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PII: S0167-7012(23)00028-3

DOI: https://doi.org/10.1016/j.mimet.2023.106694

Reference: MIMET 106694

To appear in: *Journal of Microbiological Methods*

Received date: 9 September 2022

Revised date: 28 February 2023

Accepted date: 28 February 2023

Please cite this article as: D. Pablo-Marcos, M. Siller, J. Agüero, et al., Are GES carbapenemases underdiagnosed? An allelic discrimination assay for their accurate detection and differentiation, *Journal of Microbiological Methods* (2023), https://doi.org/10.1016/j.mimet.2023.106694

ARE GES CARBAPENEMASES UNDERDIAGNOSED? AN ALLELIC DISCRIMINATION ASSAY FOR THEIR ACCURATE DETECTION AND DIFFERENTIATION

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Running title: PCR assay for GES-β-lactamases diagnostic.

Abstract

GES (Guiana Extended Spectrum) carbapenemases belong to “minor class A carbapenemases” and its prevalence could be underestimated due to the lack of specific tests. The aim of this study was to develop an easy PCR method to differentiate between GES β-lactamases with or without carbapenemase activity, based on an allelic discrimination system of SNPs that encode E104K and G170S mutations, without need of sequencing.

Two pair of primers and Affinity Plus probes, labeled with different fluorophores; FAM/IBFQ and YAK/IBFQ, were designed for each one of the SNPs.

This allelic discrimination assay allows to detect in real time the presence of all type of GES- β-lactamases, being able to differentiate between carbapenemases and extended-spectrum β-lactamase (ESBL), through a quick PCR test that avoid costly sequencing approaches and could help to decrease the current underdiagnosis of minor carbapenemases that scape of phenotypic screenings.

keywords:

Allelic discrimination, GES, PCR.

1. Introduction

World Health Organization (WHO) has declared that antimicrobial resistance is one of the main threats to global health ("World Health Organization (WHO).," 2022).

Carbapenemase-producing Organisms (CPO) have become the principal reason of carbapenem resistance(Martínez-Martínez and González-López, 2014). KPC-type enzymes are one of the most clinically relevant, and the main class A carbapenemase. Nevertheless, further KPC, exists a wide diversity of "minor class A carbapenemases" including GES-type and its prevalence could be underestimated due to the lack of specific diagnostic tests (Bonnin et al., 2020).

GES (Guiana Extended Spectrum) β-lactamase was first described in France in 1998 in a *Klebsiella pneumoniae* isolate producing GES-1, an extended-spectrum β-lactamase (ESBL) which confer penicillin and cephalosporins resistance, but has no carbapenemase activity (Poirel et al., 2000). GES-genes are horizontally transmissible between genera and species and novadays, they are reported increasingly in Gram-negative rods, including *Acinetobacter baumannii, Pseudomonas aeruginosa* and enterobacterales (Ellington et al., 2020).

GES β-lactamases confer β-lactam resistance, but not all variants have carbapenemase activity. The GES family includes 57 variants (Naas et al., 2017) that differ from each other by one to four Single Nucleotide Polymorphisms (SNPs). Some of these SNPs affect the amino acid residues Gly165 and Glu98 corresponding to the bla_{GES} genes multiple alignments, also referred as ABL170 and ABL104, respectively, according to Ambler classification (Ambler et al., 1991). Substitutions in residue ABL170 (G165N/G165S) confer carbapenemase activity, whereas Glu98Lys (E98K) mutation, is involved in improving extended-spectrum properties and in reducing efficiency against imipenem in relation to enzymes that possess wild type sequence (Kotsakis et al., 2010). Therefore, these SNPs have clinical importance as they can confer carbapenem resistance and enhance cephalosporinase activity.

Although GES- β-lactamases are quite rare, GES-producing microorganisms have emerged in many geographic areas causing nosocomial outbreaks(Nordmann and Poirel, 2014). This, together with difficulties encountered in the detection of these enzymes, makes it clue the need of an accurate diagnostic in order to successfully control them. It is interesting now to develop immunochromatographic assays or molecular test that will be able to fill the gap in the detection of rare carbapenemases.

