And the occurrence of virulence factors and antibiotic resistance in the isolates. Master Thesis. Faculty of Science, University of Kufa. Thesis.

Al-Hissnawy, D.; AL-Thahab, A.A. and AL-Jubori, S.A. (2012). Evaluation of *Citrobacter freundii* isolated in Najaf governorate as an enterotoxin producer. Medical J. Babylon, 9(1): 1-5.

Al-Jubori, S.A.; Al-Thahab, A.A.; Dhia, S. and Al-Hissnawy, D.S. (2012). Evaluation of *Citrobacter freundii* isolated in Najaf governorate as an enterotoxin producer Al-Kufa University Journal for Biology, 4(2): 256-262.

Bai, L.; Xia, S.; Lan, R.; Liu, L.; Ye, C. and Wang, Y. (2012). Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. PLoS ONE 7:e33054. 10.1371.

Dana, E.; Vanja, K.; Silvia, G.; Clement, G.; Stefan, B. and Martin, F. (2006). Evaluation of 23S rRNA PCR Primers for Use in Phylogenetic Studies of Bacterial Diversity. Applied and environmental microbiology. 72(3): 2221–2225.

Das, S. and Dash, H.R. (2015). Microbial Biotechnology-A Laboratory Manual for Bacterial Systems Springer, India.

Delgado, G.; Souza, V.; Morales, R.; Cerritos, R. and Gonza´lez-Gonza´lez, A. (2013). Genetic Characterization of A typical *Citrobacter freundii*. PLoS ONE8 (9): e74120.

Dervisoglu, E.; Yumuk, Z. and Yegenaga, I. (2008). *Citrobacter freundii* peritonitis and tunnel infection in a patient on continuous ambulatory peritoneal dialysis Journal of Medical Microbiology, 57: 125–127.

Frederiksen, W. (2004). *Citrobacter*. pp. 651-656. In: Bergey's Manual of Systematic Bacteriology, 2nd ed. Werkman and Gillen 1932, 173, part B.

Hashim, M.H. and AlKhafaji, M.H. (2018). Isolation and identification of *Citrobacter freundii* from chicken meat samples using cultural and molecular techniques Iraqi Journal of Science, 59(3A): 1216-1224.

Imran, Z.K. and Alshammry, Z.W. (2016). Molecular diagnosis of Candidemia of intensive care unit patients based on sequencing analysis of ITS regions. International Journal of Pharm Tech. Research, 9(12): 658-668.

Kurtoglu, M.G.; Opus, A.; Ozdemir, M. and Baysal, B. (2011). Isolation of *Citrobacters* in various infections and their antimicrobial sensitivity rates. J. Fac. Vet. Med. Univ. Kafkas, 17: 99-104.

Liu, L.H.; Wang, N.Y.; Wu, A.Y.; Lin, C.C.; Lee, C.M. and Liu, C.P. (2017). *Citrobacter freundii* bacteremia: risk factors of mortality and prevalence of resistance genes. J. Microbiol. Immunol. Infect. 10.1016.

Ludwig, W. and Schleifer, K.H. (1994). Bacterial phylogeny based on 16S and sequence analysis. FEMS Microbiology Reviews 15: 155-173.

Peirano, G.; van der Bij, A.K.; Freeman, J.L.; Poirel, L.; Nordmann, P.; Costello, M. (2014). Characteristics of *Escherichia coli* sequence type 131 isolates that produce extended-spectrum beta-lactamases: global distribution of the H30-Rx sublineage. Antimicrob. Agents Chemother., 58: 3762–3767.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30: 2725–2729.

Yumuk, Z.; Afacan, G.; Nicolas-Chanoine, M.H.; Sotto, A. and Lavigne, J.P. (2008). Turkey: a further country concerned by community-acquired *Escherichia coli* clone O25-ST131 producing CTX-M-15. *J.* Antimicrob. Chemother. 62: 284–288.