Shiga toxin-producing Escherichia coli (STEC) from Humans in the Netherlands

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CHAPTER 2

Assessing the Public Health Risk of Shiga Toxin-Producing *Escherichia coli* by Use of a Rapid Diagnostic Screening Algorithm

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Keywords

culture, diagnostic algorithm, gastroenteritis, real-time multiplex PCR, STEC, virulence factors

J Clin Microbiol (2015) 53:1588 –1598.

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is an enteropathogen of public health concern because of its ability to cause serious illness and outbreaks. In this prospective study, a diagnostic screening algorithm to categorize STEC infections into risk groups was evaluated. The algorithm consists of prescreening stool specimens with real-time PCR (qPCR) for the presence of *stx* genes. The qPCR-positive stool samples were cultured in enrichment broth and again screened for *stx* genes and additional virulence factors (*escV*, *aggR*, *aat*, *bfpA*) and O serogroups (O26, O103, O104, O111, O121, O145, O157). Also, PCR-guided culture was performed with sorbitol MacConkey agar (SMAC) and CHROMagar STEC medium. The presence of virulence factors and O serogroups was used for presumptive pathotype (PT) categorization in four PT groups. The potential risk for severe disease was categorized from high risk for PT group I to low risk for PT group III, whereas PT group IV consists of unconfirmed *stx* qPCR positive samples. In total, 5,022 stool samples of patients with gastrointestinal symptoms were included. The qPCR detected *stx* genes in 1.8% of samples. Extensive screening for virulence factors and O serogroups was performed on 73 samples. After enrichment, the presence of *stx* genes was confirmed in 65 samples (89%). By culture on selective media, STEC was isolated in 36% (26/73 samples). Threshold cycle (*CT*) values for *stx* genes were significantly lower after enrichment compared to direct qPCR ($P<0.001$). In total, 11 (15%), 19 (26%), 35 (48%), and 8 (11%) samples were categorized into PT groups I, II, III, and IV, respectively. Several virulence factors (*stx*2, *stx*2a, *stx*2f, *toxB*, *eae*, *efa1*, *cif*, *espA*, *tccP*, *espP*, *nleA* and/or *nleB*, *tir* cluster) were associated with PT groups I and II, while others (*stx*1, *eaaA*, *mch* cluster, *ireA*) were associated with PT group III. Furthermore, the number of virulence factors differed between PT groups (analysis of variance, $P<0.0001$). In conclusion, a diagnostic algorithm enables fast discrimination of STEC infections associated with a high to moderate risk for severe disease (PT groups I and II) from less-virulent STEC (PT group III).

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen frequently identified as causative agent of acute diarrheal disease in humans. The outcomes of STEC infections may range from asymptomatic carriage and mild diarrhea to severe disease, such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (1–3). Based on pathogenic properties, a subgroup of STEC is also designated enterohemorrhagic *E. coli* (EHEC); this subgroup of *stx*-positive strains also contains the locus of enterocyte effacement (LEE) pathogenicity island (4). EHEC belongs to certain serotypes that are frequently associated with outbreaks and life-threatening illnesses (5). Worldwide, the most common EHEC serotype both in outbreaks and in sporadic cases of severe disease is *E. coli* O157:H7 (4, 6, 7). Consequently, public health and regulatory responses have been focused mainly on this serotype. However, due to increased surveillance with tests able to target all serotypes of STEC, evidence is accumulating that 30% to 60% of EHEC infections are caused by non-O157 strains (8, 9).

To aid in assessing the public health risks associated with STEC, an empirical seropathotype (SPT) classification of strains was proposed by Karmali et al. (5), based upon the reported frequency of STEC serotypes in human illness, their known association with outbreaks, and the severity of the outcome. Serotypes classified as SPT A (O157:H7 and O157:nonmotile [NM]) or SPT B (O26:H11/NM, O103:H2, O111:NM, O121:H19, and O145:NM) have been associated with outbreaks and severe disease; however, SPT A is more frequently reported. SPT C comprises serotypes (e.g., O91:H21, O113:H21, O5:NM, O104:H21, O121:NM, and O165:H25) that have been associated with sporadic cases of severe disease but not with outbreaks. SPT D includes STEC serotypes reported to cause sporadic disease that are associated with diarrhea but not severe disease. Serotypes included in SPT E have not been associated with human illness.

The identification of non-O157 EHEC serotypes remains challenging because of a lack of phenotypical characteristics that can distinguish these strains from less-virulent STEC serotypes and other *E. coli* that share the same environment. Furthermore, of all confirmed STEC infections in the European Union during 2007 to 2010, more than 85% of the isolates were not fully serotyped (9). As SPT classification requires fully serotyped isolates, the identification of non-O157 EHEC serotypes proves to be a major obstacle. Also, the 2011 O104:H4 EHEC outbreak has demonstrated that the emergence of new virulent strains is another limitation of the SPT classification proposed by Karmali et al., as these strains cannot be assigned to a specific SPT group (10, 11).

