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INVESTIGATION OF *CITROBACTER FREUNDII* FROM SHEEP USING CULTURAL AND MOLECULAR ANALYSIS

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Abstract

Citrobacter freundii is had an important in medical and economical issues, there are few local studies about it in animals, this study aimed to isolate and identify *Citrobacter freundii* from others that have a similar biochemical and morphological characteristics. One hundred fecal samples were collected from sheep's (female and male) in Baghdad city, during december 2019 to Feburury 2020. 25 (25%) of *Citrobacter* isolates was isolated from the collected fecal samples by using culture media and identified by biochemical tests, antimicrobial susceptibility test was susceptibly to all antibiotic which tested and the identification was confirmed using Vitek 2 compact, polymerase chain reaction (PCR) and sequencing for 16S rRNA and the isolated positively identified as 98% *C. freundii* by vitke2 and 100% by sequencing when homology with references in Genbank. This study concluded that identification of *C. freundii* by PCR was in accordance with those of biochemical test and vitke2 it providing a valuable tool for rapid detection of *C. freundii* in clinical samples from sheep.

Key words: *Citrobacter freundii*, PCR, sheep, Baghdad city.

Introduction

Citrobacter, a genus of the *Enterobacteriaceae* family, Gram-negative, facultative anaerobic bacteria that look as *Coccobacilli* or rods (Abbott, 2011). They are motile using their peritrichous flagella. Their species such as *C. amalonaticus, C. koseri, and C. freundii* can ferment mannitol with making of H_2S and can use citrate as their single source of carbon (Doran, 1999; Thompson *et al.*, 2018).

Citrobacter species were considered the intestinal inhabitants of human and animals and commonly existed in sewage, water and soil (Frederiksen *et al.,* 2005) and are uncommon opportunistic nosocomial bacteria can cause respiratory tract infection, septicemia and encephalitis in sheep (Yimer- Asseged, 2018; Huisheng *et al.*, 2018). In Iraq, almost the isolation of *citrobacter* from human, while few researcher isolated from animals (Al-Hashimi, 2002). *C. freundii* isolated from some diarrhoetic cases in infants at Mosul City (Al-Muslemaw, 2007). and also, *C. freundii* was isolated from urinary tract infections, wound infections, otitis media infections, intestinal tract infections and from the environment of the ward in the hospital. Also isolated from chicken meat sample in different markets at Baghdad city (Hashim-AlKhafaji, 2018). The conventional diagnosis of bacteria has been based on clinical sings, isolation of the organism, extensive phenotyping and capsular serotyping **(**Hunt *et al.,* 2000); molecular identification improved accurateness of characterization, speed of detection, determination of taxonomic position and understanding of intra-species genetic relationships **(**Hunt *et al.,* 2000). *Citrobacter freundii* was successfully detected by using primers pairs based 16S rRNA gene that belongs to its chromosomes and produces 1500 bp from chicken meat in Baghdad city (Hashim-AlKhafaji, 2018) and the aim of this study is to isolate and identification *C. freundii* from sheep using biochemical and molecular analysis.

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Materials and Methods

Samples collection:

One hundred of sheep fecal samples were collected from different regions in Baghdad city, Information including sex, age and location were fixed on container labile, then placed in ice and transported to laboratory within period less than two hours (Quinn *et al.*, 2011).

Isolation

One gram of each fecal samples were placed in sterile

test tube containing 10 ml of normal saline, 0.1ml of each sample suspension is inoculated on the *Salmonella shigella* (SS) agar medium or MacConkey agar, and then incubated at 37°C for 24-48 hours. The suspected colonies inoculated on Xylose lysine deoxycholate (XLD) agar then are incubated at 37°C for 24-48 hours (Quinn *et al.*, 2011).

Identification

The *Citrobacter* isolates were identified to the level of species using the traditional morphological, biochemical tests, vitek2 compact system, antimicrobial susceptibility test and pathogenicity test such as PCR assay of virulence gene. Vitek 2 system (bioMérieux, Lyon, France) according to manufacturer's instructions was used to identify the bacterial isolates and Antimicrobial susceptibility test. DNA extraction to identify of *Citrobacter freundii* by PCR, DNA was extracted using Presto Mini g DNA bacteria Kit according to manufacturer's instructions (Geneaid, KOBA). The DNA concentration was measure by NANODROP-2000 spectrophotometer (Thermo Scientific Inc., USA). 16S rRNA Primer: F5'-AGAGTTTGATCCTGGCTCAG-3'R5'-TACGGTTACCTT GTTACGACTT-3' amplification size 1500bp (Hashim-AlKhafaji, 2018).

