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Phenotypic and genotypic detection of Enterobacter spp isolated from food

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***Abstract*---**The alternative sigma factor is essential for bacterial survival in harsh environments. Many *Enterobacter spp.* are opportunistic human pathogens, and their ability to adapt to changing environments may be critical to their virulence. These specialized sigma factors bind the promoters of genes appropriate to the environmental conditions and selectively increase the transcription of those genes. We used PCR to detected presence of *RpoS, fimH* and *fimA* genes in *Enterobacter Cloacae Complex* that isolated from food, (50%) of the isolates contain *RpoS* gene, (30%) of the isolates contain *fimH* and (20%) of the isolates contain *fimA. RpoS* was an important factor in cell control and bacterial persistence under stress conditions, and it contributed to virulence through a variety of mechanisms. Biofilm-producing bacteria were identified by the Congo Red Agar (CRA) method we obtained (50%) isolated of *Enterobacter Cloacae Complex (ECC)* formed Biofilm, according to laboratory investigations, 10 food samples in the current study contained *Enterobacter spp.* and were tested for antibiotic resistance. In our study, Amoixcillin-clavulanate and Ampicillin resistance was observed in all sample, while Imipenem and Levofloxacin showed high sensitivity.

***Keywords*---**Enterobacter Cloacae Complex, RpoS, fimH, fimA, Biofilm.

Introduction

Enterobacter Cloacae Complex

Members of the *Enterobacter cloacae complex*(ECC) have emerged as important pathogens frequently encountered in nosocomial infections. Several outbreaks with E. cloacae complex have been reported in recent years, especially in neonatal

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units (Vogt *et al.*,2019). Multidrug-resistant (MDR) ECC isolates have emerged and spread worldwide with the widespread use of antibiotics. (Liu *et al.*,2021). This complex includes the following species: *Enterobacter asburiae*, *E. carcinogenus*, Enterobacter *cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter nimipressuralis* and *E. mori*. All these species are genotypically very close, with more than 60% DNA-DNA homology. (Davin *et.al* 2019).

The *Enterobacter* group includes environmental species as well as opportunistic pathogens of humans and plants. It can be found in a variety of environments, including water, soil, sewage, plants, and human and animal feces. (Singh *et al.,* 2018). *Enterobacter spp.* infections can occur as a result of either exogenous or endogenous causes. This is not surprising given the organism's pervasiveness. Single-source outbreaks have been linked to contaminated intravenous solutions, blood products, distilled water, endoscopes, personnel hands, hydrotherapy water, stethoscopes, cotton swabs, cryopreserved pancreatic islet infusions, lipoidal solutions, and devices used to control intra-arterial pressure. (Reza *et al.*, 2019). Foodborne diseases have become a significant public health problem worldwide as outbreaks linked to food-borne pathogens account formillions of deaths and hospitalizations; causing colossal economic losses each and every year.(Gobbi *et al* .,2015).Vegetables can harbor human pathogens without showing signs of spoilage, raising concerns about food safety ,Vegetables can be contaminated by enteric pathogens in a variety of ways throughout the production chain, including during cultivation and harvest.(Lenzi *et al* .,2021). *Enterobacteriaceae* are considered to be the indicator bacteria for microbiological quality of food and hygiene status of a production process. Additionally, the food contaminated by *Enterobacteriaceae* poses a microbiological risk for consumers. In fact, the contamination of raw milk and meat by *Enterobacteriaceae* amid manufacturing may easily occur from various environmental sources, and this group of bacteria is frequently detected in dairy and meat products. (Mladenović *et al.*,2021). *RpoS* is an alternative sigma factor of RNA polymerase primarily found in Beta- and Gammaproteobacteria. RNA core polymerase requires a sigma factor for promoter recognition and transcription initiation. In addition to house-keeping sigma factors that control transcription of essential genes. (Dong *et al.*,2010). The alternative sigma factor *RpoS*, also known as σ38 or σs, is recognized as a key factor in the stationary phase of growth and in the survival of bacteria during exposure to stress conditions. (Liu *et al.*,2018). The *rpoS* gene is located at a highly polymorphic region of the chromosome and it has been described as a highly mutable gene in *Escherichia coli* and *Salmonella spp.* (Fernández *et al.,* 2020). The *rpoS* plays an important role in the virulence regulation of pathogenic bacteria, such as chondroitinase activity, serum resistance, biofilm production. (Huang *et al.*, 2019). Adherence which is only one step in the infectious process, is followed by the formation of microcolonies and subsequent steps in infection pathogenesis. (Brooks *et al.*, 2007) Fimbriae are adhesion factors that act on bacteria adhering to epithelial cells. Many *Enterobacteriaceae* members have fimbriae that respond to adherence on the surface of epithelial cells. (Liverelli *et al.*, 1996). Fimbriae, namely type 1, type 3, KPC and KPF-28 adhesin are essential in the establishment of infection and biofilm formation. The initial establishment of UTI requires the involvement of type 1 fimbriae. (Alcántar *et al.*, 2013).

