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Research Article

Genetic variation of hsp60 gene in *Enterobacter cloacae* isolated from frozen chicken meat in Iraq

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Abstract

This study aimed to use hsp60 genes of *Enterobacter* species from the chicken meats amplified by traditional PCR to determine its phylogenetic position compared to those registered in the gene bank. For this purpose, 120 samples of refrigerated chicken meat were collected in the province of Al-Najaf between August 2020 and April 2021. The results showed that out of 120 bacterial isolates, 30 were infected with *E. cloacae* based on Vitik tests, which is about 25%; however, 30 infected samples with *E. cloacae* were positive based on the hsp60 gene. Analysis of hsp60 sequences was using PCR and *E. cloacae* ssp. *dissolvens* was detected and its phylogenetic position compared to other *E. cloacae* gene sequences in the gene bank was provided.

Keywords: Enterobacter, Frozen chicken meat, Hsp60, PCR, Sequencing.

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Introduction

The *Enterobacter cloacae* complex (ECC) is a group of bacteria found in the environment and the human digestive tract. ECC is one of the most common pathogens in hospitals in recent decades, causing pneumonia, urinary tract infections, and sepsis (Wisplinghoff et al. 2004). Because of increased antibiotic use, multidrug-resistant (MDR) ECC strains have emerged globally. ECC infection accounts for 65–75 percent of all *Enterobacter* infections, earning it the moniker "ESKAPE" pathogen (Rice 2008). The hsp60 homolog, which codes for the 60-kDa heat shock protein and is also known as the groEL homolog, has been used successfully to classify numerous bacteria and proven to be useful in the phylogenetic analysis of *Enterobacter* spp. (Paauw et al. 2008; Morand et al. 2009). Therefore, this study aimed to use hsp60 genes of *Enterobacter* species from the chicken meats amplified by traditional PCR to determine its phylogenetic position compared to those registered in the gene bank.

Materials and method

Samples were sent to the Department of Biology at the Women's College of the Faculty of Science at the University of Kufa. From August 2020 to April 2021, 120 chicken samples were collected in AL-Najaf Province from various brands and suppliers based on Iraqi Standard Criteria No.2/2270 (2006). Standard microbiological approaches such as morphological characteristics, Gram staining, and biochemical tests were used (Overdevest et al. 2011; da Silva et al. 2013). Clinical isolates of *E. cloacae* were collected from various origins and trademarks (frozen chicken meat). Vitek, chrome agar, and a biochemical test were used to confirm the identification (Overdevest et al. 2011; da Silva et al. 2013). DNA extraction and gene detection of the Hsp60 were performed based on Hoffmann & Roggenkamp (2003). The genomic

Table 1. Genetic variation at hsp60 gene of *Enterobacter cloacae*.

No. of sample	Source: *Enterobacter cloacae*, hsp60 Gene							
	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence acc. no	Identities
1	Transition	29	C\T	GAC\GAT	Aspartic acid \Aspartic acid	Silent	KM222364	99%
	Transition	125	C\T	AAC\AAT	Asparagine\Asparagine	Silent		
2	Transvertion	140	C\G	GGC\GGG	Glycine\Glycine	Silent	KM222364	99%
	Transition	151	A\G	TAC\TGC	Tyrosine\Cysteine	Missense		
3	Transition	24	G\A	GAA\AAA	Glutamic acid\Lysine	Missense	KM222364	99%
	Transvertion	182	C\A	GGC\GGA	Glycine\Glycine	Silent		
	Transition	218	A\G	AAA\AAG	Lysine\ Lysine	Silent		