While it remains unclear which virulence factors (VF) precisely define STEC pathogenicity, the STEC serotypes that carry VF genes in addition to *stx* genes are more likely to be associated with HC and HUS (9, 12). These strains usually carry the LEE, a pathogenicity island (PAI) containing genes responsible for the characteristic attaching and effacing (A/E) lesions (4, 13). In addition, they can be

characterized by non-LEE-encoded effector (*nle*) genes, which are harbored on other PAIs in the bacterial chromosome (5, 14, 15), and virulence plasmids encoding EHEC-hemolysin (EHEC-*hlyA*) that are widely distributed among EHEC of different serotypes (16–18). Enteroaggregative *E. coli* (EAEC)-STEC hybrid strains of serotypes other than O104:H4, such as O111:H2, O86:NM, O59:NM, and Orough:NM, have also been associated with sporadic cases and outbreaks of HUS and (bloody) diarrhea, advocating the incorporation of EAEC virulence markers for the categorization of STEC (19–21). However, no single VF or combination of virulence factors precisely defines the potential of a STEC strain to cause more severe disease. While the *stx* subtypes *stx2a* and s*tx2c* and the LEE-positive strains are associated with a high risk of more serious illness (9, 22–24), other virulence gene combinations (even in *E. coli* strains that lack the *stx* genes) may also be associated with severe disease, including HC and HUS (12, 25–27). Furthermore, patient characteristics and infectious doses also determine the outcome of disease (28).

Although the current approaches for detecting STEC in clinical microbiology laboratories still mainly rely on a conventional culture (e.g., sorbitol MacConkey agar [SMAC] or cefixime tellurite [CT-SMAC]) and to a lesser extent on Stx toxin-based assays, a trend toward PCR-based methods for the rapid detection of STEC (stx_1 and stx_2 genes) has been observed in recent years, resulting in improved detection rates (29, 30). Enhanced detection and reporting of STEC infections have as a drawback an increased workload for community health services, and the clinical and public health relevance of PCR findings solely based on the detection of the *stx* genes is unclear (31). Therefore, diagnostic approaches that can categorize STEC while avoiding the limitations of the SPT classification of Karmali et al. are needed.

In this study, we describe a rapid screening algorithm, including both molecular and conventional methods, to determine the pathogenic potential of STEC. The aim is to discriminate infections with less-virulent STEC from those with clinical relevance and risk for public health.

MATERIALS AND METHODS

Patient specimens.

Our laboratory serves a population of about 1 million inhabitants, including both community and hospitalized patients. From September 2012 through December 2012 a total of 5022 stool samples were prospectively screened for presence of enteric bacterial, protozoan and viral pathogens. The samples originated from patients (n=4714) with infectious gastroenteritis (IG) included in their differential diagnosis. Their mean age was 39 years (range, 0 to 101 years) and 1985 (42.1%) patients were males. Clinical information addressing symptoms, use of antibiotics, and travelling history were obtained from the request form filled out by physicians. On receipt, all stool specimens were

routinely examined by molecular methods (real-time PCR [qPCR]) for the presence of *Campylobacter jejuni*, *Salmonella enterica*, Shiga toxin-producing *Escherichia coli* (STEC), *Shigella* spp., enteroinvasive *E. coli* (EIEC), *Cryptosporidium parvum, C. hominis*, *Dientamoeba fragilis, Giardia lamblia*, and *Entamoeba histolytica*. Upon specific request of physicians, examination for the presence of adenovirus (EIA), rotavirus (EIA), norovirus (qPCR), and *Clostridium difficile* toxins A and B (EIA) was also performed.

Design of the diagnostic algorithm and STEC risk assessment.

The algorithm consists of qPCR for detection of the *stx* genes (stx_1 and stx_2) on stool samples, as described previously (30). qPCR *stx*-negative stool samples were regarded as STEC negative. In case of a *stx*-qPCR positive result, the stool sample was enriched in brilliant green bile (BGB) broth followed by DNA extraction, and multiplex qPCR for the detection of VF (stx_1, stx_2, stx_{2f}, *escV*, *aggR* and *aat* genes), and O-serogroup determination (wzx_{O26}, wzx_{O103}, wzx_{O104}, wbd_{O111}, wzx_{O121}, ihp_{O145} and rfb_{O157}). In order to obtain an isolate, qPCR positive samples were cultured directly and after enrichment on STEC selective media. Virulence determinants and O-serogroups were confirmed by qPCR on suspicious colonies (or streaks) grown on STEC selective media, and by seroagglutination. Attempts to obtain an isolate were made up to a maximum of 5 colonies per agar plate. A schematic overview of the diagnostic algorithm is presented in Figure 1.

The risk assessment of STEC infections was performed using a molecular approach, as described previously (9). It delivers a scheme that describes the presumptive categorization of STEC according to their potential risk, using the presence of genes encoding VF additional to the presence of the *stx* genes. Categorization of *stx* PCR positive samples is based upon the presence of the VF *escV* (LEE-positive), and/or *aggR/aat* (pAA-positive) and on detection of O-serogroups that are most frequently associated with severe human disease and outbreaks, e.g. O26, O103, O104, O111, O121, O145, and O157. The potential risk for diarrhea and severe disease has been categorized as pathotype (PT) group I (high risk for diarrhea and severe disease) to PT group III (moderate risk for diarrhea/low risk for severe disease), while PT group IV consists of *stx* PCR positive samples that are not confirmed after enrichment (Table 1). In case an isolate was obtained and fully serotyped, a classification of the STEC isolate into a seropathotype (SPT) was made as described previously (5). *Stx* subtyping and genetic characterization of cultured isolates was performed in order to confirm the validity of the proposed molecular-based PT approach for risk assessment of STEC infections.

