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Molecular, biochemical, and phenotypic characterization of a newly isolated *Enterobacter hormaechei* subsp. *xiangfangensis* strain associated with diarrhea cases in Iraq

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ABSTRACT

Aims: The current study aimed to isolate and characterize bacterial strains associated diarrhea with the focus on *Enterobacter* species strains and test for susceptibility to antibiotics.
Methodology and results: A total of 400 stool samples from inpatients suffering from diarrhea in Al-Qasim Hospital at Al-Hilla City of Iraq were screened form January 2018 to January 2019. Phenotypic, molecular, and biochemical methods were used to identify the isolated bacteria. A new strain of *Enterobacter hormaechei* was obtained from two stool samples of inpatients suffering from diarrhea for more than two weeks. This strain is Gram negative, rod shaped, and facultative anaerobic. Multiple sequence alignment analysis and phylogenetic tree construction of the sequenced 16S rRNA gene of the isolated strain suggested that this strain can be identified as *E. hormaechei* subsp. *xiangfangensis*, named as *E. hormaechei* subsp. *xiangfangensis* strain AA1. This strain was resistant to augmentin, ampicillin, cephalothin, cefoxitin, ceftazidime, cefixime, ticracillin/clavulanic acid, cefotaxime, streptomycin, erythromycin, amikacin, ciprofloxacin, and chloramphenicol, while it was susceptible to meropenem along with imipenem.
Conclusion, significance and impact of study: In the present study, *E. hormaechei* subsp *xiangfangensis* was isolated for the first time in Iraq and was resistant to most of the tested antibiotics, making it an etiologic agent that is not easy to be treated.

Keywords: Enterobacter cloacae complex, *Enterobacter hormaechei*, *Enterobacter xiangfangensis*, molecular identification, 16S rRNA gene

INTRODUCTION

The *Enterobacter cloacae* complex species include a various collection of microbes that have been extensively detected in diverse environments, but they are also capable to act as human pathogens (Mezzatesta *et al*., 2012). These organisms belong to the family of Enterobacteriaceae, which has been associated with the human colonization of gastro-intestinal tract and can cause pathologic progression resulting in successive infectivity of the gut (Sanders and Sanders, 1997). They are also considered as frequent nosocomial pathogens (Gaston, 1988), which are capable to cause a huge sort of diseases, for example septicemia, cystitis, hospital-acquired bacteremia, injury contagions and pneumonia (Xiong *et al*., 2008). A lot of species of this complex showed resistance to multiple types of drugs, including the newest versions of imipenem, carbapenems meropenem, and ertapenem leading to an increased significance of them (Park *et al.*, 2007).

These organisms were known to have high heterogeneity of genomes, and they are all currently belonging to species named: *E. cloacae*, *E. ludwigii, E. asburiae*, *E. nimipressuralis*, *E. kobei*, as well as *E. hormaechei*. Among them, *E. hormaechei* showed to be the most often identified species in clinical cases (Hoffmann and Roggenkamp, 2003; Hoffmann *et al.*, 2005; Paauw *et al.*, 2008; Morand *et al.*, 2009). Research indicated first three *E. hormaechei* subspecies, which are: *hormaechei*, *oharae*, and *steigerwaltii*, depending on their specific features as well as biochemical assessment (Hoffmann *et al.*, 2005). Recently, a new subspecies was added, with a name of *E. hormaechei* subsp. *xiangfangensis* based on genomes sequence analysis of *E. hormaechei* (Sutton *et al.*, 2018). This subspecies was previously known as *E. xiangfangensis* (Gu *et al.*, 2014), then found to be linked to *E. hormaechei*. However, *E. hormaechei* is frequently misnamed to be *E. cloacae*, and this is possibly because the subspecies of *E. hormaechei* were not accurately characterized until recently. In addition, missing of names and DNA sequences of some

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species and subspecies of *Enterobacter* have been reported (Brady *et al.*, 2013; Stephan *et al.*, 2014; Doijad *et al.*, 2016) .

