DE GRUYTER OPEN

Research article

DOI:10.2478/rrlm-2018-0015

xTAG Luminex multiplex assay for rapid screening of verocytotoxin-producing *Escherichia coli* strains

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Abstract

The O26 verocytotoxin-producing Escherichia coli (VTEC)-associated outbreak of hemolytic uremic syndrome (HUS) cases in Romania during 2016 showed the need to improve the current methodology of non-O157 VTEC detection and surveillance. An in-house assay based on xTAG Luminex technology was optimized to identify seven of the most relevant diarrheagenic E.coli serogroups (O-specific wzx genes), two convenient VTEC virulence markers (eaeA and ehxA genes), and a species-specific control gene (uidA). Twenty-nine strains previously characterized in terms of serogroup and virulence genes were tested with the optimized protocol and the results were as expected. The ratio of sample signal to background varied from 66.7 (ehxA) to 7.6 (uidA) for positive samples, with a cut-off of 3. Sensitivity varied depending on the target to be amplified from approximately 10^2 genomic copies to approximately 10^4 genomic copies per reaction, respectively. The current approach seems an affordable alternative to commercially available assays that can be further exploited to improve existing autochthonous strategies to prevent future VTEC outbreaks.

Keywords: *VTEC, bead-suspension, xTAG Luminex, rapid screening*

Received: 29th January 2018; Accepted: 5th April 2018; Published: 7th April 2018

Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC) are a major group of enteric pathogenic *E. coli* represented by zoonotic pathogens with a great propensity to cause infections with systemic complications and to generate food borne outbreaks. Their key virulence strategy is based on the capacity to produce verocytotoxins which are essential bacteriophage-encoded virulence factors, but additional mechanisms involving determinants carried on pathogenicity islands, plasmids, transposons, and phages are also responsible for pathogenesis of VTEC (1). Most VTEC strains isolated from severe cases of human infections possess the locus of enterocyte effacement (LEE), a pathogenicity island also present in the strains assigned to the enteropathogenic *E. coli* (EPEC) group. LEE consists

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of genes which induce the attaching and effacing lesions in the intestinal mucosa. One of them, the intimin-coding gene *eae* has been used as a convenient marker for LEE-positive VTEC strains. The presence of large and conserved plasmids which encode accessory virulence factors among which enterohemolysin toxin encoded by *ehxA* gene is common for clinically relevant strains (2 - 4).

There are hundreds of serotypes of VTEC identified to date, but only some of them have been reported to have a high significance in human disease worldwide (5 - 8). In Europe, *E. coli* serogroups O157, O26, and O103 dominate human VTEC infections (http://atlas.ecdc.europa.eu/public/index.aspx). In Romania, the VTEO26 serogroup was the cause of anoutbreak of hemolytic uremic syndrome (HUS) among small children in 2016, making it the first major event linked to such pathogens (9 - 10).

The method used for the detection of enteric pathogenic *E. coli* in Romanian clinical microbiology laboratories, whether public or private, is still conventional serotyping performed in a slide agglutination format with commercially available O antisera against EPEC and VTEC serogroups. As this procedure can be expensive and time-consuming, the strains are usually agglutinated only with polyvalent antisera and VTEC detection is focused exclusively on O157 serogroup. As a rule, the positive strains recovered from severe cases of infection are referred to the national reference laboratory for confirmation. Prior to 2016, this was the sole autochthonous laboratory that used both culture and non-culture based assays for the detection of human pathogenic *E. coli* strains in general and of VTEC in particular. The HUS outbreak challenged clinicians, microbiologists, and epidemiologists and put pressure on the public health system to improve VTEC preparedness. Consequently, new strategies for the rapid screening of a large number of *E. coli* strains in order to distinguish pathogenic from non-pathogenic strains are needed.