The aim of this study was to develop an easy PCR method for detection and differentiation between GES β-lactamases with or without carbapenemase activity, based on an allelic discrimination system of E98K and G165S SNPs, without need of sequencing.

2. Material and methods

Nucleotide sequences of genes coding for GES-1 to 57 β-lactamases were recovered from GenBank (https://www.ncbi.nlm.nih.gov/genbank). A multiple alignment of them was performed using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). This allowed to find both nucleotide mutations, G292A and G493A, responsible of E98K and G165S aminoacidic substitutions respectively and therefore all GES-type β-lactamase with carbapenemase activity**.** Probes of allelic discrimination assay were targeted at these SNPs.

Two pair of primers (GES6_EK-F/R and GES6_GS-F/R) and Affinity Plus probes, labeled with different fluorophores (FAM/IBFQ and YAK/IBFQ), were designed through IDT (Integrated DNA Technologies Spain S.L.) for each one of the SNPs studied (G292A and G493A) **(Figure1, Table 1).** In all GES-carbapenemases, except GES-2, must be detected G165S allele. Likewise, in all GES-ESBL, except GES-13, must be detected G165G allele **(Table 3).**

A total of 130 clinical CPO isolates, all molecularly characterized by PCR and sequencing of carbapenemase-encoding genes, using primers described by Vourli et al (Vourli et al., 2004), were employed for the assay evaluation. The collection was composed of 12 different GES-type β-lactamases (GES-1, GES-2, GES-5, GES-6, GES-7, GES-11, GES-12, GES-15, GES-19, GES-27, GES-53 and GES-55) and 5 type of no-GES carbapenemases that were used as negative controls; class A (KPC), class B (NDM, VIM, IMP) and class D (OXA-48) **(Table 2).**

PCR conditions were established for the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) in a total reaction mixture volume of 10 µL, using One Step PrimeScript^{TM} RT-PCR Kit (Takara), containing 2x Takara PreMix qPCR, 0,4 µM forward and reverse primers, 0,2 µM each probe, 1 µL ROX reference dye and 1 µL of purified bacterial DNA.

Termocycling conditions were: 10s at 95ºC, followed by 40 cycles of denaturing at 95ºC for 5s, annealing and polymerization at 60ºC for 34s. Pre- and post-PCR read were performed at 42ºC for 30s.

3. Results

As a result of multiple alignment analysis, all GES β-lactamases described at the moment are listed in **Table 3**, indicating their polymorphisms at 98 and 165 positions. Their carbapenemase or cephalosporinase activity appeared thereby at β-Lactamase DataBase (BLDB) or in PubMed.

G165S allele was detected in all of GES β-lactamase producing isolates with carbapenemase activity except GES-2. Furthermore, E98K allele was detected in combination with G165S in GES-4, 6 and the

new GES-53 and 55 β-lactamases (GenBank references: UQM94989 and ON714048 respectively) **(Figure 2).**

On the other hand, G165G allele was detected in GES β-lactamase producing isolates with only cephalosporinase activity.

The E98E allele presence and the no detection of G165G/S alleles was indicative of the G165N mutation existence and this is characteristic of GES-2 carbapenemase. Furthermore, the presence of E98K and the no detection of G165G/S is indicative of GES-13.

None of other carbapenemases tested (OXA-48, VIM, IMP, KPC nor NDM), used as negative controls, amplified any of the studied alleles.

4. Discussion

Detection of CPO is a big challenge in diagnosis for Clinical Microbiology laboratories due to variable *in vitro* carbapenemases expression degree, which can even produce MICs values inside of susceptibility range to some carbapenems (Karlowsky et al., 2017).