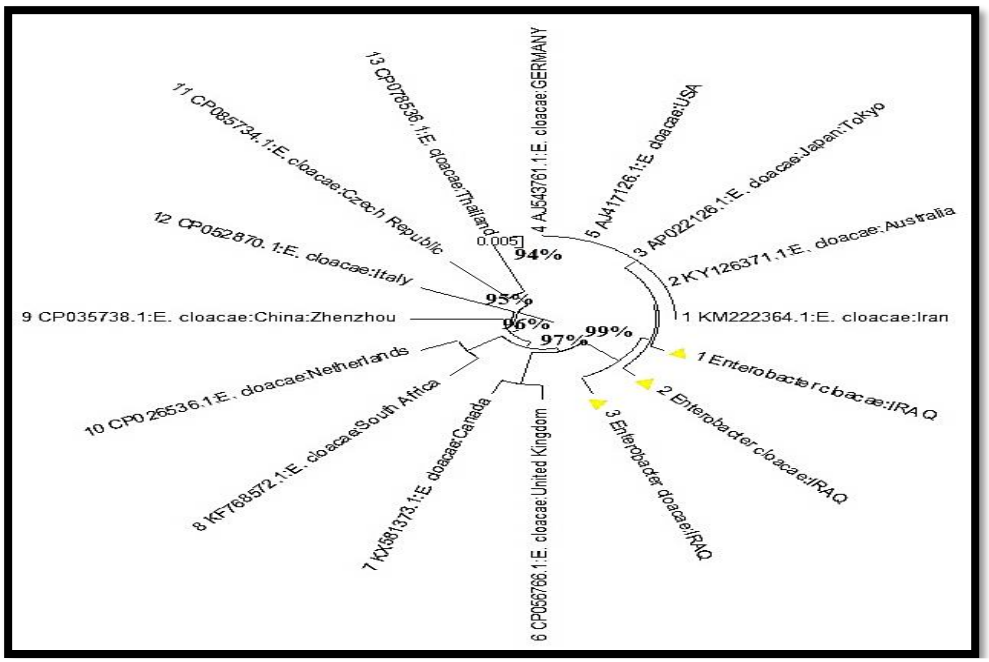

Fig.1. Phylogenic tree of Iraqi sample of *Enterobacter cloacae* (yellow numbers) based on hsp60 gene.

DNA was extracted using Intronbio/Korea kit in accordance with company instructions. For PCR, E forward GTAGAAGAAGGCGTGGTTGC and reverse primers ATGCATTCGGTGGTGATCA TCAG were used (Hoffman & Andreas 2003). In addition, conventional PCR was used to amplify HSP60. Mega 6 software was used to perform the phylogenetic analysis using NJ algorithm.

Results

The results of the standard microbiological approaches such as morphological characteristics, Gram staining, and biochemical tests are shown in Appendix 1. The results showed that out of 120 bacterial isolates, 30 were infected with *E. cloacae* based on Vitik tests, which is about 25%; however, 30 infected samples with *E. cloacae* were positive based on the hsp60 gene. The phylogenetic analysis of the hsp60 gene revealed differences in the nucleotide sequence of the hsp60 gene compared to other globally available genes in the gene bank. These differences were transition or transversion mutations and silent or missense mutations (Table 1). The Iraqi *E. cloacae* hsp60 gene was most similar to