Although *E. cloacae* complex species are implicated in hospital-acquired infections, there is lack of information about the function of every species genotype for their ability to cause diseases. Moreover, detection of all species of this taxon based on phenotypic characters is generally complicated and not always consistent, so molecular techniques are frequently utilized (Mezzatesta *et al.*, 2012). In current study, we isolated and identified *E. hormaechei* subsp. *xiangfangensis* strain AA1 for the first time in Iraq from diarrheal feces of inpatients. For microbial identification, phenotypic analysis, biochemical reactions, Vitek 2 compact system, and molecular analysis using 16S ribosomal RNA gene were carried out. Besides, susceptibility of *E. hormaechei* subsp. *xiangfangensis* strain AA1 towards antibiotics was also tested.

MATERIALS AND METHODS

Bacterial strain isolation

A total of 400 stool samples from inpatients suffering from diarrhea in Al- Qasim Hospital at Al-Hilla City of Iraq were screened form January 2018 to January 2019 for associated bacterial strains with the focus on *Enterobacter* species strains. Samples were streaked on plates containing MacConkey or Blood agar, then incubated at 37 °C for one day. Growing bacterial colonies were purified on MacConkey agar and Blood agar, then identified using routine phenotypic and biochemical tests based on O'Hara *et al.* (1989) and MacFaddin (2000), and subjected to Vitek 2 compact system, which was used according to the manufacturer's instructions; ATCC Number (700323), ID: Gram Negative card.

Antibiotics susceptibility test

The Kirby-Bauer method was utilized in order to test antibiotics sensitivity as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (Gooch, 2011). Briefly, four to five growing colonies were inoculated into 5 mL of nutrient broth then put in an incubator at 35 °C until it gets the McFarland standard turbidity (0.5). A swab from inoculated nutrient broth was cultured on Muller Hinton medium. The antibiotics disks were positioned on the inoculated Petri dishes and put in the incubator for 18 h at 37 °C. The inhibition zones formed around each antibiotic disc were then measured by a ruler and compared with standard tables to determine the sensitivity or resistance of bacterial isolate according to CLSI (2012).

Extraction of genomic DNA (gDNA)

gDNA was extracted from a bacterial culture of the isolated strain which grown overnight in 5 mL Luria-Bertani (LB) broth using bacterial RTP® extraction Kit (InVitek- Germany). The extraction was performed based on manufacturer's instructions. The extracted gDNA was validated by Nanodrop spectrophotometer. The absorbance value used to determine the purity of gDNA was A260/280.

Polymerase chain reaction (PCR)

PCR assay was done by using bacterial universal primers. The sequences of the forward and reverse primers are 5'TG ATC GTT TAC GGC GTG GAC3' and 5'A ATA CCA AGT CTC AAG AGT GT3'. The DNA fragment of 16S ribosomal RNA was produced using DNA Polymerase of Thermo Scientific™ Phusion™ High-Fidelity (Fisher scientific). PCR was carried out in a reaction mixture that contains 25 µL of 2× Phusion Master Mix, 0.5 µM for each of the primers, 100 ng/ 50 µL of DNA. The final volume of reactions was designated to be 50 µL. The thermal cycler was run for PCR was set as: denaturation for 30 sec at 98 °C, then for 10 sec (for 30 rounds), annealing at 52 °C for 30 sec, elongation at 72 °C for a period of 40 sec, and last extension at 72 °C for a period of 5 min. The resulting band was detected by 1% of agarose gels. The gels were run using electrophoresis and visualized using a Bio-Rad ChemiDoc MP after soaking with ethidium bromide stain. The bands were then extracted from the gel by the Thermo scientific Gene Extraction Kit.