Biofilms are a structured community of microorganisms enclosed in a self-produced matrix that adhere to one another and to surfaces (Ramos *et al.*,2019). Because of the biofilm matrix's protection, bacterial cells in biofilms easily develop resistance to antimicrobial agents. (Liu *et al.*,2018). Bacterial biofilm formation is widely found in natural environments with water, and also in human diseases, especially in the patients with indwelling devices for the purpose of medical treatments. (Wu *et al.*, 2015).

Methods and materials

Sample

A total of 150 Food sample were achieved during the period from October 2021 to March 2022. food sample collected from supermarket and house eat from a different region in Al-Najaf city. The sample include fresh meat, fish chicken, milk and celery. The samples were placed in separate sterile plastic bags before being immediately transported to a cool box filled with ice. All samples were transferred to the laboratory and cultured on MacConky agar medium for 24 hours at 37° C. Isolates were purified several times until pure isolates were obtained, then subjected to microscopic and special biochemical tests before being transferred to VITEK 2 for identification.

Congo Red Agar (CRA)

It was made by mixing 52 grams of Brain heart infusion agar media with a liter of distilled water and autoclaving it for 15 minutes at 121°C/15 pressure. Congo red stain (0.8 g/L) was made as a concentrated aqueous solution and autoclaved for 15 minutes at 121°C, while sugar (50 g/L) was sterilized by filtering. After cooling to 55°C, both dye and sugar were added to the agar. After that, plates were infected and incubated aerobically at 37°C for 24 hours to detect biofilm formation (Freeman *et al.*,1989). Black colonies with a dry crystalline quality indicated a positive result. Weak slime producers stayed pink most of the time, however there was some darkening in the colonies' cores. An uncertain result was indicated by the darkening of the colonies in the absence of a dry crystalline colonial morphology.

Extraction and Isolation of DNA

DNA of Enterobacter *spp.* isolates was prepared by boiling method. In brief, colonies were suspended in 100 microliters of sterile distilled water, boiled at 100C in a water bath for 15 minutes, then rapidly cooled at -20C for one hour, centrifuged, and the supernatant was saved for use in the amplification processes (Shah *et al.*, 2017).

PCR amplification was used to identify the presence of *rpoS* ***gene***. The primer used in this study was show in (Table 1)

Table 1
The sequence of Primer that were used in the present Study

Primer	Sequence	Reference
RpoS	5-CTGAATTCCCTGAGTTGCCTACGCCC-3 F: R: 5-GGGAATTCGTGCTTAATCAGGAAGGGG-3	Martinez-Garcia *et al.* (2001)
FimA	F: GCACCGCGATTGACAGC R: CGAAGGTTGCGCCATAG	Ghasemian *et al.*, (2019)
FimH	F: ATGAACGCCTGGTCCTTTGC R: GCTGAACGCCTATCCCCTGC	Fertas *et al.*, (2013)

Each 25 µl of PCR reaction mixture for PCR contained 2.5µl of upstream primer, 2.5µl of downstream primer, 2.5µl of free nuclease water, 5 µl of DNA and 12.5µl of master mix thin-walled PCR tube. The Thermal cycler conditions were as follow in (Table 2)

Table 2
PCR program that applies in the thermo-cycler

PCR gene	Temperature (c) / Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
rpoS	95 ˚C/120 sec	95 ˚C/30 se.	55 ˚C/30sec	72 C°/1min	72 C°/1min	35
Fim H	95 ˚C/4min	95 ˚C/30sec	53 ˚C/1min	72 ˚C/1min	72 ˚C/8min	35
Fim A	94 ˚C/4min	94 ˚C/1min	59 ˚C/1min	72 ˚C/30sec	72 ˚C10min	30

Results and Discussion

Entrobacter spp. were isolated from different food samples using media such as MacConkey agar. In addition to traditional biochemical tests that are used to identify *Enterobacter spp.*, The isolates were also diagnosed using the Vitek2 system by the GN / ID identification card for the diagnosis, and the results confirmed that only 20 isolates from food sample.

Congo red agar method, (Freeman *et al.,* 1989) described this method for detecting slime layer production by bacteria using a specially prepared solid medium. The positive result formation of black colonies with dry crystalline consistency while negative result formation of pink colonies. (Fig 1)

They can withstand high concentrations of antimicrobial agents. The percentage of biofilm producers in our study was 50% by CRA method which is approach compared to studies conducted by (Raksha.,2020).