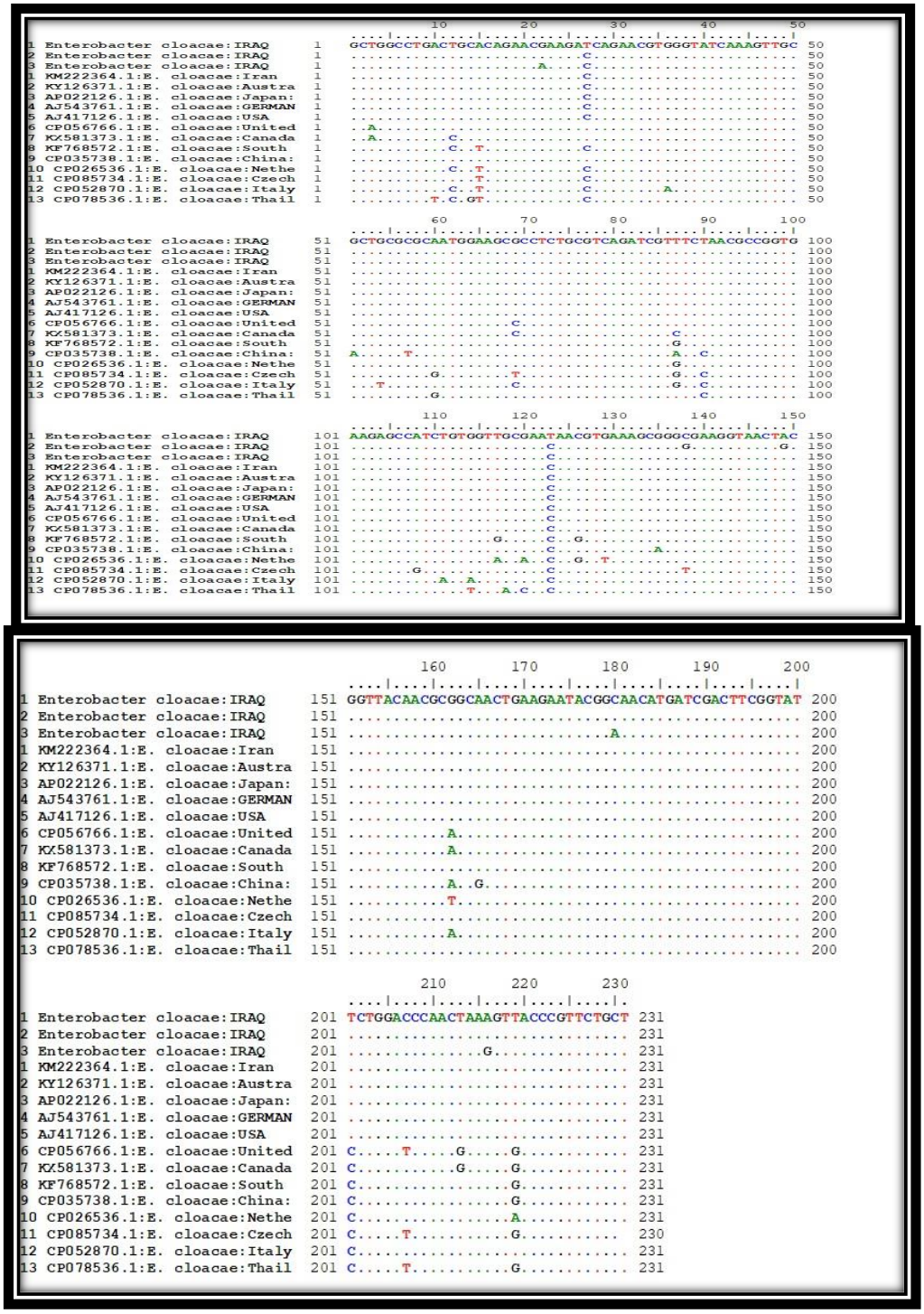

Fig.2. Alignment results of 13 sequences of 13 hsp60 gene obtained from frozen chicken meat. The color of the mutation indicated the site of each replacement mutation in the PCR products. 1-13 are retrieved sequences from NCBI, and 1-3 are our samples.

the Iranian strain (KM222364.1) with 99% and the Thailand strain (CP078536.1) with 94% homology (Table 2, Fig. 1).

Figure 2 shows the alignment of our sequences with other 13 globally available sequences obtained from frozen chicken meat with their matching reference strains of the 341 bp amplicon of the hsp60 gene DNA partial sequences. *Enterobacter cloacae* hsp60 gene of our work was registered in the GenBank with the accession number OL778832**.** In

Table 2. The retrieved *Enterobacter cloacae* hsp60 gene fron Genbank.

	Accession no.	Country	Source	Compatibility
1.	KM222364.1	Iran	*E. cloacae* (hsp60)	99%
2.	KY126371.1	Australia	*E. cloacae* (hsp60)	99%
3.	AP022126.1	Japan:Tokyo	*E. cloacae* (hsp60)	99%
4.	AJ543761.1	GERMANY	*E. cloacae* (hsp60)	99%
5.	AJ417126.1	USA	*E. cloacae* (hsp60)	99%
6.	CP056766.1	United Kingdom	*E. cloacae* (hsp60)	97%
7.	KX581373.1	Canada	*E. cloacae* (hsp60)	96%
8.	KF768572.1	South Africa	*E. cloacae* (hsp60)	96%
9.	CP035738.1	China: Zhenzhou	*E. cloacae* (hsp60)	96%
10.	CP026536.1	Netherlands	*E. cloacae* (hsp60)	95%
11.	CP085734.1	Czech Republic	*E. cloacae* (hsp60)	95%
12.	CP052870.1	Italy	*E. cloacae* (hsp60)	94%
13.	CP078536.1	Thailand	*E. cloacae* (hsp60)	94%