Luminex xTAG® technology is a high-throughput, rapid, labor-saving multiplex method offering both high sensitivity and specificity. Using this technology, up to 150 different targets can be detected in a single reaction. Also, it is a flexible technology, as additional targets may be incorporated in the assay, as needed (11). Considering the commercially available kits, the local needs and the limited financial resources, this pilot study explored the use of an *in-house* approach based on xTAG Luminex technology for the identification of seven of the most relevant diarrheagenic *Escherichia coli* serogroups (targeted through *wzx* genes encoding the O-antigen flippase enzyme), two convenient VTEC virulence markers (*eaeA* and *ehxA* genes), and *E. coli*-specific beta-glucuronidase encoding gene (*uidA*).

Material and Methods

Bacterial strains

The 29 bacterial strains used in this study originated from the bacterial collection of the Molecular Epidemiology Laboratory of Cantacuzino National Institute of Medical-Military Research – Development (CNIMMRD), Bucharest, Romania (Table 1). The *E. coli* panel included autochthonous strains isolated and identified during routine surveillance by the national reference laboratory (NRL) of CNIMMRD, as well as international *E. coli* strains with confirmed serogroups. The latter were distributed by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* from Statens Serum Institute (Denmark) to all the NRLs, including the NRL of CNIMMRD, which participated in the rounds of external quality assessment (EQA) on VTEC typing organized annually by the European Center for Disease Pre-

vention and Control (ECDC). All strains were stored at -70°C until DNA extraction.

DNA extraction and quantification

DNA extraction was performed with PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentrations of purified DNA were measured with Qubit 2.0 Fluorometer (Invitrogen, Eugene, Oregon, USA), using Qubit dsDNA HS Assay Kit (Invitrogen, Eugene, Oregon, USA). All the samples were diluted to

Table 1. Bacterial strains used in this study, their serogroup and target gene content

	Strain	**Serogroup**	***eaeA***	***ehxA***	***uidA***
1	*Escherichia coli* 3096*	O26	+	+	+
2	*Escherichia coli* 3099*	O26	+	+	+
3	*Escherichia coli* 193151*	O26	+	+	+
4	*Escherichia coli* 3868	O26	+	+	+
5	*Escherichia coli* 12107	O45	-	-	+
6	*Escherichia coli* 8789	O103	+	+	+
7	*Escherichia coli* 115	O103	+	+	+
8	*Escherichia coli* 2206	O103	+	+	+
9	*Escherichia coli* 9842	O111	+	-	+
10	*Escherichia coli* 3501	O111	+	+	+
11	*Escherichia coli* 2393	O111	+	+	+
12	*Escherichia coli* 3390	O121	+	+	+
13	*Escherichia coli* 2771	O121	+	+	+
14	*Escherichia coli* 8263	O145	+	-	+
15	*Escherichia coli* 179	O145	+	-	+
16	*Escherichia coli* EDL933	O157	+	+	+
17	*Escherichia coli* 213666*	O157	+	+	+
18	*Escherichia coli* 5017	O157	+	+	+
19	*Escherichia coli* 4062	O157	+	+	+
20	*Escherichia coli* 117	O146	-	+	+
21	*Escherichia coli* 172	O91	-	+	+
22	*Escherichia coli* 181	O171	-	-	+
23	*Escherichia coli* 3467	O104	-	-	+
24	*Escherichia coli* 8145	O166	-	-	+
25	*Escherichia coli* 356	O139	-	+	+
26	*Escherichia coli* 262*	unknown	-	-	+
27	*Escherichia coli* 211986*	unknown	-	-	+
28	*Campylobacter jejuni* 223251 *	NA	-	-	-
29	*Staphylococcus aureus* 92*	NA	-	-	-

* Autochthonous bacterial strains

10^6 genomic copies/µl and serial dilutions in water were performed until 1 genomic copy/µl.