PCR guided culture.

For culture of STEC selective media SMAC and CHROMagar STEC (CHROMagar Microbiology, Paris, France) were used (24h at 35°C), directly and after enrichment in BGB broth for approximately 16h at 35°C. Identification of STEC and/or EHEC O157- suspicious colonies (non-sorbitol fermenting colonies on SMAC and mauve non-fluorescent colonies on CHROMagar STEC) and STEC/EHEC non-O157 (mauve fluorescent colonies on CHROMagar STEC) was carried out by detection of virulence genes and serogenotyping with qPCR, performing an indole reaction and serological typing (serogroup O157 only). All genotypically/biochemically identified *E. coli* were confirmed using the VITEK 2 system (bioMérieux, Boxtel, The Netherlands). All culture and identification media were produced by Mediaproducts BV, Groningen, The Netherlands, whereas the *E. coli* O157 agglutination serum was from Oxoid, Basingstoke, Hampshire, England. Resistance profiling was performed with the VITEK 2 system. Furthermore, of qPCR positive samples with Ct<35, five *E. coli* colonies cultured on SMAC agar were sub-cultured and sent to the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands) for genotying (*stx*$_1$, *stx*$_2$, *stx*$_{2f}$, *eae*, EHEC-*hlyA*, and O157) and O:H-serotyping of isolates, as part of STEC national surveillance.

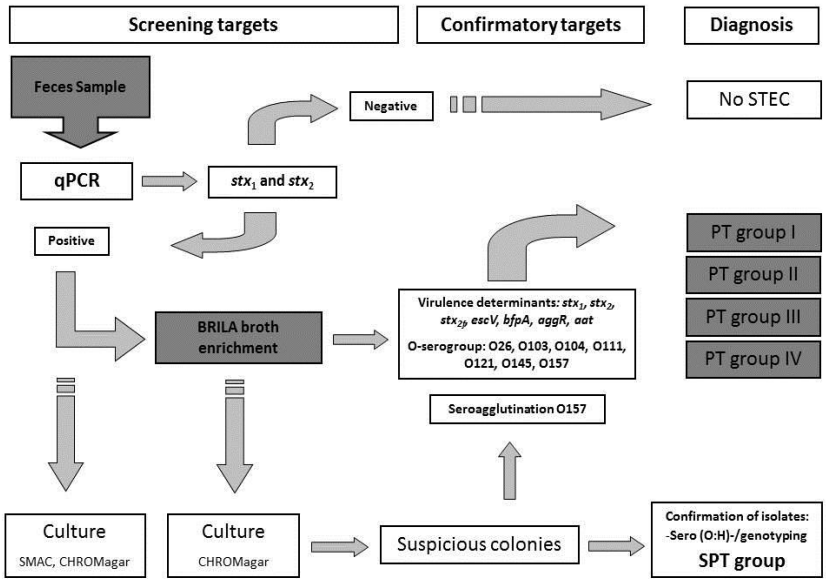

Figure 1 The STEC diagnostic algorithm consists of both molecular and conventional methods; when *stx* genes are detected with direct qPCR, the stool sample is enriched. DNA is isolated from the enriched broth and screened for the presence of *stx* genes and additional VF and O serogroups. VF and O serogroups are confirmed by qPCR on suspicious colonies grown on selective media and by seroagglutination. STEC isolates were fully serotyped (O:H typing) at the RIVM.

Table 1. Proposed molecular approach for the presumptive categorization of STEC based on enriched BGB PCR results

PT group	Direct PCR *stx* genes present	Enriched BGB PCR *stx* genes present	Additional genes[a]	Serogroups	Potential risk	
					Diarrhea	HUS/HC
I	Yes	Yes	*escV*-positive or *aggR and/or aat* positive	O26, O103, O104, O111, O121, O145, O157	High	High
II	Yes	Yes	*escV*-positive or *aggR and/or aat* positive	Any other serogroup	High	Moderate
III	Yes	Yes	*escV* negative and *aggR* and/or *aa t* negative	Any serogroup	Moderate	Low
IV	Yes	No	NA	NA	NA	NA

NA; not applicable

[a] *escV* gene, marker for presence of the LEE PAI; *aggR*/*aat* genes, markers for the presence of the pAA plasmid carried by EAEC; NA, not applicable.

Molecular assays

(i) ***Specimen preparation and DNA extraction***

Specimen preparation followed by DNA extraction using the automated NucliSens easyMAG (bioMérieux, Boxtel, The Netherlands) according to the manufacturer's instructions was performed as previously described (30). Briefly, for DNA extraction from stool, 100 µl fecal suspension and 50 µl of enriched selenite broth was used as input. For DNA extraction from enriched BGB broth, 100 µl was used as input. In addition, approximately 6000 copies of the Phocine herpes virus 1 (PhHV), which served as an internal control (IC), were co-purified. DNA was eluted in 110 µl of elution buffer. For confirmation of suspicious colonies by qPCR, DNA from isolates was extracted by heat lysis for 10 min at 95°C in NucliSens easyMAG elution buffer. For genetic characterization by microarray, the DNA extraction was performed using a DNeasy blood and tissue kit (QIAGEN, GmbH Germany) from the overnight culture of the pure isolates according to the manufacturer's instructions.