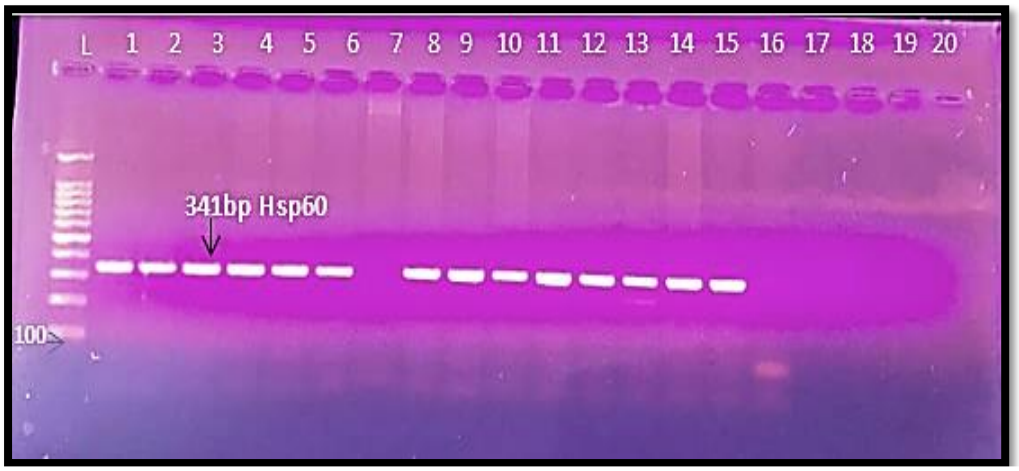

Fig.3. The Electrophoresis of positive hsp60 gene with 341 bp isolated from *Enterobacter cloacae* of frozen chicken meat. The 100bp DNA Ladder is found in lane L.

addition, the gel electrophoresis results of the hsp60 gene isolated from *E. cloacae* of frozen chicken meat in our study are shown in Figure 3.

Discussions

Based on the results, the bacterial strain sequences in our study were clustered with other available sequences of *E. cloacae* with proper similarity. Infection types can be traced back by identifying strains with an aggressive reaction or low antibiotic susceptibility (Ranjbar et al. 2014). Distinguishing species with minor genetic changes by a simple and quick repeatable procedure help their variations. The hsp60 gene sequencing is a cheap and less time-consuming method than traditional techniques and it is suggested to be assessed due to the growing antibiotic resistance (Hoffman & Roggenkamp 2003). Hoffman & Roggenkamp (2003) were described the *E. cloacae* complex group by discovering 12 clusters hsp60 genotyping. According to the results, the most common bacteria found in the clinical isolates of the chicken meat were *Enterobacter* spp. This finding agrees with the previous findings (Morandet al. 2009; Ohad et al. 2014; Guerin et al. 2016). The sequencing of the hsp60 gene is neither quick nor high-throughput (Ohad et al. 2014). However, *E. cloacae* 16s rRNA DNA (rDNA) did not provide a cohesive cluster, but a patchy tree with *E. coli*, *Enterobacter aerogenes*, *Citrobacter* spp., and *Leclercia* spp.. This does not help their classification; *E. dissolvens* is the closest species to *E. cloacae* with a DNA genetic similarity of 82% (Hoffmann & Andreas 2003).

Enterobacter cloacae hsp60 gene of our study was submitted to NCBI with accession number OL778832. Because the original genomic assessment for strains like *Enterobacter* is crucial to understand their taxonomy to the species level, along with traditional bacteriological identification. *Enterobacter* species have unstable taxonomic status because it is a polyphyletic genus, according to the 16S rRNA gene. Multilocus sequence typing (MLST) analysis is proper for the taxonomic identification of *Enterobacter* species (Khennouchi et al. 2015; Akbari et al. 2016; Singh et al. 2018).