Primers for targets detection

PrimerPlex vers. 2 software was used to design the 10 pairs of specific primers for the 10 targets (Table 2), following the instructions from Luminex (11). The xTAG assays require modified specific primers. The forward specific primers were modified by a unique 24-base oligonucleotide "TAG*"* sequence at the 5' terminus that is used to connect the amplicon to a specific MagPlex-xTAG microsphere (Luminex Corporation, Toronto, ON, Canada). The MagPlex-xTAG microspheres are 6.5-micron superparamagnetic beads where each bead region is covalently pre-coupled with a different 24-base oligonucleotide "anti-TAG" sequence (complementary to the "TAG"sequence). A 12-carbon spacer was incorporated between the "TAG" sequence and primer. All reverse primers were biotinylated at the 5' terminus. The primer sequences, their target genes, the size of the resulting amplicons and the corresponding bead region are shown in Table 2.

Multiplex PCR optimization

Seven *E. coli* strains were used for optimization of multiplex PCR (i.e. *E. coli* 3096, *E. coli* 12107, *E. coli* 8789, *E. coli* 9842, *E. coli* 3390, *E. coli* 8263, *E. coli* EDL933) selected tocover all the targets. First, the specificity of primers was confirmed in simplex PCR reactions. The multiplex PCR was influenced by many factors such as annealing time, annealing temperature, and primer concentrations. These conditions were optimized in order to assure specific amplification and sensitive detection of the targets. Finally, the multiplex reaction was set as follows: 12.5 µl GoTaq HotStart MasterMix (Promega, Madison, WI, USA), 0.6 µM uidA and O26wzx primers, 0.2 µM ehxA primers, 0.1 µM the other primers, and 1 µl DNA, in a total volume of 25 µl. Two negative controls (water) were included in each set up. To determine the sensitivity of the multiplex reaction, multiple reactions with different DNA concentrations (1-10^6 genomic copies/reaction) were performed. PCR reactions were performed with the following parameters: initial denaturation 95°C – 2min, 35 cycles with denaturation at 94° – 30 sec, annealing at 50°C – 90 sec, extension at 72°C – 30 min and final extension at 72°C – 7 min. The PCR products were then subjected to hybridization on microspheres, no more than 48h later.

Hybridization on MagPlex-xTAG beads

The assay was conducted according to Luminex (11). Shortly, the hybridization mixture was set in a 96-well low-profile plate and consisted for each sample of: 2500 MagPlex-xTAG microspheres of each set, 1 µl PCR product and 70 µl Streptavidin, R-Phycoerythrin Conjugate (SAPE) (Invitrogen, Carlsbad, CA, USA) to 10 **µ**g/mL, as indicator, in a final volume of 95µl. For PCR negative controls, 2 wells for each control were assigned, leading to 4 wells with negative controls. SAPE was supplemented with BSA, to 0.1% in final volume. Hybridization was performed in a thermocycler (Veriti 96-Well Thermal Cycler, Applied Biosystems). Different hybridization parameters were tested, the final tests being performed at 40°C, 30 minutes.

Luminex 200 analyses

Immediately after the hybridization reaction, the products were analyzed on the Luminex 200 analyzer (Luminex Corporation, Toronto, ON, Canada) according to the system manual, using xPONENT vers. 3.1 software (Luminex Corporation, Toronto, ON, Canada). The sample size was set to 70µl, with a minimum of 100 beads per target to be analyzed. Reading was performed at 40°C, with 90 s timeout, and DD (Doublet