Phenotypic methods such as CIM (Carbapenem Inactivation Method) or the Hodge test are often used as screening for carbapenemase detection. Nevertheless, recent studies have shown 50% of false negatives detecting GES-6 producing isolates with CIM method (Aguirre-Quiñonero et al., 2017). Concerning the Hodge test, several studies have also shown the presence of false positives and false negatives results (Girlich et al., 2012).

The low sensitivity of phenotypic screening methods could be due to the low carbapenem hydrolytic profile of GES-CPO. For this reason, in hospitals with a high prevalence of this type of carbapenemases, a negative phenotypic screening method is not enough to discard the presence of one of them, being needed a molecular confirmation method with higher sensitivity for GES family carbapenemase detection.

Given the current concern about the spread of carbapenemases, different diagnostic tests have emerged. Most of commercial assays available for resistance genes detection are limited to the “Big Five” carbapenemase families (KPC, OXA-48-like, NDM, IMP and VIM). Only few of them include GES- β-lactamases detection such as Amplidiag CarbaR+MCR, CRE (AusDiagnostics), LightMix Modular Carbapenemases (TibMolBiol/Roche) or PANA RealTyper CRE Kit (Panagene) (“Public Health England. Commercial assays for the detection of acquired carbapenemases.,” 2021; Rösner et al., 2019). However, a positive result for GES by whatever of these methods do not discriminate between GES with carbapenemase or cephalosporinase activity.

There are some GES-β-lactamases whose hydrolyzing activity is no classified (GES-27, 29, 35, 36, 37, 42, 43, 46, 55, 56 and 57), probably because more *in vitro* studies are needed. However, the presence of G165S mutation was always described in GES-variants with demonstrated carbapenemase activity. We can suppose that GES- 27, 29, 36, 37, 42, 43 and 55 might have carbapenemase activity due to all of them have G165S mutation. In the same way, GES-35, 46, 56 and 57 might have cephalosporinase activity because of the presence of G165G.

This allelic discrimination assay allows to detect in real time the presence of all type of GES-β-lactamases, being able to differentiate between carbapenemases and ESBL, through a quick PCR test that avoid costly sequencing approaches and could help to decrease the current underdiagnosis of minor carbapenemases that scape of phenotypic screening methods. This advance could have important implications in the establishment of effective targeted treatments as well as in the control of the spread of these microorganisms. This approach is compatible with the integration of genomic sequencing in surveillance, which allows the detection of new gene variants that will further help to develop/modify genetic tools such as the one described in this study.

Conflict of interests

The authors have no conflict of interests.

Acknowledgments

We would like to acknowledge routine laboratory technicians for their assistance in the production of the data.

Funding

This research was supported by grant from the Instituto de Salud Carlos III (number PI18CIII/00030)
CIBER – Consorcio Centro de Investigación Biomédica en Red (CB21/13/00068 and CB21/13/00095) (CIBERINFEC) and Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea – NextGenerationEU also supported this work.

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Author contributions

Daniel Pablo-Marcos: Supervision, conceptualization, investigation, data curation, validation, writing original draft.

María Siller: Investigation, data curation, validation, review and editing of draft.

Jesús Agüero: Supervision, conceptualization, Investigation, data curation, validation, review and editing of draft.

Alba Álvarez-Justel: Investigation, data curation, validation, review and editing of draft.

Sergio García-Fernández: Investigation, data curation, validation, review and editing of draft.

Silvia Velasco de la Fuente: Investigation, data curation, validation, review and editing of draft.

Jesús Rodríguez-Lozano: Investigation, data curation, validation, review and editing of draft.

Jesús Lucas-Fernández: Investigation, data curation, validation, review and editing of draft.

Carmen Fariñas: Investigation, data curation, validation, review and editing of draft.

Belén Aracil: Investigation, data curation, validation, review and editing of draft.

Jesús Oteo-Iglesias: Investigation, data curation, validation, review and editing of draft.

Jorge Calvo: Supervision, conceptualization, investigation, data curation, validation, review and editing of draft.

Table 1

Sequence of primers and probes designed for allelic discrimination assay of E98K and G165S SNPs.