(ii) ***Real-time PCR***

Real-time amplification was carried out on an AB 7500 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) as described previously (30). Each 25 µl reaction consisted of 5 µl template DNA, 1x TaqMan Universal PCR Master Mix, and 2.5 µg bovine serum albumin (Roche Diagnostics Netherlands B.V., Almere, The Netherlands). The primers and probes used for detection of virulence determinants and O-serogroup specific gene targets are listed

in the supplementary table S1. Reactions were run under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. In every PCR run a negative extraction control (NEC) and positive extraction control (PEC) or PCR mix control (PMC) was included. A real-time PCR was considered inhibited when the *Ct* value for the PhHV exceeded the mean *Ct* value for uninhibited specimens + 2 standard deviations.

(iii) *Stx subtyping*

DNA isolates of the BGB broth and/or confirmed STEC isolates were sent to the University Medical Center Groningen (UMCG, Groningen, The Netherlands) for *stx* subtyping. Subtyping of the stx_1 and stx_2 gene was performed as described previously (32). Briefly, for stx_1 subtyping, a triplex PCR was performed, each 25 µl reaction consisted of 2.5 µl of PCR buffer 10x (Qiagen), 1 µl of $MgCl_2$ 25 mM (Qiagen), 0.5 µl of 10 mM dNTP mix (Applied Biosystems), 0.25 µl Hotstar Polymerase 5U/µl (Qiagen), 2 µl of each of two primers for stx_{1a}, 1 µl of each of the four primers for stx_{1c} and stx_{1d} (stock solution of all primers was 5 µM), and 5 µl of template DNA.

For stx_2 subtyping PCR, each 20 µl reaction consisted of 2.5 µl of PCR buffer 10x (Qiagen), 0.8 µl of MgCl2 25 mM (Qiagen), 0.4 µl of 10 mM dNTP mix (Applied Biosystem), 0.2 µl Hotstar Polymerase 5U/µl (Qiagen), 1.25 µl of each of the primers and 5 µl of template DNA. Stx_{2c} and stx_{2e} subtyping PCR was performed as a duplex PCR as well as stx_{2f} and stx_{2g}. Reactions were run under the following conditions: 95°C for 15 min followed by 35 cycles of 94°C for 50 s, 64°C (hybridization was at 66°C for stx_{2d}) for 40 s and 72°C for 60 s, with a final extension at 72°C for 3 min.

(iv) *Genetic characterization by DNA microarray*

Confirmed STEC isolates were sent to the University Medical Centre Groningen for genetic characterization using an *E. coli* genotyping combined assay kit according to the manufacturer's protocol (Clondiag, Alere Technologies, GmbH, Jena, Germany). The *E. coli* oligonucleotide arraystrips contain gene targets for the identification of virulence genes, antimicrobial resistance genes and DNA-based serotyping genes. Briefly, multiplex linear DNA amplification and labeling was performed in a total volume of 10 µl containing 3.9 µl of 2x labeling Buffer, 1 µl of *E. coli* labeling primer mix, 0.1 µl of DNA Polymerase and 5 µl of genomic DNA (100-200 ng/µl). Reactions were run under the following conditions: 96°C for 5 min followed by 45 cycles of 50°C for 20 s, 72°C for 30 s and 96°C for 20 s.

The hybridization and washing steps were performed using the Hybridization plus kit according to the manufacturer's protocol. Visualization of hybridization was achieved using the ArrayMate instrument (CLONDIAG GmbH, Jena, Germany) and signals of the array spots were analyzed automatically. Ambiguous called signals were re-checked visually in order to obtain a definite interpretation when possible. In case any signal remained inconclusive, they were regarded as negative.

Statistical analysis.

We used the Fisher exact method to test if the presence or absence of VF was associated to certain PT groups and whether growth of suspicious colonies on CHROMagar STEC was associated with presence of the *escV* gene (LEE-positive) (JavaStat). Median *Ct* values of subgroups were compared using the Wilcoxon rank sum test with NCSS version 2007 (NCSS statistical software, Kaysville, UT, USA). One-way analysis of variance (ANOVA) was used to compare the total number of VF present in isolated strains that were assigned to PT groups. For all tests statistical significance was indicated by a two-tailed $p<0.05$.

Furthermore, cluster analysis of VF with construction of dendrograms was performed with Bionumerics version 4.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) using the Dice correlation and the unweighted-pair group method using average linkages (UPGMA).

RESULTS

Detection frequency of *stx* genes in patient specimens.

A total of 5,022 stool specimens from 4,714 patients were examined, using direct qPCR for detection of the *stx* genes. In total, 90 samples (84 patients) were positive for the *stx* genes (1.8%). The diagnostic algorithm was applied on all samples, but for only 73 samples (70 patients) all screening data were available; therefore the remaining 17 samples were excluded for analysis. Direct qPCR for the *stx* genes was confirmed by qPCR on "enriched BGB" in 65 samples (89%). In the remaining 8 samples (11%) no *stx* genes could be detected after broth enrichment, although in one sample the virulence factors *aggR/aat/escV* and O104 serogroup were detected. These 8 samples initially had a relatively high Ct-value (Ct $\geq$34) in the direct qPCR.