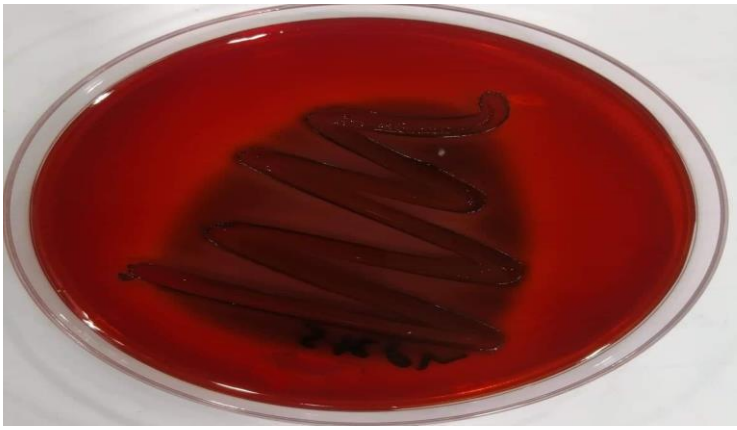

Figure 1. Biofilm formation of *Enterobacter spp.* in Congo-Red Agar Method (CRA)

The susceptibility of antibacterial agents for food sample isolates of *Enterobacter* spp. to traditional drugs suggested through CLSI (2021) had been estimated are shown in Table (3).

Table 3
Antimicrobials sensitivity test of Enterobacter Cloacae Complex isolates

Antibiotic	R	I	S
Amikacin	1(10%)	3(30%)	6(60%)
Amoxicilllin – clavulanic acid	10(100%)	0(0%)	0(0%)
Ampicillin	8(80%)	2(20%)	0(0%)
Aztreonam	3(30 %)	2(20 %)	5(50%)
Cefepime	7(70%)	3(30%)	0(0%)
Cefotaxime	7(70%)	3(30%)	0(0%)
Cefoxitin	8(80%)	2(20%)	0(0%)
Ceftazidime	7(70%)	3(30%)	0(0%)
Ceftriaxone	5(50%)	2(20%)	3(30%)
Ciprofloxacin	2(20 %)	3(30 %)	5(50%)
Imipenem	0(0%)	2(20%)	8(80%)
Levofloxacin	0(0%)	3(30%)	7(70%)
Penicillin G	10(100%)	0(0%)	0(0%)
Piperacillin	7(70 %)	3(30%)	0(0%)
Chloramphenicol	3 (30 %)	1(10%)	6 (60 %)
Tetracycline	3(30%)	2(20%)	5(50%)

According to laboratory investigations, 10 food samples in the current study contained *Enterobacter spp.* and were tested for antibiotic resistance. In our study resistance to Amoixcillin-clavulanate, Ampicillin and Penicillin G have been seen in all cases. In Egypt study done by Sadek *et al* (2021) that isolate Enterobacter *cloacae complex (ECC)* from food showed all isolated resistance to Amoxicillin-clavulanate and Ampicillin. The findings revealed concerning multi-resistance to at least four or more of the tested antibiotics. The public is concerned because these foods may act as a reservoir for resistant strains that can be transmitted to humans through ingestion of contaminated food. Identical patterns of other authors have reported resistance. The present study observed Tetracycline 30% resistance of ECC. The findings are in agreement with those of Haryani *et al.*, (2008). The present study observed low resistance of ECC to Ciprofloxacin (20%) and no resistance to Imipenem and Levofloxacin, this result agreement with Nyenje *et al.*, (2012) who isolated 33 *Enterobacter cloacae* from various ready-to-eat foods sold in Alice, South Africa.

Molecular Detection of rpoS gene in Enterobacter spp.

Eight (40%) of the twenty *Enterobacter spp.* isolates tested positive for the *rpoS* gene. For templates, a discrete PCR product of the expected size (800 bp) was observed. (Fig 2)

RpoS was an important factor in cell control and bacterial persistence under stress conditions, and it contributed to virulence through a variety of mechanisms. Our research was carried out in the presence of the *rpoS* gene in *Enterobacter spp.*

This result agrees with Hussain and Alammar's (2013) study, which found 31% of *Enterobacter spp.* isolates have the *rpoS* gene, while this result disagrees with (Naba'a *et al.*,2018), who did not get a positive result.

