Detection of extended-spectrum beta-lactamase cefotaxime resistance and virulence genes in *Escherichia coli* by duplex quantitative real time PCR and melt curve analysis

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RUNNING HEAD

Beta Lactamase Resistance and Virulence in *E. coli*

SIGNIFICANCE AND IMPACT OF THE STUDY

We developed a duplex real time qPCR assay coupled with melt curve analysis using commercially available qPCR mixes based on SYBR Green and EvaGreen for simultaneously detecting ESBL *bla*$_{\text{CTX-M}}$ and virulence genes in *E. coli* isolated from food and irrigation water**.** A conventional PCR assay was adopted for detecting large amplicons (375 to 1580 bp). This may facilitate development of tailor-made assays for rapid and accurate detection plus characterization of emerging foodborne and environmental pathogens within low resource settings.

22 **ABSTRACT**

Emerging virulent and antibiotic resistant pathogens present a global public health risk. Routine monitoring of prevalence within the clinical, environmental and food production setting is vital. Real time qPCR (qPCR) coupled with melting curve analysis can rapidly and accurately characterize pathogens. We evaluated commercial qPCR mixes based on SYBR Green l and EvaGreen for developing an assay for simultaneously detecting antibiotic resistance (Extended Spectrum beta-lactamase, ESBL and *bla*$_{\text{CTX-M}}$) and virulence (*stx1*, *stx2* and *eae*) genes in *E. coli* (n=12) isolated from irrigation water and irrigated vegetables. SYBR Green and EvaGreen detected two amplicons (*stx1* and *bla*$_{\text{CTX-M}}$) and (*stx2* and *eae*) in a single reaction. A higher mean melting temperature (T_m) separation between targeted amplicons and smoother melting curves were observed with the EvaGreen suggesting better performance when targeting multiple amplicons. Through simple stepwise optimization of DNA, cycling, primers, reaction volume and melting curve scanning rate, we adopted a conventional PCR assay for detection of large amplicons (375 to 1580 bp) for qPCR. This may facilitate development of cost-effective tailor-made assays for rapid and accurate monitoring of emerging foodborne and environmental pathogens in resource constrained regions.

Key words: *Escherichia coli*, Duplex real time-qPCR, melt curve analysis, virulence, extended- spectrum beta-lactamase

INTRODUCTION

Pathogenic foodborne *E. coli* is clinically categorized into Diarrheagenic *E. coli* (DEC) pathotypes. DEC pathotypes commonly associated with food and water include; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC) (Croxen *et al.*, 2013).

These groups can be identified by molecular characterization of virulence genes in isolated strains such as toxins (Shiga toxin, *stx1* and *stx2*) and attachment proteins (intimin, *eae*), that are commonly used to identify EHEC (Croxen *et al.*, 2013). Closely linked to virulence is the spread of antibiotic resistance among pathogenic bacteria such as *E. coli* which has been listed as one of the greatest risks posed to humanity in the 21st century (The Review on Antimicrobial Resistance, 2014). The rise of extended-spectrum beta-lactamase (ESBL)- producing bacteria within the last 20 years has increased this risk (Cantón, González-Alba & Galán, 2012; Shi *et al.*, 2015). Similarly, within the last decade, high prevalence of Cefotaxime resistant ($bla_{\text{CTX-M}}$) ESBL bacteria has increased the risk of spreading pathogens resistant to third generation cephalosporins, usually a line of last treatment for acute bacterial infections (Shi *et al.*, 2015). Global dissemination of the highly virulent and antibiotic resistant *E. coli* ST131 strain exemplifies this emerging public health threat.

We have previously shown that food (Ntuli, Njage & Buys, 2016, 2017; Ntuli *et al.*, 2018; Aijuka *et al.*, 2018) and irrigation water (Aijuka *et al.*, 2015) sources in South Africa carry pathogenic and antibiotic resistant *E. coli* and therefore pose a risk to national food safety and public health. Our work suggests that both the informally sold food products and environmental sources near the food chain are reservoirs of potentially pathogenic and antibiotic resistant *E. coli*. Similarly, this high prevalence of DEC may be associated with the high diarrheal disease burden (Chola *et al.*, 2010; Moshabela *et al.*, 2012; Gray & Vawda, 2016) among infants and immune compromised adults.