The *stx* ΔCt values for "enriched BGB" PCR and direct qPCR ($\Delta Ct = Ct_{BGB} - Ct_{direct}$) ranged from Ct +9 to –21. In 55/65 (85%) of the "enriched BGB" samples the $\Delta Ct \leq 0$, indicative for the presence of viable STEC; Ct values of "enriched BGB" PCR (mean Ct value = 23.1) were significantly lower compared to Ct values of direct qPCR (mean Ct value = 29.6) (Wilcoxon rank sum, $p<0.001$) (Figure 2A).

The additional virulence genes *escV*, *aggR*/*aat* and *bfpA* were detected in 49% (n=36), 6% (n=4), and 6% (n=4) of qPCR positive samples, respectively. The O145, O26, O157, O104, O121 and O111 serogroups were detected in 11.0% (n=8), 8% (n=6), 4% (n=3), 3% (n=2), 3% (n=2), and 1% (n=1) of qPCR positive samples, respectively (Table 2).

PCR guided culture.

The PCR guided culture yielded a positive result in 42.5% (31/73) of direct qPCR positive samples. A STEC isolate was obtained in 35.6% (26/73) of the samples; from one sample two STEC isolates were

obtained. Two additional samples (3%) were streak PCR positive for *stx* genes. The serotypes that were identified are listed in Table 2. Using the Karmali seropathotype concept, one STEC isolate (O157:H7) could be assigned to SPT group A, six STEC isolates (4x O26:H11; 2x O145:NM) to SPT group B, and two STEC isolates (O117:H7 and O146:H21) to SPT group D. The other 17 STEC isolates (65.4%) could not be assigned to a SPT group. One isolate and one streak PCR positive sample could not be serotyped.

From the remaining three culture positive samples an enteropathogenic *E. coli* (EPEC) (n=2; O88:H25 and ONT:H31) or EAEC (n=1; O104:H4) was isolated. The isolation yield of the SMAC medium was higher (21/73) compared to the CHROMagar STEC medium (15/73), although 5 isolates and one streak PCR positive sample were only identified with CHROMagar STEC. Furthermore, the growth of suspicious colonies on CHROMagar STEC was highly associated with presence of the *escV* gene (LEE-positive) detected by the "enriched BGB" PCR (19/23 vs 17/50) (Fisher exact; $p<0.0001$).

Ct values (stx_1/stx_2) of samples in which the PCR guided culture remained negative were significantly higher than in samples with positive guided culture (Wilcoxon rank sum, $p<0.0003$). This difference in Ct value between the PCR guided culture negative and positive group remained significant, when comparing Ct values of enriched BGB PCR (Wilcoxon rank sum, $p<0.001$). The distribution of Ct values of enriched BGB PCR on which guided culture was performed are shown in Figure 2B.

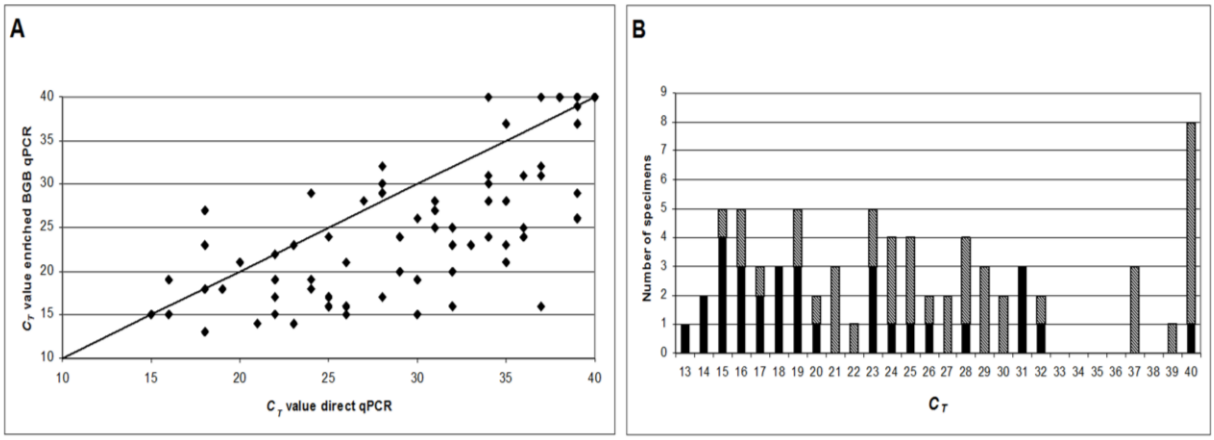

Figure 2. Direct comparison of *stx CT* values for direct qPCR versus *stx CT* values for enriched BGB PCR (A). The solid line represents the hypothetical identical performance between both methods. The s*tx CT* values for enriched BGB PCR were significantly lower (Wilcoxon rank sum, *P*0.001). Distribution of *CT* values for STEC isolates that were positive according to enriched BGB qPCR (B). The black bars represent the number of stool specimens positive in the PCR-guided culture. The dashed bars represent the additional qPCR-positive stool specimens.