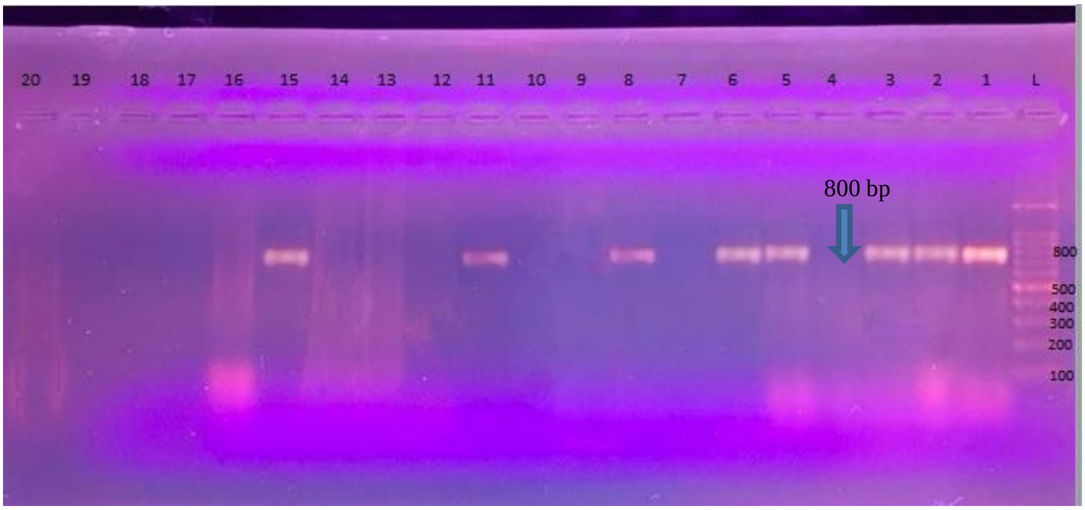

Figure 2. Gel electrophoresis of PCR amplified product of *Rpos* gene primers with product 800 bp of Enterobacter *spp.* isolates. Lanes (1, 2, 3, 5, 6, 8,11,15) food sample show positive result. Lane (L) DNA molecular size marker (100-2000 bp ladder).

E. cloacae stationary-phase cells showed an increased resistance to several stress conditions, a behavior that was initially described in *E. coli.* The role of rpoS in the stress response was investigated in *E. cloacae* by creating a mutant *rpoS* strain through insertional inactivation. Disruption of *rpoS* resulted in a decrease in cell survival following exposure to heat, extreme pH, and high osmolality. Resistance to high salt concentrations may be important in the processes of survival, colonization, and infection. (Martínez-García *et al.*,2001). Isolates losing *rpoS* gene were resulted a decrease in cell survival following exposure to heat and osmolarity (Dong and Schellhorn, 2009).

Detection of fimH and fimA Genes

The molecular detection of *fimH* gene by using specific primer that encodes fimbria type 1 in *Enterobacter spp.* 6/20(30%), (Figure; 3), while fimA gene detection in *Entrobacter spp.* 4/20(20%) (Figure;4). Fimbriae are thought to play an important role in epithelial cell attachment. Fimbriae mediate bacterial colonization and host cell signaling by binding to specific host receptors. Fimbriae adhesions control both the fate of the bacterial pathogen in the host and the progression of the disease process. The organism's virulence is also determined by the type-1 of fimbriae (Al-Kraety *et al.*,2020). *FimH* adhesin mediates both bacterial adherence to and invasion of host cells and contributes to the formation of intracellular bacterial biofilms by uropathogen (Wright ,2007).