Developing countries in the BRICS (Brazil, Russia, India, China and South Africa) economic block and specifically South Africa, Brazil and India have a two-tier food production system characterized by a formal and informal sector.

The formal sector has a modern and highly controlled food chain while the informal side lacks these checks and balances. The lack of a controlled and monitored informal sector puts the population being served at risk of foodborne illness thereby necessitating strategies for routinely monitoring foodborne pathogens. Quantitative real time PCR (qPCR) presents an avenue for quick diagnostics and informed treatment options. Real time qPCR is the most powerful tool for quantitative nucleic acids analysis (Kubista *et al.*, 2006). QPCR enables simultaneous amplification and detection of specific DNA sequences with the amount of amplified product formed, monitored by fluorescence emitted by an intercalating dye or hydrolysis probe (Navarro *et al.*, 2015). Additionally, qPCR can be used to detect multiple virulence genes in food and environmental pathogens (Mendes *et al.*, 2007; Chassagnea *et al.*, 2009; Singh & Mustapha, 2014; Ahberg, Manz & Neuzil, 2015). This is because most pathogens have varying pathogenic traits making the method ideal for wide use in detection and epidemiological studies (Singh & Mustapha, 2014). Furthermore, qPCR using intercalating dyes such as SYBR Green and (EvaGreen) coupled with melting curve analysis (MCA), presents a fast, accurate and efficient means of detecting pathogenic characteristics associated with foodborne bacteria (Guion *et al.*, 2008)

Therefore, the objective of this study was to develop a qPCR assay using commercially available qPCR mixes based on SYBR Green 1 and EvaGreen fluorescence binding, coupled with melt curve analysis. The assay was aimed at enabling the simultaneous detection of *stx1*, *stx2*, *eae* and $bla_{\text{CTX-M}}$ genes in *E. coli* isolated from food sources and irrigation water.

RESULTS AND DISCUSSION

Duplex real time qPCR conditions

The optimization of these variables included varying the volume of DNA, primer pairs, $MgCl_2$ (only Kapa HRM fast PCR mix) for each 20 µL reaction. The thermocycling conditions were adjusted (gradient temperature range) to get the best annealing temperatures for all targeted amplicons for each duplex. To target all amplicons at their T_m during MCA, the slowest ramp rate (with 0.01^0C/s increments) was used. Conditions were considered optimal when; i) melting temperature of each amplicon in monoplex and duplex corresponded ii) a smooth melting curve and peak lacking non-specific products over the threshold was observed in monoplex and duplex. The curves in monoplex and assay conditions have been supplied as supplementary material (Sup).

SYBR Green l and EvaGreen based qPCR based kits

Two qPCR mixes, A (SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix containing SYBR Green l dye and B (KAPA HRM Fast PCR mix) provided the most reproducible results (T_m and C_q values) for detection of the target genes. Mixes A and B were only able to provide ample T_m separation for each target gene within a duplex reaction (Table 1). The selection of the genes for duplexing was based on size (bp) of the amplified product with each pair of targeted genes consisting of a large and small amplicon. The two genes detected in each duplex reaction had ample mean T_m separation based on MCA. T_m separation is considered ample when ≥ 2˚C (Mendes *et al.*, 2007; Chassagnea *et al.*, 2009; Bai, Shi & Nagaraja, 2010). The duplex reactions from each qPCR mix targeted two pairs of genes; *stx1* and $bla_{CTX\text{-}M}$ and *stx2* and *eae* respectively.

Melt Curve Analysis

With qPCR mix A, mean T_m separation between *stx1* (80.5˚C) and *bla*$_{CTX-M}$ (85.85˚C) was 5.35˚C in monoplex. Similarly, a large T_m separation of 4.4 ˚C was noticed between *stx2* (86.8˚C) and *eae* (82.4˚C). This distinct T_m separation enabled the gene pairs' use in designing the duplex assay (Figure 1 and 2). Duplex single tube reactions yielded melting peaks with T_m's similar (not statistically different $p \leq 0.05$) to monoplex reactions (data not shown).