Table 2. Primers used in this study and corresponding MagPlex - xTAG bead region

Gene target	Primer	Primer sequence 5'-3'	Amplicon size	Corresponding MagPlex-xTAG bead region
O26	O26wzxF	CAAATACATAATCTTACATTCACT[1)]-C12[2)]-GCTA-AAATTCAATGGGCGGAAA	131 bp	A13
	O26wzxR	biotin - GCAACAGGCGAAGCAAGA		
O45	O45wzxF	CATAATCAATTTCAACTTTCTACT-C12-GAGC-CGAGATGGTAATTCCTAA	110 bp	A12
	O45wzxR	biotin -GCACAACGCAACGAAAGTC		
O103	O103wzxF	AATTTCTTCTCTTTCTTTCACAAT-C12-AGGCT-TATCTGGCTGTTCTTAC	127 bp	A14
	O103wzxR	biotin - GCGAGCGGTACAACAATACA		
O111	O111wzxF	TACTTCTTTACTACAATTTACAAC-C12-TTCAAT-TAACTGGTGGCGTCTC	102 bp	A15
	O111wzxR	biotin - AGTTGAAGGCAAGGGACATAAG		
O121	O121wzxF	CAAACAAACATTCAAATATCAATC-C12-GACAAT-GACAGTGCTGGACTA	113 bp	A22
	O121wzxR	biotin - CCGAAATGATGGGTGCTAAGA		
O145	O145wzxF	TCAAACTCTCAATTCTTACTTAAT-C12-AAGGTG-TATGGTGTTGGTTAGC	121 bp	A21
	O145wzxR	biotin - AGCCACTCCAAGCACACT		
O157	O157wzxF	CTTTCTCATACTTTCAACTAATTT-C12-CATGCAC-GCAATGATACTCAAT	117 bp	A20
	O157wzxR	biotin - AACGACTTCACTACCGAACAC		
eaeA	eaeAF	ACACTTATCTTTCAATTCAATTAC-C12-ATG-GAACGGCAGAGGTTAATC	149 bp	A18
	eaeAR	biotin -CGTAAAGCGGGAGTCAATGT		
ehxA	ehxAF	CTTTCTTAATACATTACAACATAC-C12-AGAATG-GATATGACGCAAGACA	139 bp	A25
	ehxAR	biotin - GCAAGTTCACCGATCTTCTCA		
uidA	uidAF	ATACTTTACAAACAAATAACACAC-C12-AATG-GTGATGTCAGCGTTGAA	105 bp	A19
	uidAR	biotin - TTGCCAGAGGTGCGGATT		

[1)]TAG sequences – underline
[2)]12 Carbon spacer - HEG (hexaethylene glycol)

Discriminator) gate 7500–13500 with the High PMT (PhotoMultiplierTube) selected to increase the reporter gain level. The results were reported as median fluorescence intensity (MFI) of each microsphere region. From the MFI, signal to background ratios were calculated (signal_MFI/background_MFI), where background MFI value is obtained from the 4 negative control MFIs using the formula: Background MFI = $MFIMean_{NC} + 1.645(SD_{NC})$. Samples were considered to be positive when signal to background ratio was greater than 3.0.

The evaluation of sensitivity

To determine the sensitivities of the multiplex assay, serial dilutions (1-10^6genomic copies/µl) of the 7 bacterial DNA samples used for optimization were tested. The sensitivity results were confirmed in three different experiments.

The evaluation of specificity

To assess the specificity of the assay, all 29 bacterial strains considered for this study were tested. Approximately 5x10^6 genomic DNA copies were introduced in each reaction.

Results

Specificity of the multiplex reaction

The antigenic and virulence characteristics of the 29 bacterial strains tested with the optimized protocol were as expected. The ratio of sample signal to background varied from 66.7 (*ehxA*) to 7.6 (*uidA*) for positive samples. Table 3 presents the signal to background ratio values of the positive targets. When multiple samples were positive for the same target, the ratio values are indicated as the mean ± standard error of the mean. When the samples were negative, the signal to background ratio was below 3.0.

Sensitivity of the multiplex reaction

The sensitivity varied depending on the target to be amplified: 10^2 genomic copies (about 5pg. of DNA) for O45*wzx*, O111*wzx*, O145*wzx* and O157*wzx*, 10^3 genomic copies for O26*wzx*, O103*wzx*, O121*wzx, ehxA*, *eaeA*, and 10^4 genomic copies for *uidA* target. The sensitivity was calculated plotting the median of positive sample to background ratio of each target to a cut-off of 3.0 (Figure 1).

Discussion

Although there is a wide variety of laboratory assays available for VTEC identification, the problems related to the diagnostic of VTEC infections are still challenging (5, 12 -14). The choice of the methods utilized is not easy to make, which is the reason why there is a lack of standardization in the approach of identification of human VTEC strains, especially for non-O157 VTEC.

