Detection of Quorum Sensing Signal Molecules and Identification of *esp*B and Crt4 genes among Biofilm Forming of *Citrobacter freundii*

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Abstract

150 samples from different clinical sources were collected from October 2018 to March 2019 from three hospitals in Maysan: Al Sadr General Education Hospital, Al Zahrawi Surgical Hospital and Maternity and Child Hospital to demonstrate the spread and distribution of *Citrobacter freundii* in several hospitals in Maysan. All isolates were identified based on morphological characteristics and biochemical tests. Results were confirmed by Api 20 E and Vitek 2 compact. A total of 14 isolates (9.3%) of 150 were found to be Citrobacter freundii. The isolates were considered as acute diarrhea in children, UTI, burns, wounds and most frequent ear swabs. PCR results showed that the LuxR 428bp genes were present in the Citrobacter freundii bacteria identified by previous diagnostic methods and this confirms the accuracy of the tests and methods used to determine this type.

Keywords: *Citrobacter freundii; espB and Crt4 genes; Quorum sensing signal*

Introduction

Citrobacter, a genus of the Enterobacteriaceae family, Gram-negative, facultative anaerobic bacteria that look as coccobacilli or rods (1) . Citrobacter spp. are motile using their peritrichous flagella, can ferment mannitol with making of H2S, and can use citrate as their single source of carbon (2),(3). 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 (4) ,(5). Citrobacter spp. infections can be mortal with 33-48% overall death rates being reported including 30% for children(6),(7). Children and immune deficiency, elderly, or weakened patients are at risk of infection (2), (9). Citrobacter spp. is prevailing worldwide, as it is a part of the normal intestinal flora of humans (10),(11). Less well known species that have also been implicated in foodborne disease like some strains of Citrobacter spp. (notably C. freundii), Klebsiella spp., Providencia spp. Enterobacter spp. and Proteus spp., may occasionally cause what is often described as opportunistic gastroenteritis (12), this study aimed to isolation and identification of C. freundii from chicken meat samples using cultural and molecular techniques.

Method and materials

Samples collection

150 samples from different clinical sources were collected from October 2018 to March 2019 from three hospitals in Maysan.

Isolation

each sample was inoculated on the Salmonella shigella (SS) agar medium, the plates were left to solidify at room temperature, and then were incubated at 37 °C for 24-48 hours. Later the grown colonies were further investigated

Identification

The Citrobacter isolates were identified to the level of species using the traditional morphological and biochemical tests [(13)]. The identification of isolates was confirmed by vitek2 compact system.

Cultural characteristics on selective and differential media.

SS, MacConkey and Xylose lysine deoxycholate (XLD) agar

The organisms were cultured on S.S agar media and incubated overnight at 37°C. The colonies of C. freundii appear with black center after 24hrs incubation period, The suspected colonies of C. freundii cultured on MacConky media, the positive result appears pink (Lactose fermenters) after 24hrs incubation period, pale colonies further incubated for 24hrs to identify the (late lactose fermenters). The selected colonies were cultured on Xylose lysine deoxycholate agar, after 24hrs, the positive result appeared as yellow colonies [13].

Eosin Methylene Blue (EMB) agar

In order to differentiate Citrobacter from E.coli, the lactose fermenter isolates were subcultured on EMB for 24hr. at 37Co. Brown colonies were the positive result[14].

Identification of bacteria by Vitek 2 compact system.

Vitek 2 compact was used to identify the bacterial isolates. It is a compact system of two parts, Instrument and computer. The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinisation, enzyme hydrolysis, and growth in the presence of inhibitory substances.

Identification of Bacteria by PCR

DNA Extraction

Genomic DNA was isolated from Bacteria according to the protocol of Wizard Genomic DNA Purification Kit, Intron. A PCR reaction with a specific primer (Table-1).

Table 1-Primers sequences

Primer Name	Sequences	Tm˚C	Size (bp)
LuxR-F	GCACGGATTACATCATTA	49.3	428
LuxR-R	GCACGGATTACATCATTA	49.3	

For **LuxR** gene was performed to identify *C. freundii* (Table-2).

Table 2-Reaction mixture

PCR master mix	Volume
DNA template	5 µL
Green master mix	12.5 µL
Forward Primer10pmol	2.5 µL
Reverse Primer10pmol	2.5 µL
Free nuclease water	2.5 µL
Total	25 µL

(25µl) of PCR amplification mixture contained (12.5 µl) Master mix, (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 40 sec., extension 72 °C for 1 min. and final extension 72 °C for 7min.