DNA sequencing method

The extracted 16S rRNA PCR fragment from one of the isolates of *E. hormaechei* was sent to Bioneer (Korea) to execute the base sequencing. The base sequence of the isolated strain was deposited in the NCBI GenBank records, given an accession number of MH893736. Homologous sequences for 16S rRNA gene were obtained from NCBI using the BLASTn tool. Homologs from *E. hormaechei* strains were chosen with accession numbers: (KX016591.1, KF254597.1, KF254588.1, MG731572.1, MH384426.1, MH790305.1, MN208166.1, KU851252.1, KF254587.1, MH790303.1, MH346244.1) as well as homologs from strains belonging to other *Enterobacter* spp. with accession numbers: (LC170018.1, MG871247.1, KY660471.1, KY810738.1, KM885186.1, MG557804.1, KM279704.1, HG003647.1). Sequences were aligned using clustal Omega website, and phylogenetic tree analysis was done using neighbor-joining method.

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RESULTS

Bacterial identification

A total of 400 stool samples from inpatients suffering from diarrhea in Al-Qasim Hospital at Al-Hilla City of Iraq were screened for associated bacterial strains. Table 1 summarizes the total numbers and percentages of bacterial species isolated and identified throughout the period of the study. Bacterial growth was found in 84.5% (338 out of 400) of the stool samples, while no growth was seen in 15.5% (62 out of 400) of the samples.

In this study, we focused on characterization of *E. hormaechei* as it has not reported previously to be associated with diarrhea. Two isolates of *E. hormaechei* were obtained from inpatient women suffering from diarrhea for more than two weeks (Table 1). The primary plate culture of the stool samples growing on MacConkey agar at 37 °C for 24 h showed circular, smooth, pink colonies with regular edges of 2-4 mm diameters. These colonies were purified and identified using Gram's stain which showed blue short bacilli. The phenotypic and biochemical properties of the isolate are summarized in Table 2. According to the microscopic appearance, morphology of colonies on growth media, as well as biochemical tests, the isolated bacterial strain was associated to the family of Enterobacteriaceae, and it revealed a pattern similar to *E. hormaechei* (O'Hara *et al.*, 1989), while Vitek 2 compact system identified the isolated bacterium as *Yersinia enterocolitica.*

Antibiotics susceptibility

The antibiotics susceptibility of the identified bacterial strain was tested against 15 antibiotics (Table 3). The results showed that the strain was resistant to β-Lactams, whether penicillins or cephalosporins, including resistance to augmentin, ampicillin, cephalothin, cefoxitin, ceftazidime, cefixime, ticracillin/clavulanic acid, cefotaxime, streptomycin, erythromycin, amikacin, ciprofloxacin, and chloramphenicol. On the other hand, it was susceptible to meropenem along with imipenem.

Table 1: Numbers and percentages of the isolated bacteria species from patients throughout the study period.

Bacterial species	Number of isolates	Percentage (%)
Salmonella typhimurium	112	33.13
Staphylococcus aureus	98	28.99
Escherichia coli	40	11.83
Pseudomonas aeruginosa	38	11.24
Clostridium perfringens	32	9.46
Klebsiella oxytoca	16	4.73
Enterobacter hormaechei	2	0.59
Total	338	100.00

Table 2: Phenotypic and biochemical properties of the isolated strain.

Tests	Reactions
Growth on MacConkey agar	Pink colonies
Gram stain	Gram negative short bacilli
Oxidase	-
Catalase	+
Nitrate	+/-
Methyl red	+
Voges proskaur	+
Citrate	+
Indole	-
Urease	+
Motility	+
TSI (triple sugar iron)	A / A
Maltose	+
D-Mannose	+

(+): positive reaction; (-): negative reaction; A / A: acid/acid (yellow slant/yellow butt) reaction

Table 3: Antibiotics susceptibility of the isolated bacterial strain.