Name of primer	Sequence 5'-3'	Concentration (µM)
GES6_EK-Fw	TCA TAT GGG CCT GAC AT	10
GES6_EK-Rv	TGT GTC CCG ATG CTA GAA	10
E98_FAM/IBFQ	AGACC+AT+T+C+GA+CG	5
K98_YAK/IBFQ	AC+C+AT+T+T+GA+CGA	5
GES6_GS-Fw	CTC TCT GAG TCG GCT AGA	10
GES6_GS-Rv	GCA ATA GGC GTA GTT GTA TCT	10
G165_ FAM/IBFQ	AT+G+G+GCG+ACA	5
S165_ YAK/IBFQ	AG+AT+G+A+GCGA+CA	5

Fw = Forward primer

Rv = Reverse primer

Table 2

Isolates used in the evaluation of the allelic discrimination assay for GES β-lactamases detection.

Microorganism	β-lactamase
Enterobacter cloacae complex (n=3)	GES-1
Pseudomonas aeruginosa (n=5)	
Klebsiella pneumoniae (n=1)	GES-2
P. aeruginosa (n=12)	GES-5
E. cloacae complex (n=65)	GES-6
Serratia marcescens (n=10)	
P. aeruginosa (n=5)	
P. aeruginosa (n=3)	GES-7
E. cloacae complex (n=2)	
Acinetobacter baumannii complex (n=8)	GES-11
Acinetobacter baumannii complex (n=2)	GES-12
P. aeruginosa (n=1)	GES-15
P. aeruginosa (n=1)	GES-19
P. aeruginosa (n=1)	GES-27
E. cloacae complex (n=1)	GES-53
Serratia marcescens (n=1)	GES-55
Negative controls	
K. pneumoniae (n=2)	OXA-48
E. cloacae complex (n=2)	IMP
Pseudomonas putida (n=2)	VIM
K. pneumoniae (n=1)	KPC
Klebsiella oxytoca (n=1)	
Escherichia coli (n=1)	NDM

Table 3

Adaptation of Naas T 2016 table actualized with β-lactamase database (BLDB), including GES-53 to 56, recently described. Amino acid substitutions in the main GES-type β-lactamases. In red, GES carbapenemases with E98K and G165S mutations. In blue, rest of GES-β-lactamases with carbapenemase activity described. In green, GES- β-lactamases with probably carbapenemase activity.

Amino acid position / GES-type	98	165	Amino acid position / GES-type	98	165
GES-1#	E	G	**GES-30***	**E**	**S**
GES-2*	**E**	**N**	GES-31#	K	G
GES-3#	K	G	GES-32#	K	G
GES-4*	**K**	**S**	**GES-33***	**K**	**S**
GES-5*	**E**	**S**	**GES-34***	**K**	**S**
GES-6*	**K**	**S**	GES-35#	E	G
GES-7#	K	G	**GES-36**	**E**	**S**
GES-8#	E	G	**GES-37**	**E**	**S**
GES-9#	E	G	GES-38#	K	G
GES-10#	E	G	**GES-39***	**G**	**S**
GES-11#	E	G	**GES-40***	**E**	**S**
GES-12#	E	G	**GES-41***	**E**	**S**
GES-13#	K	N	**GES-42**	**E**	**S**
GES-14*	**E**	**S**	**GES-43**	**E**	**S**
GES-15*	**E**	**S**	GES-44#	E	G
GES-16*	**E**	**S**	GES-45#	E	G
GES-17#	K	G	GES-46	K	G
GES-18*	**E**	**S**	**GES-47***	**E**	**S**
GES-19#	E	G	**GES-48***	**E**	**S**
GES-20*	**E**	**S**	**GES-49***	**K**	**S**
GES-21*	**E**	**S**	**GES-50***	**E**	**S**
GES-22#	E	G	**GES-51***	**E**	**S**
GES-23#	E	G	GES-52#	K	G
GES-24*	**E**	**S**	**GES-53***	**K**	**S**
GES-25*	**E**	**S**	**GES-54***	**K**	**S**
GES-26#	E	G	**GES-55***	**K**	**S**
GES-27	**E**	**S**	GES-56#	E	G
GES-28*	**E**	**S**	GES-57#	E	G
GES-29	**E**	**S**			