Results and Discussion

Bacterial Isolation and Identification

Twenty five chicken meat samples were collected from local markets in Baghdad city. Citrobacter was detected in 3 samples, were all samples cultured on S.S. agar for initial isolation, after incubation at 37°C for 24 hr ; different types of bacterial isolates appeared on S.S. agar, of them: small pale flattened colonies with

black center due to their ability to produces H2S on S.S agar, then these colonies sub-cultured on MacConkey, XLD and EMB to differentiate Citrobacter from Salmonella because both of them are H2S, Citrobacter is lactose fermenter on MacConkey agar appeared as pink colonies while Salmonella is pale colonies (Non lactose fermenter) on XLD Citerobacter appeared as yellow colonies while Salmonella appeared as red colonies with black center . After incubation period; lactose fermenter (pink) on MacConkey and yellow colonies on XLD while on EMB they were brown in colour, these were depended as Citrobacter. To confirm the primary identification Gram stain was performed to examine the microscopic properties which were Gram negative bacilli. The ability of Citrobacter to produce urease enzyme was detected using urease test in order to differentiate it from the genus Proteus which was urease producer while Citrobacter isolates were non urease producers. Thus depending on colonial morphology; bacterial isolates were identified as Citrobacter Figure-1 (A, B, C, D) and (Table-3) showed these biochemical tests used to identify Citrobacter as described by[15], [16].

Figure1-Different selective and differential media cultured with Citrobacter spp. after incubation at 37°C for 24 hr.

A. Pale colonies with black center on S.S. agar

B. Small pink (Lactose fermenter) colonies on MacConkey agar

C. Yellow colonies on XLD agar

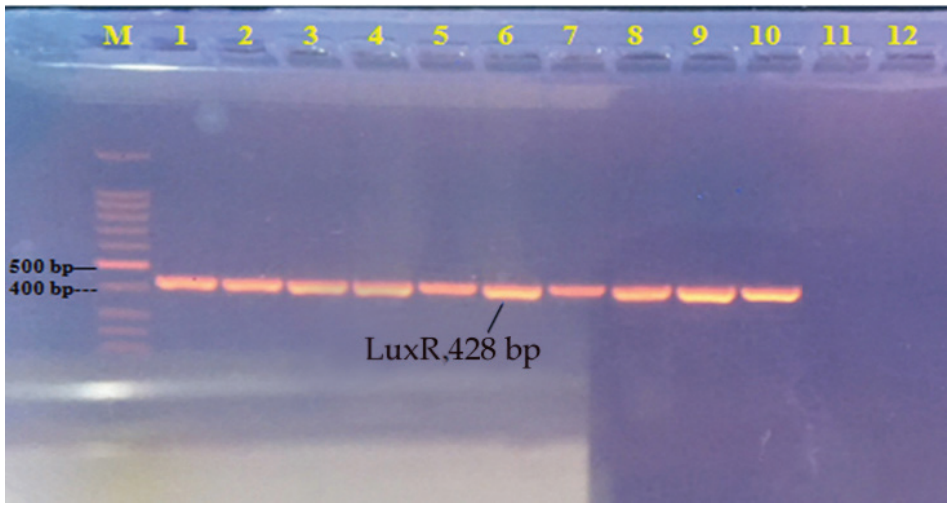
D. Brown colonies on EMB.

Table 3-Result of biochemical tests

Test	Result
Growing on MacConkey agar	Dry Pink colonies
Growing on EMB	Not forms green metallic sheen
Gram stain reaction	Gram negative bacteria
Urease	Non urease producer
S.S agar	Pale colonies with black center
XLD agar	Yellow colonies

To confirm the identification of Citerobacter spp. Vitek 2 compact system was depended and the result showed that the isolated bacteria in this study was Citerobacter and the species freundii

In order to confirm the identification of Citrobacter to species level LuxR gene amplification was performed using monoplex PCR technique, 1.5 % agarose gel electrophoresis was used to detect the positive result as shown in Figure-2.



(Figure 2) Amplified PCR products of LuxR gene (428 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 LuxR of C10 isolate of *Citrobacter freundii*.

One of the most gorgeous likely uses of 16Sr RNA gene sequence informatics is to offer genus and species or tax identification for isolates [17]. Although 16SrRNA gene sequencing is highly valuable in regards to bacterial classification [18]. PCR products were exposed to direct sequencing, both strands of PCR products were sequenced with an automatic sequencer. Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI).

Conclusion

The following conclusions were obtained from this study:

• The prevalence and distribution of Citrobacter spp. in some Maysan hospitals shows a relatively low percentage in its distribution.