Antibiotics	Result
Augmentin	R
Ampicillin	R
Cephalothin	R
Cefoxitin	R
Ceftazidime	R
Cefixime	R
Cefotaxime	R
Ticracillin / Clavulanic acid	R
Streptomycin	R
Erythromycin	R
Amikacin	R
Ciprofloxacin	R
Chloramphenicol	R
Meropenem	S
Imipenem	S

R: Resistant; S: Sensitive

Detection of 16S rRNA gene and sequence analysis

The target fragment of 16S rRNA were detected from the gDNA of the two *E. hormaechei* isolates with about 900-1000 bp as shown in Figure 1. The 16S rRNA gene sequence of the isolated strain of the current study can be found under the accession number MH893736 at NCBI–GenBank submissions with the name of *E. hormaechei* subsp *xiangfangensis* strain AA1. The BLAST results of NCBI–GenBank database showed that the base sequence of the 16S rRNA was only 92% identical to the

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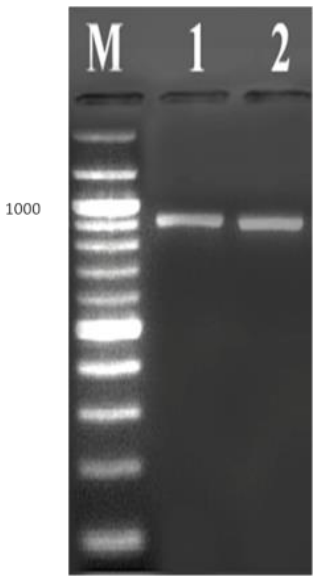

Figure 1: The target fragment of 16S rRNA obtained from the characterized *E. hormaechei* strain. M: 2000 bp marker of DNA , Lanes 1 and 2: The target amplification of 16S rRNA gene (size 900-1000 bp).

type strain 16S rRNA sequence of *Yersinia enterocolitica*, while it was 99% identical with strains of *E. hormaechei* and *E. cloacae*. Table 4 represents homology sequence identity for 16S rRNA gene of *E. hormaechei* subsp *xiangfangensis* strain AA1 with others *Enterobacter* spp.

Phylogenetic tree analysis

Analysis of the phylogenetic relationship (Figure 2) was done using the sequenced 16S rRNA gene from *E. hormaechei* subsp *xiangfangensis* strain AA1 with all sequences listed in Table 4. The results revealed that *E. hormaechei* subsp *xiangfangensis* strain AA1 is closely related to *E. hormaechei* subsp *xiangfangensis* strain S15 (MH384426.1), named here as *E. xiangfangensis* strain S15 (MH384426.1).

DISCUSSION

The *E. cloacae* complex contains significant organisms clinically as well as environmentally. They can be described as emerging pathogenic microorganisms, among them, *E. hormaechei* is the most frequently identified bacterium that causes hospital-acquired infections (Paauw *et al.*, 2008). This species is revealed to have a medical importance in previous reports of a number of epidemic cases recorded in rigorous care units of different countries include Venezuela, United States and Brazil (Wenger *et al.*, 1997; Campos *et al.*, 2007; Rodulfo *et al.*, 2016). Furthermore, *E. hormaechei* was previously isolated from bronchial discharge, blood and wounds (Campos *et al.*, 2007), and it was found to be susceptible to contaminate hospitalized patient and can be spread from infected person to another when contamination management methods are insufficient (Wenger *et al.*, 1997). *Enterobacter hormaechei* is often defined mistakenly as *E. cloacae* of due to the lack of some information about names and DNA sequences of some of *Enterobacter* species and subspecies (Brady *et al.*, 2013; Stephan *et al.*, 2014; Doijad *et al.*, 2016). Here, we used different ways to identify our newly isolated strain, *E. hormaechei* subsp *xiangfangensis* strain AA1, including phenotypic, biochemical, and molecular techniques.