With qPCR mix B, a mean T_m difference of 7.85˚C was observed between *stx1* (79.85˚C) and *bla*$_{CTX-M}$ (87.7˚C). Additionally, a similarly large mean T_m difference of 7.5˚C was noticed between *stx2* (88.15˚C) and *eae* (80.65˚C). Using mixture B containing EvaGreen (7.68˚C) as opposed to mixture A having SYBR Green 1 (4.88˚C) provided a larger mean T_m difference between amplicons suggesting a larger temperature range for amplicon detection. This larger mean T_m separation observed with mixture B allowed simultaneous detection of the target genes with similar T_m's as in the monoplex reactions. The melting curve peaks generated using mix B (EvaGreen) for monoplex and duplex reactions were smoother and generating more fluorescence than mix A (SYBR Green). The volume of DNA in monoplex and duplex varied more when using mix A than B (Table 1).

Limit of detection (LOD) and analytical specificity (AS)

LOD for template DNA in both assays was 0.02 ng with a linear dynamic range of 0.02 ng to 100 ng. Both assays detected all relevant genes from each test strain used for assay development (Table 2). We acknowledge the few strains used for assay validation. Our aim was to show potential of easily adopting conventional PCR into qPCR for rapid and routine diagnostics. The assay development requires minimum optimization and basic technical skills for easy adoption in resource constrained environments.

The differences in specificity of the two assays when using each qPCR mix in terms of; DNA template, smoothness of the melt curves and mean T_m separation may be linked to the DNA binding mechanism of each dye. SYBR Green l is an asymmetric cyanine compound binding to the minor groove of DNA leading to increase in formation of non-specific products thereby limiting its applicability in real time PCR over a broad range of temperatures (Gudnason *et al.*, 2007). Additionally, it has a high affinity for G-C rich amplicons regardless of size that might interfere with the PCR reaction process (Gudnason *et al.*, 2007). Contrastingly, the symmetrical cyanine structure of EvaGreen aids formation of fewer non-specific products since the binding of double stranded (dsDNA) occurs evenly over the whole molecule. This observation has previously been observed in saturating dyes of the SYTO family that showed a broader range of T_m's based on their saturation ability once bound to dsDNA thereby providing suitable conditions for qPCR multiplexing compared to SYBR Green dyes (Monis, Giglio & Saint, 2005; Eischeid, 2011).

The robustness of qPCR coupled with MCA in diagnostic testing makes the above drawbacks easy to circumvent. In this study, we optimised the template concentration, ramp rate of T_m acquisition step, primer pair and $MgCl_2$ volumes within each duplex reaction. Previous studies have shown the robustness of qPCR in detecting amplicons using different strategies. For instance, a low scanning rate for intercalating dyes helps to control PCR inhibition since every system (thermocycler) exhibits a delay between temperature at the sensor and the sample, therefore such a rate will precisely determine T_m's (Ahberg *et al.*, 2015). Additionally, when using commercial qPCR mixes, the dye concentration is constant, therefore varying primer pair concentration can provide optimization of the assay (Monis *et al.*, 2005). Furthermore, the assays were able to simultaneously detect 6 Metallo-beta-lactamase encoding genes with amplicons ranging from 72 to 1449 bp in different *Enterobacteriaceae* species through optimization of primer pair concentration, sequence (designed to have 1˚C Tm separation) and

volume (total reaction volume 48µL)(Mendes *et al.*, 2007). Similarly, Ahberg et al. (2015) used MCA to design a qPCR assay that quantified Avian flu at each melting scan step rather than at the end of the reaction.

These studies depict how robustness of qPCR enables tailor-made solutions laboratories. Commercial qPCR mixes provide standardization of many assay reagents and consumables. Therefore, laboratories only need to vary few conditions to develop assays that are specific thereby saving time and resources. We have developed duplex qPCR assays with MCA using commercial mixes for detecting antibiotic resistance and virulence genes in *E. coli*. Simple optimization of reagents and conditions for this assay highlights robustness for application in food safety and public health.

MATERIALS AND METHODS

E. coli source and characterization

E. coli isolates were previously isolated from irrigation water (Aijuka et al. 2015) and irrigated lettuce (Aijuka, 2014) in South Africa. Isolates, n=12 (Table 3) had previously been characterized for possession of $bla_{\text{CTX-M}}$ ESBL including cefotaxime resistance (Njage & Buys, 2015) and *stx1, stx2* and *eae* (Aijuka *et al.*, 2015). All isolates were stored in Tryptone Soy broth (TSB) (Biolab, South Africa) containing 30% glycerol at -80˚C in the Department of Consumer and Food Sciences, University of Pretoria.