• The dominance species of Citrobacter was Citrobacter freundii.

• All isolates of Citrobacter freundii produced Bioflim formation by Congo red agar, Christensen method and micro-titer plate assay.

• The effect of different temperature and pH values on Citrobacter freundii growth showed that best growth temperature was 37°C and the best growth pH for growth was 7.

• These data are of great significance as the signal molecules aid in biofilm formation which in turn confer various properties of pathogenicity to the clinical isolates including drug resistance. The use of quorum sensing signal blockers to attenuate bacterial pathogenicity is therefore highly attractive, particularly with respect to the emergence of multi antibiotic resistant bacteria.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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References

1. Abbott, S. L. Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas, and other Enterobacteriaceae. In Manual of Clinical Microbiology, 10th Edition, *American Society of Microbiology*. 2011. 639-657.
2. Doran, T. "The Role of Citrobacter in Clinical Disease of Children: Review." *Clinical Infectious Diseases*, 1999. 28(2): 384-394.
3. Knirel, Y., Kocharova N., Bystrova, O., Katzenellenbogen, E. and Gamian, A. Structures and serology of the O-specific polysaccharides of bacteria of the genus Citrobacter. , *rchivum Immunologiae et Therapiae Experimentalis*, 2002. 50(6): 379-392.
4. Thompson, R.,; Perry, J., Stanforth, S. and Dean, J. Rapid detection of hydrogen sulfide produced by pathogenic bacteria in focused growth media using SHS-MCC-GCIMS. *Microchemical Journal*, 2018. 140: 232-240.
5. Ryan, K., Ray, C. and Sherris, J. 2004. Sherris medical microbiology: an introduction to infectious diseases, McGraw-Hill. 237
6. Raphael, E. and Riley, L. W. Infections caused by antimicrobial drug-resistant saprophytic Gram-negative bacteria in the environment. *Frontiers in medicine*, 2017. 4: 183
7. Pepperell, C., Kus, J., Gardam, M. Low-virulence Citrobacter species encode resistance to multiple antimicrobials. *Antimicrob Agents Chemother*, 2002, 46(11): 3555-3560
8. Owoseni, M. and Okoh, A. Assessment of chlorine tolerance profile of Citrobacter species recovered from wastewater treatment plants in Eastern Cape, *South Africa. Environmental monitoring and assessment*, 2017; 189(4): 201.
9. Chen, Y., Wong, W, Fung, C., Yu, K. and Liu, C. Clinical features and antimicrobial susceptibility trends in *Citrobacter freundii* bacteremia. *Journal of microbiology, immunology, and infection*, 2002. 35(2): 109-114.
10. Holmes, B. and Aucken, H. 1998. Citrobacter, Enterobacter, Klebsiella, Serratia and other members of the Enterobacteriaceae, in Collier L, Balows A, Sussman M (eds):Microbiology and Microbial Infections: Systematic Bacteriology (ed 9). London, Arnold, (999-1033).
11. Card, R., Mafura, M., Hunt, T., Kirchner, M., Weile, J., Rashid, M. amd Anjum, M. Impact of ciprofloxacin and clindamycin administration on Gram-negative bacteria isolated from healthy volunteers and characterization of the resistance genes they harbor. *Antimicrobial agents and chemotherapy*, 2015. 59(8): 4410-4416.
12. Lawley, R., Curtis, L. and Davis, J. 2012. The food safety hazard guidebook. *Royal Society of Chemistry*.
13. MacFaddin, J. 2000. Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott Williams and Wilkins, Philadelphia, PA.
14. Bettelheim, K., Evangelidis, H., Pearce, J., Sowers, E. and Strockbine, N. A. Isolation of a *Citrobacter freundii* strain which carries the *Escherichia coli* O157 antigen. *Journal of clinical microbiology,* 1993. 31(3): 760-761.
15. Janda, J., Abbott, L., Cheung, W. and Hanson, D. Biochemical identification of Citrobacteria in the clinical laboratory. *Journal of Clinical Microbiology,* 1994. 32(8): 1850-1854
16. Gwida, M., Hotzel, H., Geue, L. and Tomaso, H. Occurrence of Enterobacteriaceae in raw meat and in human samples from Egyptian retail sellers. *International scholarly research notices*. 2014. (2014):1-6.
17. Janda, J. and Abbott, S. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, 2007. 45(9): 2761- 2764.

18. Bosshard, P., Zbinden, R., Abels, S., Böddinghaus, B., Altwegg, M. and Böttger, E. "16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory." *Journal of clinical microbiology*, 2006. 44(4): 1359-1366.