Table 2. Overall results of risk categorization of STEC-positive stool samples

No. of samples by PT group	qPCR		Stx subtyping	Serotyping of cultured isolates[a]	Total no. of virulence factors by DNA array
	Serogenotype	Additional virulence factors			
I (n=11)					
1	O157	*escV*	*stx*$_{1a}$ + *stx*$_{2c}$	O157:H7	32
1	O157	*escV, aggR/aat*	*stx*$_{2c}$	Not cultured	
1	O26	*escV*	*stx*$_{1a}$ + *stx*$_{2b}$ + *stx*$_{2c}$	O26:H11	35
1	O26	*escV*	*stx*$_{2a}$	O26:H11	31
2	O26	*escV* (n=2)	*stx*$_{1a}$	O26:H11	29/29
1	O26 / O121 / O145	*escV*	*stx*$_{2b}$ + *stx*$_{2c}$	O26 (streak)[b]	-
1	O145	*escV*	*stx*$_{1a}$ + *stx*$_{2a}$	O145:NM	28
1	O145	*escV*	*stx*$_{2}$ not typable	O145:NM[c]	34
1	O157 / O26 / O145	*escV*	*stx*$_{2a}$ + *stx*$_{2d}$ + *stx*$_{2e}$	Not cultured	-
1	O121 / O111 / O145	*escV*	*stx*$_{1a}$	Not cultured	-
II (n=19)					
2		*escV* (n=2)	*stx*$_{1a}$ + *stx*$_{2a}$	O165:NM	33
				O182:H25	25
4		*escV* (n=4), *aggR/aat* (n=1)	*stx*$_{1a}$	Not cultured	-
1		*escV*	*stx*$_{2a}$	O182:H25	27
1		*escV*	*stx*$_{2a}$ + *stx*$_{2c}$	Not typed[d]	33
1		*escV*	*stx*$_{2c}$	Not cultured	-
1		*escV*	*stx*$_{2f}$	O63:H6 (n=1)	18
1		*escV*	*stx*$_{2f}$	O125:H6 (n=1)	16
1		*escV, bfpA*	*stx*$_{2f}$	O88:H25 (EPEC)[e]	15
6		*escV* (n=6)	*stx*$_{2f}$	Not cultured	-
1		*escV*	*stx*$_{1c}$	Not cultured	
III (n=35)					
3		*escV* (n=1), *bfpA* (n=1)	*stx*$_{1a}$	O91:NM (n=3)[f]	9/9/10
2			*stx*$_{1a}$	O91:H14 (n=2)	9/11
1			*stx*$_{1a}$	ONT:NM	8
1		*escV*	*stx*$_{1a}$	O117:H7[f]	4
1			*stx*$_{1a}$	Culture positive (streak)	-
11	O145 (n=1)		*stx*$_{1a}$	Not cultured	-
1		*escV, bfpA*	*stx*$_{1c}$ + *stx*$_{2b}$	O128:H2[f]	17
1			*stx*$_{1c}$ + *stx*$_{2b}$	O76:H19	20
1		*aggR/aat*	*stx*$_{1c}$ + *stx*$_{2b}$	O146:H21[f]	19
1			*stx*$_{1c}$ + *stx*$_{2b}$	Not cultured	-
1			*stx*$_{2b}$	O7:H6	2
1			*stx*$_{2b}$	ONT:H31 (aEPEC)[e]	12
1			*stx*$_{2b}$	Not cultured	-
2			*stx*$_{2c}$	ONT:H28 (n=1)	11
1			*stx*$_{2d}$	Not cultured	
1			*stx*$_{2e}$	Not cultured	
1			*stx*$_{1c}$	Not cultured	
1			*stx*$_{1}$ not typable	O16:H5	13
3	O145 (n=1)	*escV, bfpA* (n=1)	Not typable	Not cultured	
IV (n=8)					
1	O104	*escV, aggR and/or aat*	Not typed	O104:H4 (EAEC)[e]	10
7	O145/O104 (n=1)		Not typed	Not cultured	-

[a] NM, non-motile; ONT, O-serogroup O1 to O187 negative.
[b] PCR-positive culture by screening DNA isolated from a loopful of bacterial growth of the first streaking area of culture plates.
[c] Coinfection with an ONT:H45 (*stx*2f positive) isolate. This isolate was not genetically characterized.
[d] The isolate could not be recultured after transportation to the RIVM for genotyping/serotyping.
[e] Isolates did not contain *stx* genes and were designated EPEC (O88:H25; *escV*, *bfpA*), atypical EPEC (aEPEC [ONT:H31; *escV*]), and EAEC (O104:H4; *aggR* and/or *aat*).
[f] The isolates did not contain the additional virulence factors *escV* or *aggR* and/or *aat*.

***Stx* subtyping of clinical samples.**

Subtyping of *stx* genes was performed on DNA isolates of enriched BGB broths that were PCR positive for *stx* genes. Of these 65 positive samples, 30 (46%) were stx_1 positive, 26 (40%) were stx_2 positive, and 9 (14%) were stx_1 and stx_2 positive. Two stx_1 subtypes (stx_{1a} and stx_{1c}) and six stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2f}) were detected with a total of 15 different stx_1 and stx_2 subtype combinations. The most frequently detected subtype variants were stx_{1a} (40%), stx_{2f} (14%), stx_{1c} + stx_{2b} (6%), stx_{2c} (6%), stx_{2b} (5%), and stx_{1a} + stx_{2a} (5%), accounting for 49 samples (75%). For three samples subtyping results remained negative, although the DNA load seemed to be sufficient. For two of these samples subtyping remained negative after DNA isolation from the obtained STEC isolate. For an additional three samples no *stx* subtype could be obtained due to low DNA load (all Ct ≥32 in enriched BGB PCR).

Risk categorization of STEC and distribution of virulence factors between PT groups.