PCR amplification was performed final volume (25µl) containing (12.5 µl) Master mix (promega), (1 µl) forward primer, (1 µl) reverse primer, (8.5 µl) nuclease free water, and (2 µl) DNA template. The protocol for PCR condition was initial denaturation 95°C for 5 min. denaturation 95°C for 30 sec., annealing 60°C for 30 sec. extension 72°C for 1 min. and final extension 72°C for 7min. The PCR product tubes of sample with forward and reverse primer of 16S rRNA were sent for DNA sequencing.

Results

Results of one hundred fecal samples (normal feces and diarrhea) of female were 6(12%) and 12(24%) respectively, while, 0(0%) and 7(14%) *C. freundii* isolates were isolated from male (normal feces and diarrhea) respectively, table 2.

Table 2: Source and isolation rate of *Citrobacter freundii.*

Studies\ months	No. of examined samples	Sexes and age	Type and source of sample	No. of positive isolates	Positive percentage %
November 2019 December 2019 January 2020 February 2020	100	Female 12 moth	Normal feces	6	12%
			diarrhea	12	24%
		Male 9 month	Normal feces	0	0%
			diarrhea	7	14%
Total	100			25	25%

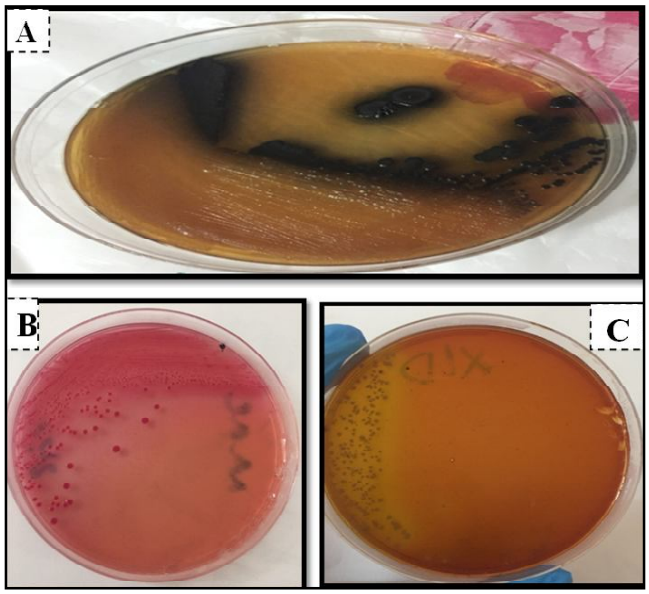

Fig. 1: *Citrobacter* isolates on (a) SS agar (b) M.A agar (c) XLD agar.

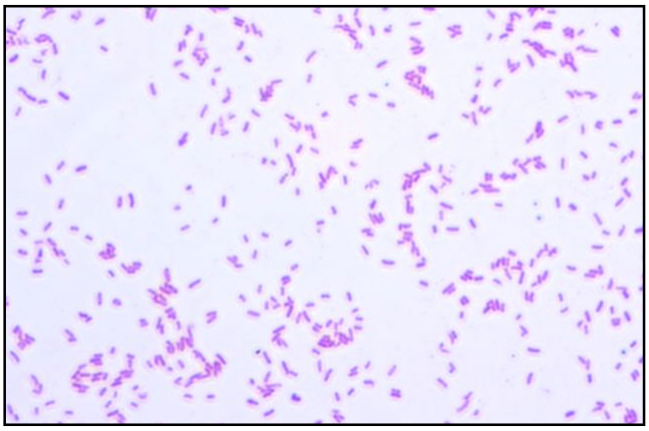

Fig. 2: Gram negative stain *of Citrobacter* isolates.

Identification of *C. freundii* was done by study colonial morphology on SS, M.A and XLD agar, all isolates showed similar appearance on SS agar as small or large pale flattened colonies with black center due to their ability to produces H_2S on S.S agar after 24 hrs Fig. 1. *Citrobacter* isolates were appeared as pink colonies on MacConkey agar due to lactose fermenter while *Salmonella* is pale colonies (Non lactose fermenter) MacConkey agar, on XLD *Citerobacter* appeared as yellow colonies while Salmonella appeared as red colonies with black center. To confirm the primary identification Gram stain was performed to examine the microscopic properties which were Gram negative bacilli Fig. 2. All isolates and standard strain examined by biochemical tests table 3.

To confirm the identification of *Citerobacter* spp.

Table 3: Biochemical test of *Citrobacter* isolates.

Results	Test
Pink colonies	Growing on MacConkey agar
Black center	S.S agar
Yellowish colonies	XLD agar
G-	Gram stain reaction
-	Urease
+	Catalase
-	Oxidase
-	Gelatinase

Vitek 2 compact system ad antimicrobial susceptibility were depended and the result showed that the isolated bacteria in this study was *Citrobacter* and the species *freundii* as shown in table 4.