#Extended-spectrum β-lactamase (ESBL)

* Carbapenemase

Figure 1

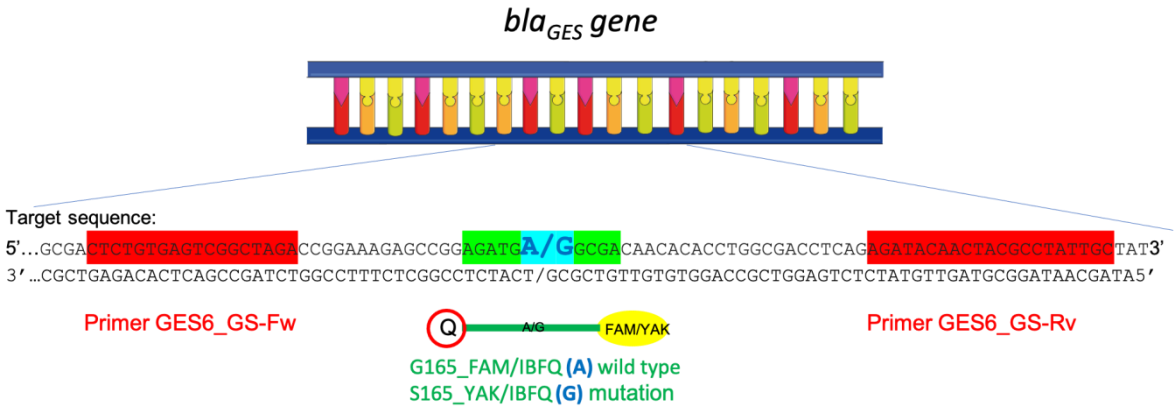


Figure 1. Preparation of allelic discrimination assay at G165 position. Primers designed in red. Wild type and mutated probe in green. Only the probe with exactly the same sequence will bind to the target.

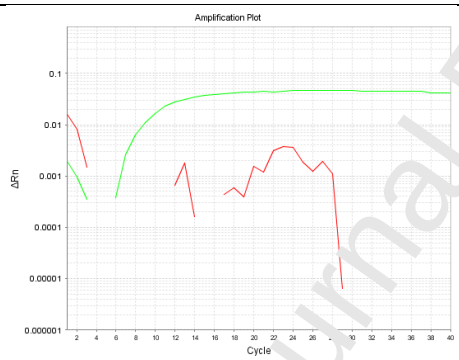
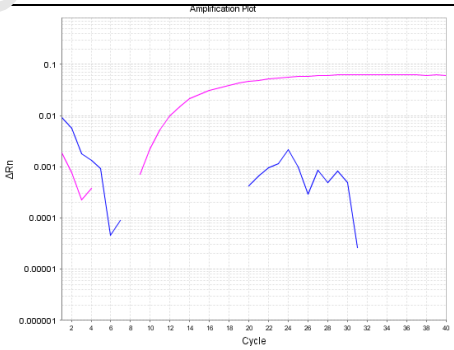
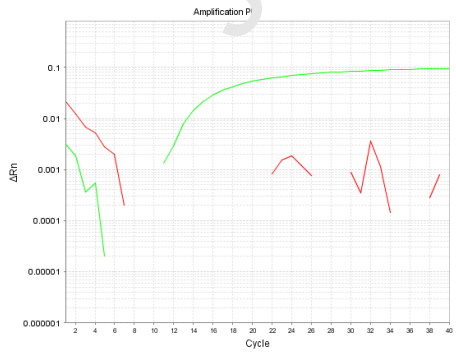
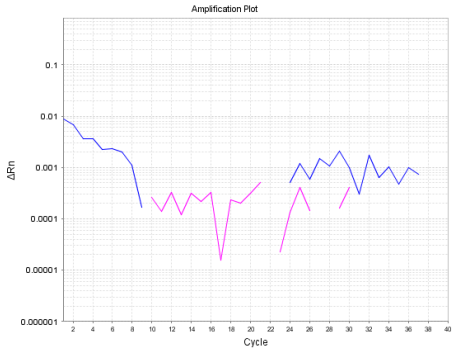
Figure 2