In the ECDC zoonosis reports published annually, Romania has a very low reported incidence of VTEC (15). Although this incidence is most likely to be real, the reported epidemiology might be undoubtedly influenced by the efficiency of the laboratory practice. The outbreak of VTEC infections that occurred in 2016 disquieted the public health system revealing the inability to quickly recognize and respond to such diseases. In the attempt to optimize the diagnosis for VTEC in Romania, we considered the advantages of using the Luminex xTAG® platform as the equipment was available but not previously used for diagnostic assays for infectious diseases.We were aware of the existence of the commercially available kits that screened for VTEC using Luminex platform. Luminex xTAG® Gastrointestinal Pathogen Panel (xTAG® GPP) (16) was one of them, which was able to identify major gastrointestinal pathogens, VTEC included, and to discriminate members of VTECO157 serogroup. Another one, more recently introduced, xMAP® Molecular STEC Serotyping Assay, screened not only for *E. coli* strains of serogroup O157, but also of other non-O157 serogroups, frequently associated with VTEC, along with the

Table 3. Sample signal to background ratio for positive samples

Target	O26 *wzx*	O45 *wzx*	O103 *wzx*	O111 *wzx*	O121 *wzx*	O145 *wzx*	O157 *wzx*	*eaeA*	*ehxA*	*uidA*
Sample signal/ background	11.2 ± 1.6	48.7	12.8 ± 1	52.1 ± 1.2	54.4 ± 3.3	53.2 ± 0.2	42 ± 1.4	22.1 ± 1.3	54.0 ± 9.6	10.6 ± 2.3