The isolate was tested to present 16 s rRNA. Hence, the isolate was positive for 16SrRNA gene amplification using monoplex PCR technique, 1.5% agarose gel electrophoresis Fig. 4.

Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The *Citrobacter freundii* isolate shared 100% homology with reference strains in GenBank

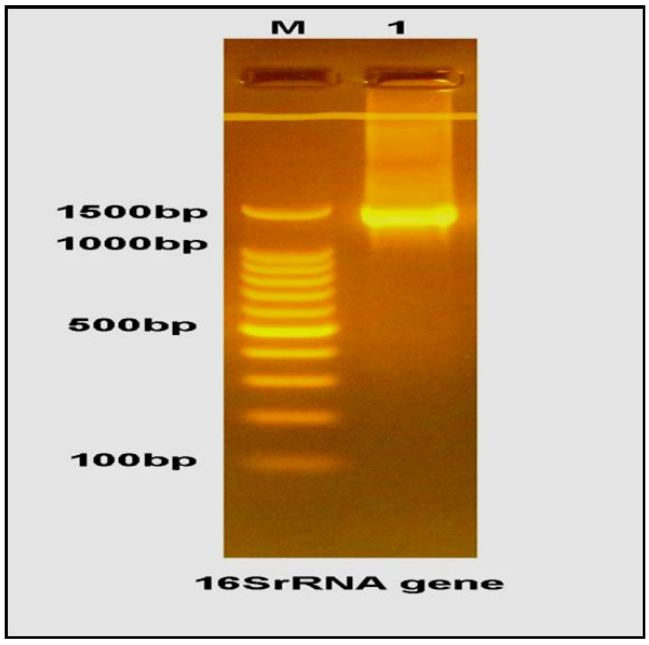

Fig. 4: Amplified PCR products of 16SrRNA gene (1500 bp): Agarose gel electrophoresis, ethedium bromide stained, 1.5 % agarose, electrophoresed in 75 volt for 2 hrs and photographed under ultraviolet trans-illuminator. M: The DNA molecular weight marker (100 bp ladder) and 1: the amplified PCR product of 16SrRNA of *Citrobacter freundii*.

Table 4: results of Vitek 2 compact system ad antimicrobial susceptibility.

Identification Information			Analysis Time:	3.85 hours		Status:	Final
Selected Organism			98% Probability Bionumber:	Citrobacter freundii 4415611754421210			
ID Analysis Messages							

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	+	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Susceptibility Information	Analysis Time: 8.48 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	<= 8	S	Amikacin	<= 2	S
Ticarcillin/Clavulanic Acid	<= 8	S	Gentamicin	<= 1	S
Piperacillin	<= 4	S	Tobramycin	<= 1	S
Piperacillin/Tazobactam	<= 4	S	Ciprofloxacin	<= 0.25	S
Ceftazidime	<= 1	S	Pefloxacin		
Cefepime	<= 1	S	Minocycline	4	S
Aztreonam	<= 1	S	Colistin		
Imipenem	0.5	S	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	<= 20	S

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

Table 5: Result of *Citrobacter freundii* homology with Genbank.

Citrobacter sp. strain Tue2_1 16S ribosomal RNA gene, partial sequence

Sequence ID: MN548424.1 **Length:** 1462 **Number of Matches:** 1

Range 1: 631 to 1408 GenBank Graphics ▼ Next Match ▲ Previ

Score	Expect	Identities	Gaps	Strand
1496 bits(778)	0.0	778/778(100%)	0/778(0%)	Plus/Plus

```
Query  1     TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  631   TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG

Query  61    CGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  691   CGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG

Query  121   AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  751   AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT

Query  181   CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACC
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  811   CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACC

Query  241   AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  871   AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC

Query  301   TTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCA
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  931   TTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCA

Query  361   TTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCT
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  991   TTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCT

Query  421   AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCC
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1051  AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCC

Query  481   CCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAA
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1111  CCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAA

Query  541   CATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAAT
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1171  CATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAAT

Query  601   GACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAG
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1231  GACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAG

Query  661   CTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCA
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1291  CTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCA

Query  721   TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1351  TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
```

(Accession No. MN548424.1) table 5.