One of the conventional techniques used for bacterial identification is characterizing microorganisms phenotypically. However, the complex of *E. cloacae* normally encompasses a strong phonotypical inconsistency, making the naming of these bacteria challenging (Ohad *et al.*, 2014). Therefore, this technique cannot be utilized alone for identification of bacteria with uncommon phenotypic traits. The conventional identification tests showed that the isolated bacterial strain was associated with the family of Enterobacteriaceae. It revealed a pattern similar to *E. hormaechei* (O'Hara *et al.*, 1989), while Vitek 2 compact system identified the isolated bacterium as *Yersinia enterocolitica.* Our results are consistent with the previous research showing that differentiation between some members of Enterobacteriaceae family is difficult, and the Vitek system was only able to identify 80.1–94.4% of the members of this family on top of the non-fermenting, Gram negative bacilli (Woo *et al.*, 2000; O'Hara and Miller, 2003). In addition, misidentification of some strains by VITEK 2 was also reported in previously published studies (Schaberg *et al.*, 1991; Leven *et al.*, 1995; Spanu *et al.*, 2003).

The discovery of molecular methods leads to gain a vast amount of bacterial genome. Among molecular methods, analyzing variable regions of bacterial 16S rRNA gene sequences is the most regular tool in taxonomic studies (Ibal *et al.*, 2019). By employing this molecular sequence, phylogenetic tree can be constructed depending on differences of nitrogenous bases between species, and re-classification of bacteria may be possible (Woo *et al.*, 2000; Jenkins *et al.*, 2012). Therefore, the 16S rRNA DNA sequencing can be considered as the golden technique for bacterial identification. Analysis of this sequence in our newly isolated strain showed that it is 99% identical with a type strain of *E. hormaechei* as well as *E. cloacae*. However, the phylogenic analysis revealed that the isolated strain can be identified as *E. hormaechei* subsp *xiangfangensis*. This strain was isolated previously just once in the world, particularly in China, and not a lot is known about the role of it as a pathogen since it was obtained from the habitual sourdough (Gu *et al.*, 2014). The results also showed that the currently isolated strain was resistant to most types of the tested antibiotics, and this may explain why the inpatients suffer of diarrhea for prolonged time and can also indicated that infections with *E. hormaechei* is difficult to be treated.

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Table 4: Homology sequence identity for 16S rRNA gene of *E. hormaechei* subsp *xiangfangensis* strain AA1.

No.	Accession number	Name of species sequence	Identity
1	KX016591.1	*E. hormaechei* strain ESN22	99.33%
2	KF254597.1	*E. hormaechei* strain 10 (C4Plas(D))	99.33%
3	KF254588.1	*E. hormaechei* strain 8 (C2P2)	99.33%
4	MG731572.1	*E. hormaechei* strain EB8D	99.49%
5	MH384426.1	*E. hormaechei* subsp. *xiangfangensis* strain S15	99.33%
6	MH790305.1	*E. hormaechei* subsp. *xiangfangensis* strain GW32I	99.16%
7	LC170018.1	*E. cloacae* strain A27	99.23%
8	MG871247.1	*E. cloacae* strain FC1766	99.16%
9	KY660471.1	*E. cloacae* strain UFLA WFC733	99.32%
10	KY810738.1	*E. cloacae* strain SO4-E36	99.16%
11	KM885186.1	*E. cloacae* strain BSI2	99.16%
12	MG557804.1	*E. cloacae* strain 309-2	99.16%
13	KM279704.1	*E. cloacae* strain WSYJ-SW-YL-14-06-1	99.16%
14	HG003647.1	*E. cloacae* strain IRQBAS2	99.16%
15	MN208166.1	*E. hormaechei* strain 112-a blue	99.49%
16	KU851252.1	*E. hormaechei* strain YAnl_w2	99.49%
17	KF254587.1	*E. hormaechei* strain 184A (VM(e))	99.33%
18	MH790303.1	*E. hormaechei* subsp. *xiangfangensis* strain GW15I	99.16%
19	MH346244.1	*E. hormaechei* strain KP119816.1	99.16%