Samples were presumptively categorized in four pathotype (PT) groups based on the "enriched BGB" PCR results. A total of 11 samples (15%), 24 samples (33%), 30 samples (41%), and 8 samples (11%) were categorized in PT group I, group II, group III, and group IV respectively. However, based on the presence of the additional virulence factor *bfpA* and screening of VF in the cultured isolates, a total of 5 samples (7%) were re-categorized from PT group II to PT group III; 4 STEC isolates did not contain the *escV* gene (O91:NM, O117:H7, O128:H2 and ONT:H31) and in one sample there was no correlation in *Ct* value for *stx* (*Ct*=39) and the other VF *escV* (*Ct*=19) and *bfpA* (*Ct*=18). The final risk categorization was 11 samples (15%), 19 samples (26%), 35 samples (48%), and 8 samples (11%) for PT group I, group II, group III, and group IV, respectively (Table 2). The presence of *stx* genes for samples categorized in PT group IV could not be confirmed after enrichment, thereby excluding them from further analysis.

The studied virulence factors (VF) differed with respect to their distribution among the different pathotype groups (Table 3). Compared to the PT group that is associated with a moderate risk for diarrhea and low risk for severe disease (III), PT groups that are associated with a high risk for diarrhea and higher risk for severe disease (I + II, combined) exhibited a significant higher prevalence of various VF analyzed (specifically, stx_2, stx_{2a}, stx_{2f}, *toxB*, *eae*, *efa1*, *cif*, *espA*, *tccP*, *espP*, *nleA/B*, and the *tir* cluster). Although not significant, stx_{2c} was more prevalent in PT group I + II (OR 4.1 [95%CI = 0.7 – 32.7]). Inversely, stx_1, the *mch* cluster, *ireA* and *eaaA* were significantly more prevalent in PT group III (Table 3). Interestingly, the adhesion-encoding gene *iha* was present in all PT group I isolates and almost all isolates in PT group III (92%), however there was no significant association between

presence of *iha* and PT groups. Furthermore, all LEE-positive STEC isolates contained the EHEC-*hlyA* gene, with exception of the two stx_{2f} STEC isolates. Noteworthy, certain VF were also highly associated with PT group I (specifically, stx_{2c}, *toxB*, *eae*, *efa1*, *cif*, *tccP*, *nleA* and/or *nleB*, *katP* and the *tir* cluster).

The total number of VF present in STEC isolates also showed a significant non-random distribution between PT groups (Table 2); the number of VF differed significantly between PT group I (VF_{mean} 31 [95%CI = 27 – 35], PT group II (VF_{mean} 25 [95%CI = 21 – 30]), and PT group III (VF_{mean} 11 [95%CI = 8 – 13]) (ANOVA, $p<0.0001$, F = 38.5). Interestingly, the total number of VF present in the two cultured stx_{2f} STEC isolates (O63:H6, and O125:H6) that were categorized in PT group II, were considerably lower compared to other 4 STEC isolates categorized in this PT group (Table 2). By cluster analysis of potential VF, *escV*-positive and *escV*-negative isolates were separated into two main clusters (Figure 3). The *escV*-negative cluster included all PT group III isolates and a *stx*-negative isolate EAEC O104:H4 (PT group IV). The *escV*-positive cluster included all PT group I + II isolates and two *stx*-negative EPEC isolates (O88:H25 and ONT:H31) that clustered in a distinct branch with the two stx_{2f} positive STEC isolates.

Clinical symptoms of patients.

Diarrhea was reported by 80%, 44%, 57%, and 75% of patients in PT groups I, II, III, and IV, respectively. Bloody diarrhea was reported by 20%, 6%, 3%, and 0% of patients in PT groups I, II, III, and IV, respectively. Patients in PT group I presented significantly more often with (bloody) diarrhea compared to PT groups II + III (Fisher exact test, $P = 0.006$). One patient categorized in PT group I developed HC (serotype O26:H11), and family members of another patient categorized in PT group I (serotype O26:H11) also had gastrointestinal complaints. Interestingly, symptoms reported by patients that are not associated with acute disease, such as persistent diarrhea and/or abdominal complaints without loose stools, were absent in patients categorized in PT group I (0%) but present in patients in PT groups II, III, and IV (33%, 29%, and 25%, respectively). The age distribution of patients did not differ between PT groups (PT group I: mean age = 27 years; 95% CI = 10 to 44 years; PT group II: mean age = 36 years; 95% CI = 23 to 48 years; PT group III: mean age = 41 years; 95% CI = 32 to 50 years) (ANOVA, $P = 0.33$; $F = 1.1$), although the median age of patients in PT group I was considerably lower (15 years) compared to PT group II (33 years) and PT group III (39 years).