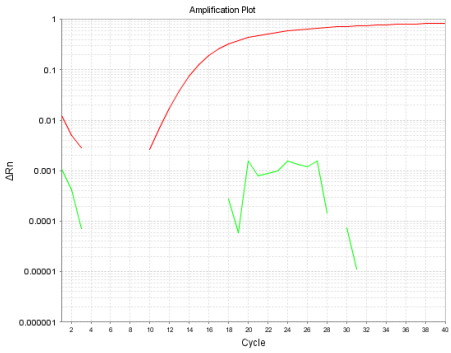
A. ESBL GES with no carbapenemase activity (GES-1): Detection of wild type E98E and G1765G alleles.

B. GES-2 carbapenemase: Detection of E98E allele and none of G165G/S allele due to G165N mutation.

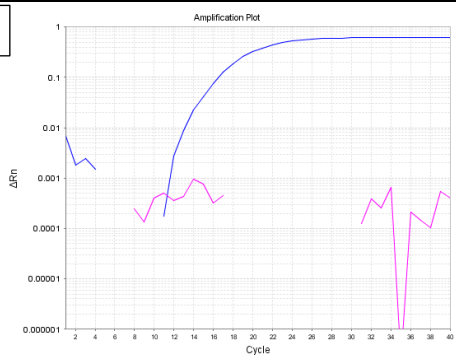
C. GES-6 carbapenemase: Detection of mutated E98K and G165S alleles.

D. GES-5 carbapenemase: Detection of E98E and G165S alleles.

RESULTS OF ALLELIC DISCRIMINATION ASSAY	
Extended-spectrum β-lactamase (GES-1)	
E98E allele detection	G165G allele detection
	A 
GES-2 carbapenemase	
E98E allele detection	No detection of G165G/S alleles
	B 
GES-6 carbapenemase	
E98K allele detection	G165S allele detection

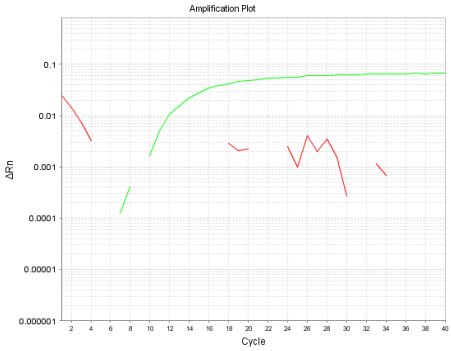


C

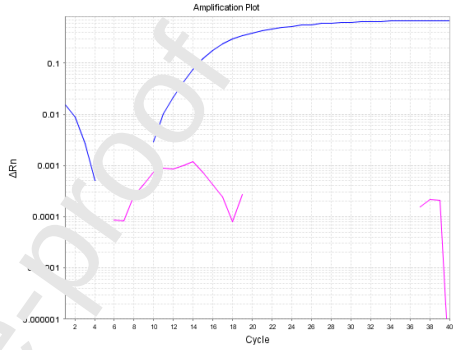


GES-5 carbapenemase

E98E allele detection	G165S allele detection



D



Green: E98E allele detection
Red: E98K allele detection
Magenta: G165G allele detection
Blue: G165S allele detection

Legend

Highlights

- Substitutions in residue ABL170 confer GES-carbapenemase activity.
- Substitutions in residue ABL104 enhance GES-cephalosporinase activity.
- Most commercial kits for carbapenemases detection do not include GES carbapenemases.
- This allelic discrimination assay allows to detect and differentiate all type of GES beta-lactamases and their carbapenemase or cephalosporinase activity.