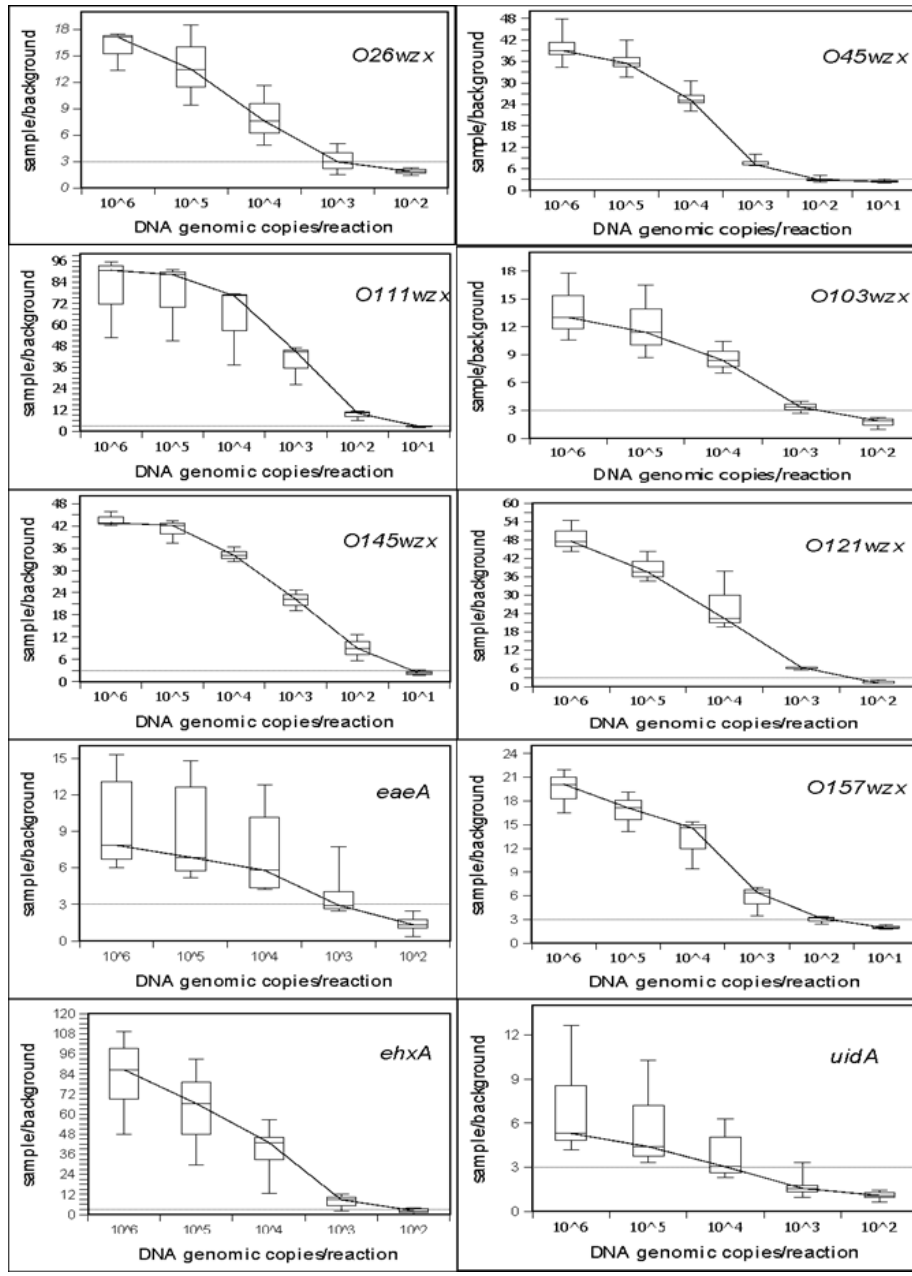

Figure 1. Sensitivity of detection for each target from multiplex assay. Cut-off for sample signal to background ratio was set to 3.0

eae and *aggR* (transcriptional regulator) genes. However, to our knowledge, there was no laboratory-based evaluation of this kit, in terms of specificity and sensitivity, available before our report about this *in-house* assay. Thus, based on the results presented here, our xTAG assay has a great advantage over the already available commercial kits at least in terms of flexibility, be-

ing able to be adjusted (e.g. expanded to include more targets), as needed, in order to increase its diagnostic capability. Moreover, as no target specific labels are added to the stock xTAG beads, these could be used in other xTAG assays as well, thus diminishing the cost of the assays.

Convinced by the results of previously published studies about the added value of bead-based multiplexed molecular testing (17 - 20) we chose to develop an *in-house* protocol for the rapid screening of the O serogroups known to most frequently include VTEC strains with epidemiological relevance. Seven VTEC relevant serogroups were targeted in the presented assay. Considering the heterogeneity of these *E. coli* serogroups, a predictive screening for two *E. coli* virulence genes was associated in order to distinguish pathogenic from non-pathogenic strains. Additionally, the *uidA* gene target was included in order to provide an opportunity if necessary to skip preliminary tests for the *E. coli* species identification.

The number of strains included in the panel used for the validation of our *in-house* protocol was small which was considered a limitation to our study. Yet, these first accurate and reproducible results obtained in less than 4 hours led us to consider the assay reliable. In terms of sensitivity, the detection limit calculated to be from 10^2 to 10^4 genomic copies for the various gene targets was considered acceptable for our needs, as the assay was intended for testing *E. coli* strains/cultures and not stool specimens. Whereas the validation could be further expanded on a higher number of clinical isolates in order to be sure of the assay specificity, the cost associated with the microspheres used in such a complex multiple detection could still be a limitation for the future use in routine diagnostic. Nevertheless, this drawback could be overcome when large batches of strains are tested, as may be the case in an outbreak.

Overall, the current approach seems to be an affordable alternative to commercially available assays that can be further exploited to improve existing autochthonous strategies to prevent future VTEC outbreaks.

Funding

This work was supported by The Research Institute of the University of Bucharest, Romania (ICUB Fellowships for Young Researchers awarded to M. O., November 2016) and by Cantacuzino National Institute of Medical-Military Research - Development, Bucharest, Romania.

Conflict of interest disclosure

None of the authors has any conflict of interest to declare.

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