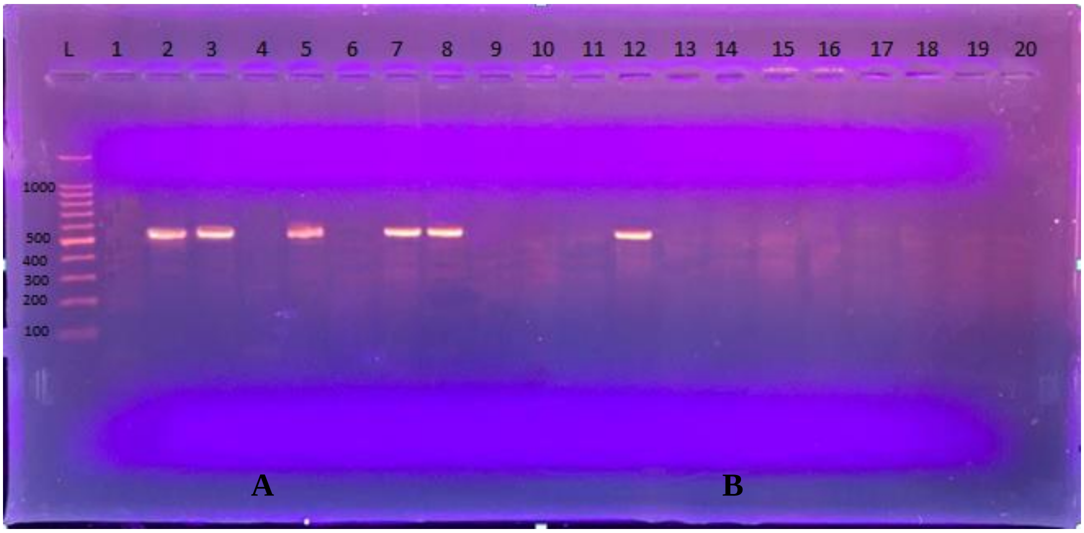

Figure 3. Gel electrophoresis of PCR amplified product of *fimH* gene primers with product 508 bp of *Enterobacter spp*. isolates. Lanes (2, 3 ,5, 7, 8, 12) food isolates positive result of *Enterobacter spp* (L), DNA molecular size marker (100-2000 bp ladder)

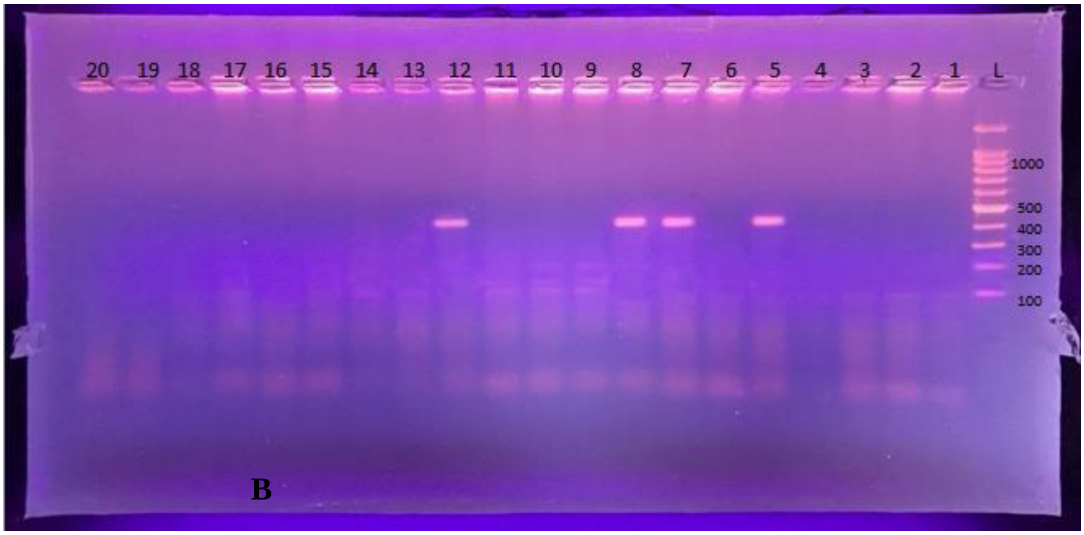

Figure 4. Gel electrophoresis of PCR amplified product of *fimA* gene primers with product 434 bp of *Enterobacter spp.* isolates. Lanes (5, 7, 8, 12) food isolates positive result of *Enterobacter spp* (L), DNA molecular size marker (100-2000 bp ladder)

The *fimH* gene results of *Enterobacter spp.* in present study are agreement with Hassan *et al.*, (2011) obtained that 32 *Enterobacter spp.* isolated from clinical urine specimens and identified the *fimH* gene in 40% of the isolates. Additionally, Brust *et al.*, (2019) isolated 8 *E. cloacae* identified the *fimH* gene, while the Abdul and Univers, (2013) show high result 18(75%) of *Enterobacter spp.* who collected 24 isolates from *Enterobacter spp.* in Babylon city.

The presence of the *fimA* gene, which codes for type 1 fimbria adhesive structures, allows bacteria to colonize and develop biofilm while also preventing antibiotic entry into the cells. The attachment of *Enterobacteriaceae* to host epithelial and endothelial cells is aided by type one and type three fimbria. (Murphy *et al.*,2013; Reisner *et al.*,2014). The virulence factor, such as the adhesin factor named Fimbriae type 1 (*FimA*), which plays a role in inducing adhesion to host epithelial cells, is an important factor in the early stages of biofilm formation as well as *PapC*, which forms pili formation to attach to host cells or catheter materials. (Gunardi *et al.*,2021). The *fimA* gene results of *Enterobacter spp.* in present study are agreement with Liu *et al.*, (2022) that show (53%) isolated.

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