Different genes were used for the identification of Enterobacter strains, including oriC (Roggenkam 2007; Delmas 2006), gyrB and rpoB (Paauw et al. 2008), and hsp60 (Hoffmann & Andreas 2003). The hsp60 gene is simple for *Enterobacter* spp. identification. The rpoB and gyrB genes were used in *E. cloacae* complex, but their accuracy of hsp60 to determine sub-species and provide correct clusters are superior (Delmas 2006). There must be appropriate methods to identify bacteria causing urinary tract infection and *E. cloacae* complexes are one of them (Kremer & Hoffmann, 2012).

Husain & Aziz (2022) concluded that local meats are less infected than imported ones, revealing that frozen meat products (such as chicken chops and associated products) contain many bacteria. In addition, slaughterhouses, meat processing herbs, tools, and employees are contaminated. Hence, the pathogens bacteria pose a serious threat to public health due to their zoonoses transfer to humans and food spoilage by changing its flavor and odor, which has led to economic setbacks in many states (Husain & Aziz 2022). Therefore, their identification using extensive multilocus sequencing and A-DNA hybridization is crucial.

Acknowledgment

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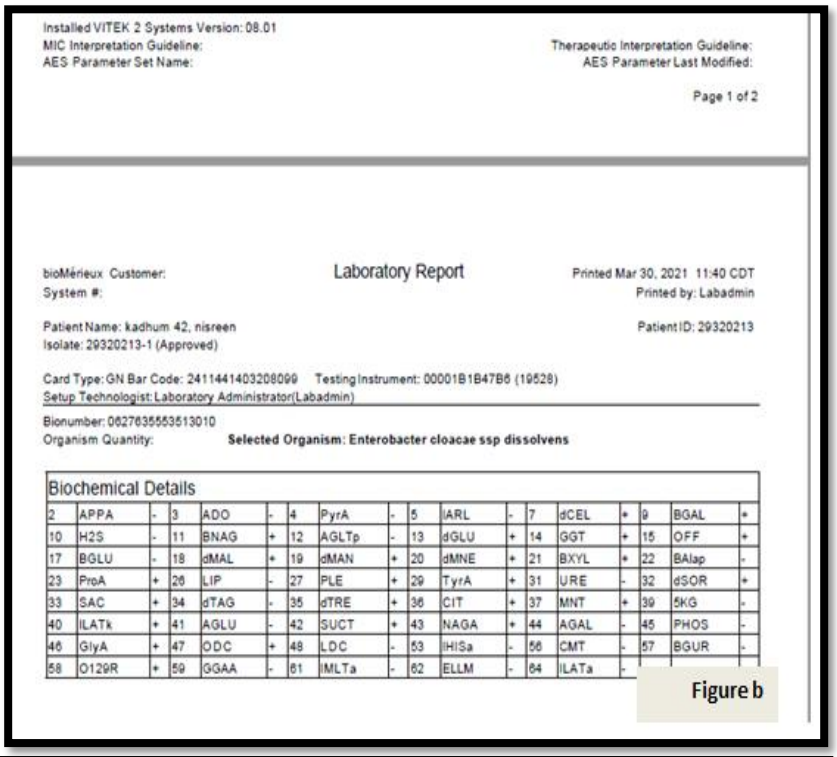
Appendix 1: a) the report of VITEK 2 Systems Version: 08.01 test for *Enterobacter cloacae* ssp. dissolves, and b) biochemical test results of *E. cloacae* ssp. dissolves.



Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 2

bioMérieux Customer:
System #:

Laboratory Report

Printed Mar 30, 2021 11:40 CDT
Printed by: Labadmin

Patient Name: kadhum 42, nisreen
Isolate: 29320213-1 (Approved)

Patient ID: 29320213

Card Type: GN Bar Code: 2411441403208099 Testing Instrument: 00001B1B47B6 (19528)
Setup Technologist: Laboratory Administrator(Labadmin)

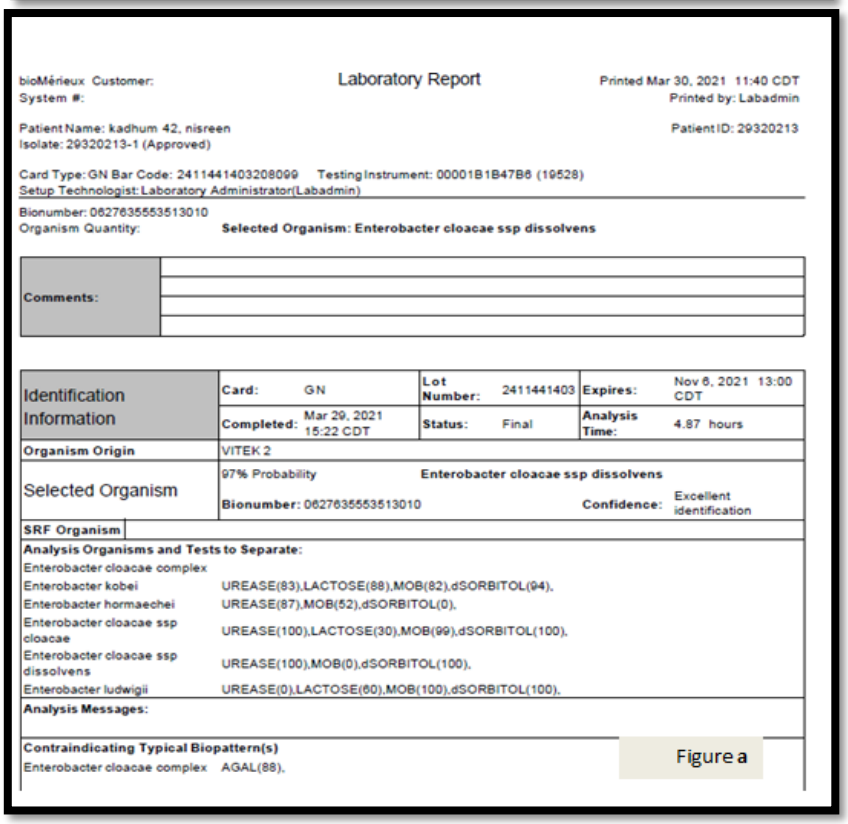
Bionumber: 0627635553513010
Organism Quantity:

Selected Organism: Enterobacter cloacae ssp dissolvens

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	-			

Figure b



bioMérieux Customer:
System #:

Laboratory Report

Printed Mar 30, 2021 11:40 CDT
Printed by: Labadmin

Patient Name: kadhum 42, nisreen
Isolate: 29320213-1 (Approved)

Patient ID: 29320213

Card Type: GN Bar Code: 2411441403208099 Testing Instrument: 00001B1B47B6 (19528)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0627635553513010
Organism Quantity:

Selected Organism: Enterobacter cloacae ssp dissolvens

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411441403	**Expires:**	Nov 6, 2021 13:00 CDT
	Completed:	Mar 29, 2021 15:22 CDT	**Status:**	Final	**Analysis Time:**	4.87 hours
Organism Origin	VITEK 2					
Selected Organism	97% Probability		**Enterobacter cloacae ssp dissolvens**			
	Bionumber: 0627635553513010				**Confidence:**	Excellent identification
SRF Organism						

Analysis Organisms and Tests to Separate:

Enterobacter cloacae complex	
Enterobacter kobei	UREASE(83),LACTOSE(88),MOB(82),dSORBITOL(94),
Enterobacter hormaechei	UREASE(87),MOB(52),dSORBITOL(0),
Enterobacter cloacae ssp cloacae	UREASE(100),LACTOSE(30),MOB(99),dSORBITOL(100),
Enterobacter cloacae ssp dissolvens	UREASE(100),MOB(0),dSORBITOL(100),
Enterobacter ludwigii	UREASE(0),LACTOSE(60),MOB(100),dSORBITOL(100),

Analysis Messages:

Contraindicating Typical Biopattern(s)
Enterobacter cloacae complex AGAL(88),

Figure a