DNA extraction and purity determination

Isolates were grown in TSB at 37 ºC for 24 h and harvested (centrifugation at 10 000 rpm, 10 min). The bacterial pellet was resuspended with 200µL of Triton™ X-100 buffer (Sigma Aldrich, St. Louis, USA) according to (Xue *et al.*, 2009). The mixture was incubated at 95 ºC for 30 min with agitation, cooled to 4 ºC for 10 min and centrifuged (13000 rpm for 10 min). DNA purity (260/280) was measured using Nanodrop 2000 (ThermoFisher, Wilmington DE,

USA). DNA stock solutions were diluted (1:10) with sterile double distilled water (0.02 to 0.1 g/L).

Primers and real time qPCR

We optimized reagents, consumables and qPCR thermocycling and subsequent melt curve conditions (Sup 1 and 4). Primer sequence homology and specificity was tested *in silico* and synthesised (Whitehead Scientific (Pty) Ltd/ (Brakenfell, South Africa)). Primers were subsequently tested for specificity in monoplex and duplex. Four commercial qPCR kits were evaluated. Two of each contained SYBR Green 1 or EvaGreen dyes. The dyes bind double stranded DNA differently thereby facilitating their adoption in qPCR and MCA (Jansson *et al.*, 2017). SYBR Green 1 dye-based mixes were KAPA SYBR®Fast qPCR Kit Master mix (2X) Universal (KAPA SYBR Fast DNA polymerase, ROX reference dye, Flourescein dye (10nM), SYBR Green I, $MgCl_2$) (KAPA Biosystems, South Africa) and SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Antibody-mediated hot start Sso7d fusion polymerase, dNTPs, $MgCl_2$, SYBR Green 1, enhancers, stabilizers and blend of reference dyes (ROX and Flourescein)) (Bio-Rad Laboratories Inc. Hercules, USA). EvaGreen mixes included KAPA HRM Fast PCR Kit (EvaGreen, Taq DNA polymerase) (KAPA Biosystems) and Precision melt Supermix (Hot start iTaq™ DNA polymerase, dNTPs, $MgCl_2$, EvaGreen dye, enhancers and stabilizers) (Bio-Rad).

Final quantities and concentrations of optimized reagents and consumables in each 20 µL closed tube reaction are shown in (Sup 1). The harmonized protocols for each qPCR mix are shown (Sup 4). All qPCR reactions were carried out in 0.2 mL PCR tubes **(**Bio-Rad). The optimised PCR reactions and subsequent MCA were carried out using the CFX96 Touch™ Real Time PCR detection system (Bio-Rad) and fluorescent analysis in the FAM/SYBR Green 1 Channel.

Limit of detection (LOD) and analytical specificty (AS)

Determining LOD involved preparing serial dilutions ($l0^{-1}$ to 10^{-8}) of DNA from isolates and running qPCR reactions with the commercial qPCR kits. A plot of quantification cycle (C_q) versus log starting quantity of DNA was generated. LOD for each duplex assay was expressed (detection per log DNA concentration). The plot curve (C_q v/s Log starting DNA) and standard curve were calculated using the CFX Manager™ software Industrial Diagnostics Version 2.0 (Bio-Rad). AS was determined using 12 isolates (Table 3).

Data analysis

QPCR data (means and standard deviations) for T_m, C_q and melt curves for duplexes were calculated using CFX Manager™ (Bio-Rad). A student T-test (Microsoft Excel 2016, Microsoft Corp, Redmond, USA) compared T_m's of target amplicons in monoplex and duplex at 95% confidence level ($p \leq 0.05$). All analyses were repeated at least three independent times.