Discussion

Citrobacter spp. are uncommon opportunistic nosocomial bacteria can cause urinary tract, hematologic, or neonatal infections (e.g. meningitis, sepsis, general bacteremia); intra-abdominal sepsis; brain abscesses; or pneumonia (Ryan *et al.*, 2004; Raphael-Riley, 2017). The positive results for isolation (25%) of *C. freundii* were observed from one hundred fecal samples (normal feces and diarrhea) of female and male. these results were in agreement with (Al-Muslemaw, 2007). Which isolated eight *C. freundii* isolate from 250 of clinical samples such as fecal and urine in Baghdad city and *C. freundii* was the most common type, occupying 75% of clinical isolates. These results also in agreement with (Hashim-AlKhafaji, 2018). Which isolated (3) *C. freundii* from 25 chicken meats sample in local market also in Baghdad city. The results of antimicrobial susceptibility was in agreement with (AL–Gannabee, 2006). Who pointed the isolates of *C. freundii* were sensitive (100%) to Ciprofloxacin, Imipenem and Gentamycin. The results of identification of *Citrobacter* isolate by PCR analysis, the isolate was tested to present 16 s rRNA. Hence, the isolate was positive for 16SrRNA gene amplification at 1500bp. This results was in agreement with (Hashim-AlKhafaji, 2018). Who found the *C. freundii* was identification by PCR analysis and positive to the presence 16 s rRNA gene. The results was also in agreement with (El-Barbary-Hal, 2017**)** which pointed the use of universal primer 27F was succeeded to amplify the 16S rRNA gene in the PCR reaction and the resulting sequences covered variable regions of 16S rRNA in bacterial isolates in order to accurately identify the bacterial species. Comparing the nucleotide sequences of 16S rRNA gene using BLASTN, showed that the similarity of studied bacterial isolates was a 99% match with that of *C. freundii* (accession No. KX156769).

Conclusion

The results of this study concluded that identification of *C. freundii* by PCR was in accordance with those of biochemical test and vitke2 it providing a valuable tool for rapid detection of *C. freundii* in clinical samples from sheep in Baghdad city for the first time.

References

Abbott, S.L. (2011). *Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas* and other Enterobacteriaceae. In Manual of Clinical Microbiology, 10th Edition American Society of Microbiology. 639-657.

AL–Gannabee, Z.S.M. (2006). The effect of the probiotic *Saccharomyces boulardii* on *Citrobacter* infections in mice. M.Sc. thesis. college of science, University of Baghdad, Baghdad, Iraq.

Al-Hashimi, E.S.N.H. (2002). Pathogenicity of *Citrobacter freundii* bacterium and toxins isolated from some diarrheic cases in infant at Mosul City. MSc thesis. College of Science. Mosul University, Iraq.

Al-Muslemawi, T.A.J. (2007). Study of some biochemical, biological and pathological properties of lipopolysaccharide extracted from *Citrobacter freundii*. Ph.D. Thesis. Baghdad University, Baghdad, Iraq.

Doran, T. (1999). The Role of *Citrobacter* in Clinical Disease of Children: Review. *Clinical Infectious Diseases,* **28(2):** 384-394.

El-Barbary, M.I. and A.M. Hal (2017). Molecular identification and pathogenicity of *Citrobacter* and *Serratia* species isolated from cultured *Oreochromis niloticus*. *Egyptian Journal of Aquatic Research,* **43:** 255–263.

Frederiksen, W., D.J. Brenner and N.R. Krieg (2005). Bergey's manual of systematic bacteriology, 2nd edn. *Proteobacteria,* **2:** 651-656.

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

Huisheng, L., Z. Zhanqin, X.I. Yun, D. Ke and X. Qiao (2018). Fatal cases of *Citrobacter freundii* septicemia and encephalitis in sheep. *J.V. Diagno. Investi.,* **30(2):** 245-248.

Hunt, M.L., B. Adler and K.M. Townsend (2000). The molecular biology of *Pasteurella multocida*. *Veterinary Microbiology,* **(72):** 3-25.

Quinn, P.J., B.K. Markey, F.C. Leonard, P. Hartigan, S. Fanning and P.E.S. Fitz (2011). Veterinary microbiology and Microbial Disease. 2nd ed. UK: *Wiley - Blackwell West Sussex,* **978(1):** 118-30215.

Raphael, E. and L.W. Riley (2017). Infections caused by antimicrobial drug-resistant saprophytic Gram-negative bacteria in the environment. *Frontiers in medicine,* **4:** 183.

Ryan, K., C. Ray and J. Sherris (2004). Sherris medical microbiology: an introduction to infectious diseases, McGraw-Hill. 237.

Thompson, R., J. Perry, S. Stanforth and J. Dean (2018). Rapid detection of hydrogen sulfide produced by pathogenic bacteria in focused growth media using SHS-MCC-GCIMS. *Microchemical Journal,* **140:** 232-240.

Yimer, N. and B. Asseged (2018). Aerobic bacterial flora of the respiratory tract of healthy sheep slaughtered in Dessie municipal abattoir, northeastern Ethiopia. Revue 10 *Remaniee,* **42:** 473.