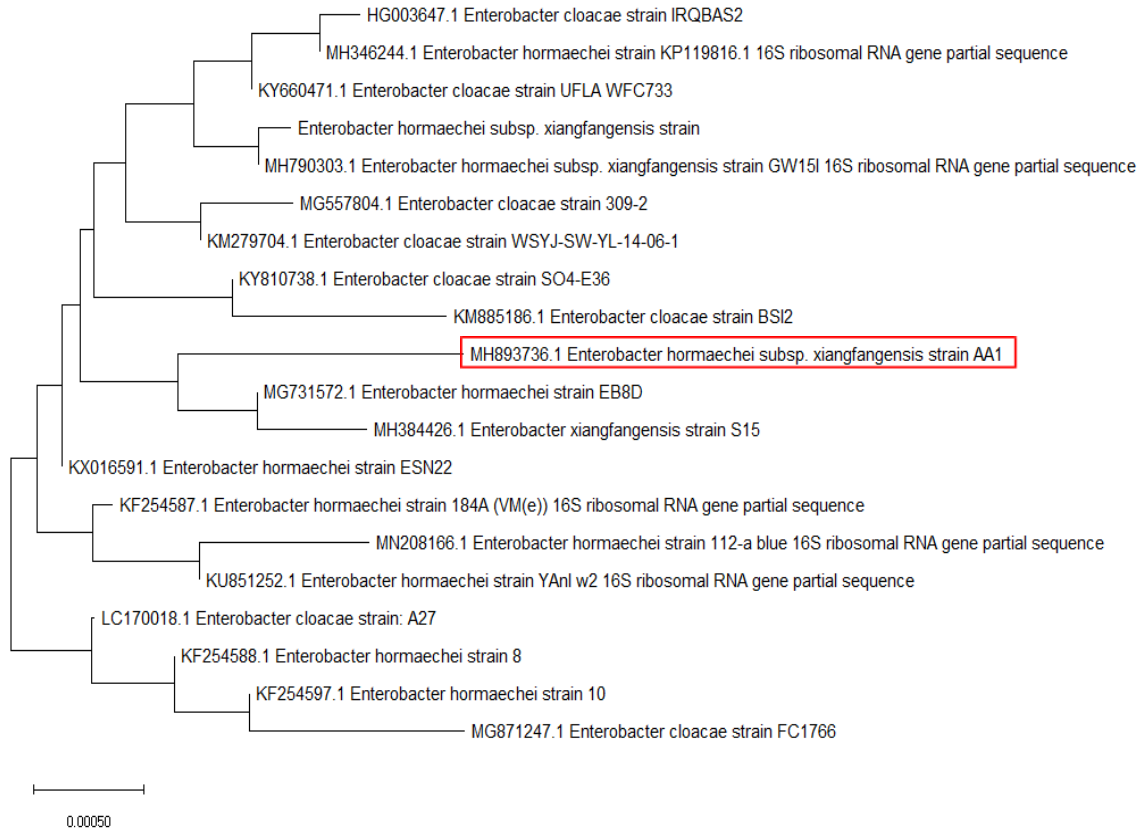

Figure 2: Phylogenetic tree analysis of the sequenced 16S rRNA gene of *E. hormaechei* subsp *xiangfangensis* strain AA1, accession number (MH893736). The history of evolution was deduced by utilizing neighbor-joining method.

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CONCLUSION

For the first time in Iraq, we isolated and characterized a new strain of *E. hormaechei* named as *E. hormaechei* subsp *xiangfangensis* strain AA1 that was associated with diarrhea cases.

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None.

CONFLICT OF INTEREST

There is no conflict of interest.

DATA AVAILABILITY

All data created or explored in this study are incorporated in the published manuscript.

ETHICS STATEMENT

There is no experiment with human contributors or animals executed by any of the researchers in the current study.

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