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CONFLICT OF INTEREST

None

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Table 1 Volumes and melting temperatures of amplicons in monoplex and duplex qPCR assays

	Quantitative real time PCR super mix			
Monoplex/ Duplex reaction	SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix		KAPA HRM Fast PCR Kit	
Targeted gene (s)	DNA quantity (µL)	Melting Temperature (˚C)	DNA quantity (µL)	Melting Temperature (˚C)
eae	1.5	82.4±0.07	1.5	80.65±0.07
stx1	0.9	80.5±0.07	1.5	79.85±0.07
$bla_{\text{CTX-M}}$	0.5	85.85±0.07	1.5	87.7±0.21
stx2	0.5	86.8±0.02	1.5	88.15±0.07
stx1 and $bla_{\text{CTX-M}}$	1.0	80.4±0.07 and 84.40±0.01	1.0	79.35±0.07 and 87.50±0.07
eae and stx2	0.5	82.8±0.07 and 85.70±0.07	0.5	81.10±0.07 and 87.80±0.07

Targeted genes: *stx1*-shiga toxin 1; *stx2*-shiga toxin 2; *eae*- intimin; $bla_{\text{CTX-M}}$- general class of cefotaxime resistant extended-spectrum beta-lactamase (ESBL) producing *E. coli*

Table 2 Genes and primer sequences for developing duplex qPCR assays

Gene	Primers	Primer sequence (5'-3')	Target gene product size (bp)	GC content (%)	Reference
Shiga toxin 1	*stx1*-F	TGT CGC ATA GTG GAA CCT CA		50	
	Stx1-R	TGC GCA CTG AGA AGA AGA GA	655	50	
Shiga toxin 2	*stx1*-F	CCA TGA CAA CGG ACA GCA GTT		52.4	
	stx2-R	TGT CGC CAG TTA TCT GAC ATT C	477	45.5	
Intimin	*eae*-F	CAT TAT GGA ACG GCA GAG GT		45.5	(Bai *et al.*, 2010)
	eae-R	ACG GAT ATC GAA GCC ATT TG	375	45	
[1]CTX-M type-ESBLs	bla_{CTX}-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	1354	48.1	(Hasman *et al.*, 2005; Hendriksen *et al.*, 2009)
	bla_{CTX}-R	TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	1580	51.7	

1- Targets the whole group of this class of antibiotics that is categorized into 5 groups based on their amino acid sequence identity

bla_{CTX-M}- general class of Cefotaxime resistant extended-spectrum beta-lactamase (ESBL) producing *E. coli*

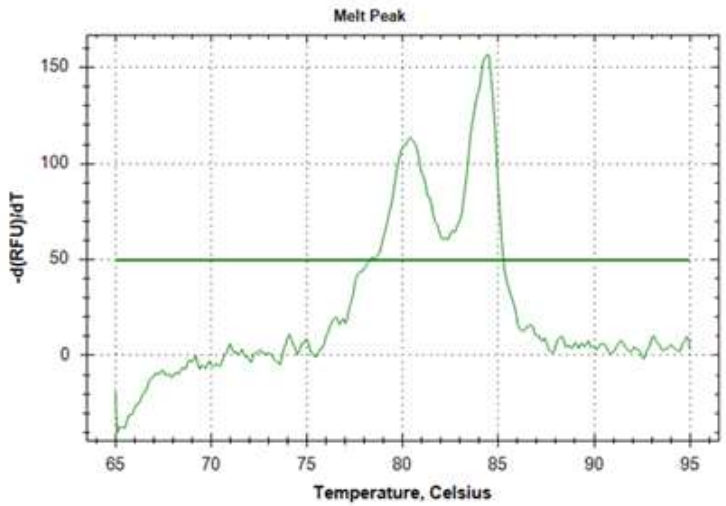
Table 3 Source and characteristics of *E. coli*

Source of isolation	Isolate code	*bla*$_{CTX-M}$	Virulence genes (*stx 1*, *stx 2* and *eae*)			DNA concentration (g/L)
			stx 1	*stx 2*	*eae*	
Irrigation water	MPU(W)8(3)	+	+	+	-	0.020
Irrigation water	MPU(W)9(1)	+	+	+	+	0.050
Irrigation water	NW(W)6(1)	+	+	+	-	0.055
Irrigation water	NW(W)9(1)	_	+	+	+	0.095
Irrigation water	NW(W)9(2)	_	+	+	+	0.095
Irrigation water	NW(W)5(1)2	_	-	-	+	0.074
Irrigation water	NW(W)5(3)	_	+	+	+	0.098
Irrigation water	MPU(W)5(7)	_	+	+	-	0.024
Irrigation water	MPU(W)5(1)	_	-	-	-	0.033
Irrigated lettuce	NW(V)5(3)	_	-	-	-	0.036
Irrigated lettuce	NW(V)7(3)	+	+	+	-	0.096
Irrigated lettuce	NW(V)3	+	+	+	+	0.099

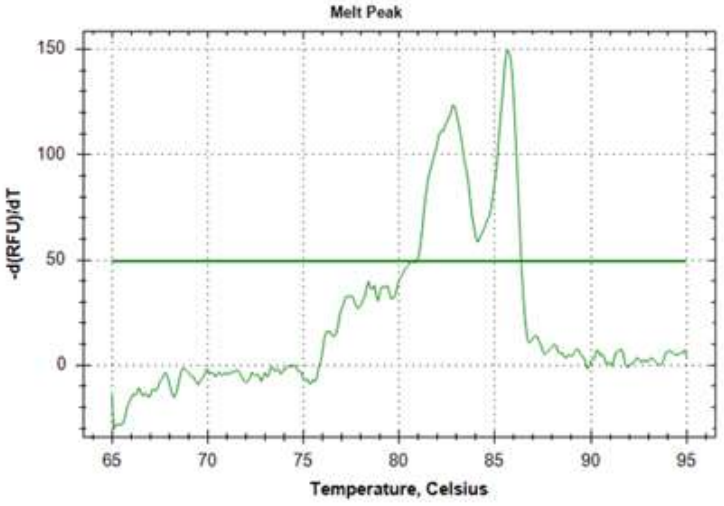
bla$_{CTX-M}$- cefotaxime resistant extended-spectrum beta-lactamase (ESBL) producing *E. coli; stx 1*-shiga toxin 1; s*tx 2*-shiga toxin 2; *eae*-intimin;

+: positive for gene

-: negative for gene

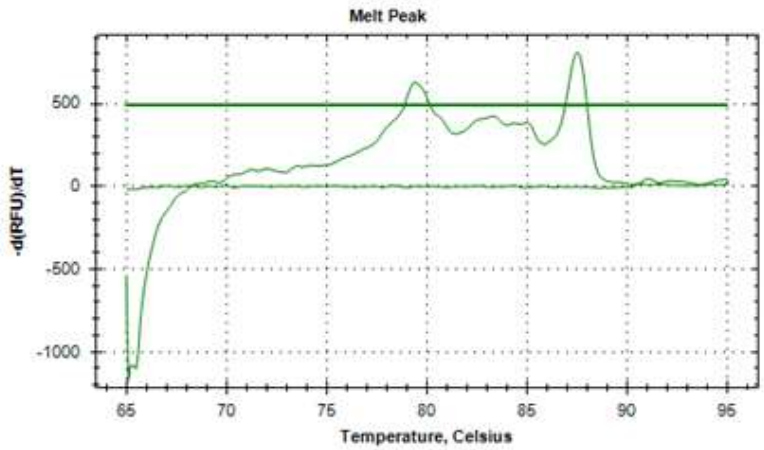


1.) ***stx1 and bla*** $_{\text{CTX-M}}$

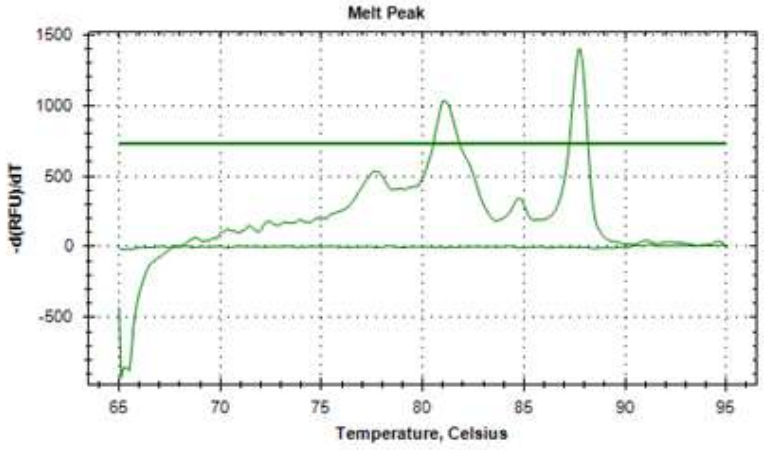


2.) ***stx2*** **and** ***eae***

Figure 1



1.) ***stx2*** **and** ***bla*****$_{\text{CTX-M}}$**



2.) ***stx2*** **and** ***eae***

Figure 2