Table 3. Pathotype distribution of virulence factors and *stx* subtypes

Virulence genotype	Total no. (%)	No. (%)[a] of isolates or enriched BGB broths for PT: I	II	III	Statistical comparison of PT I + PT II vs PT III[b] P^c	OR (95% CI)
Enriched BGB broths (n=65)						
stx_1 (all)	39 (60)	6 (55)	8 (42)	25 (71)	0.021	0.3 (0.08 – 0.9)
stx_{1a}	31 (48)	6 (55)	7 (37)	18 (51)		
stx_{1c}	6 (9)	0	1 (5)	5 (14)		
stx_2 (all)	35 (54)	8 (73)	14 (74)	13 (37)	0.006	4.7 (1.4 – 15.6)
stx_{2a}	7 (11)	3 (27)	4 (21)	0	0.003	∞(1,7 – inf)
stx_{2b}	9 (14)	2 (18)	0	7 (20)		
stx_{2c}	8 (12)	4 (36)	2 (11)	2 (6)		4.1 (0.7 – 32.7)
stx_{2d}	2 (3)	1 (9)	0	1 (3)		
stx_{2e}	2 (3)	1 (9)	0	1 (3)		
stx_{2f}	9 (14)	0	9 (47)	0	< 0.0001	∞ (2.5 – inf)
stx_{2a} or stx_{2c}	14 (22)	7 (64)	5 (26)	2 (6)	0.002	11.0 (2.0 – 80.5)
Isolates (n=26)						
astA	7 (27)	3 (43)	3 (50)	1 (8)		
EHEC-*hlyA*	20 (77)	7 (100)	4 (67)	9 (69)		
toxB	7 (27)	6 (86)	1 (17)	0	0.005	∞ (1.9 – inf)
mch cluster	9 (35)	0	0	9 (69)	< 0.0001	< 0.0001 (0 – 0.3)
ireA	8 (31)	0	0	8 (62)	0.002	< 0.0001 (0 – 0.4)
eae	13 (50)	7 (100)	6 (100)	0	< 0.0001	∞ (18.4 – inf)
efa	7 (27)	6 (86)	1 (17)	0	0.005	∞ (1.9 – inf)
iha	20 (77)	7 (100)	1 (17)	12 (92)		
lpfA	16 (62)	3 (43)	3 (50)	8 (62)		
iss	15 (58)	5 (71)	0	10 (77)		
cif	9 (35)	6 (86)	3 (50)	0	< 0.0001	∞ (3.5 – inf)
espA	17 (65)	6 (86)	6 (100)	5 (38)	0.011	19.2 (1.5 – 537.4)
tccP	12 (46)	7 (100)	5 (83)	0	< 0.0001	∞ (10.4 – inf)
eaaA	10 (39)	0	0	10 (77)	< 0.0001	< 0.0001 (0 – 0.2)
espP	11 (42)	5 (71)	4 (67)	2 (15)	0.015	12.4 (1.4 – 142.1)
nleA/B	12 (46)	7 (100)	5 (83)	0	< 0.0001	∞ (10.4 – inf)
etpD	4 (15)	2 (29)	2 (33)	0		
katP	7 (27)	5 (71)	1 (17)	1 (8)		
tir cluster	10 (39)	6 (86)	4 (67)	0	< 0.0001	∞ (4.8 – inf)

[a] Total no. of enriched BGB broths: PT I, 11; PT II, 19; PT III, 35. Total no. of isolates: PT I, 7; PT II, 6; PT III, 13.
[b] PT I and II combined (associated with high risk for diarrhea and high/moderate risk for severe disease) are compared to PT III (lower risk for diarrhea and severe disease).
∞, infinite.
[c] *P* values (from the Fisher exact test) are shown only if the *P* value was <0.05.

Figure 3. Cluster analysis of potential virulence genes in cultured strains. The *escV*-negative cluster included all PT group III isolates and an *stx*-negative isolate, EAEC O104:H4 (PT group IV). The *escV*-positive cluster included all PT groups I_II isolates and two *stx*-negative EPEC isolates (O88:H25 and ONT:H31) that clustered in a distinct branch with the two *stx*2f-positive STEC isolates. Spots depicted in gray represent ambiguous results that were regarded as negative.

DISCUSSION

We here present the first prospective study that uses a diagnostic algorithm directly applied on stool samples of patients presenting with gastrointestinal complaints to assess the public health risk of STEC. Although the disease severity and incidence of STEC is not solely based on the pathogenic potential of the organism but also on host-associated and environmental factors, enough

information has accumulated that the presence of virulence factors (VF) carried additional to the *stx* genes varies considerably between STEC strains, and could therefore be used to categorize the potential risk of STEC (5, 17, 33-36).

The detection frequency of the *stx* genes observed in this study (1.8%) was comparable with previous studies performed in The Netherlands (30, 37). The diagnostic algorithm enabled categorization of STEC infections into 4 pathotype (PT) groups. The majority of the initial *stx*-PCR positive samples (48%) were categorized in PT group III, while 15% and 26% of *stx*-PCR positive samples were categorized in PT group I and PT group II, respectively, both having a high risk for diarrhea and moderate to high risk for severe disease. The presence of *stx* genes could not be confirmed after enrichment in 11% of samples and these were categorized in PT group IV. *Stx* subtyping and genetic characterization was performed in order to confirm the validity of the proposed categorization of STEC infections.

Previous studies have indicated that the subtype of shiga toxin produced may influence the clinical outcome of STEC infections (23, 24). STEC harboring stx_{2a} or stx_{2c} are associated with HUS and bloody diarrhea, while strains carrying stx_{1c} or stx_{2b} have often been isolated from patients with milder infections (38). Although STEC carrying stx_{2d} usually predict a milder disease, strains that produce elastase-activatable Stx2d may predict a severe clinical outcome of the infection (39). Other variants, such as stx_{2e} and stx_{2f}, have been associated with animals and are rarely isolated from humans (24, 40).

In our study, there was a strong association between presence of stx_2, in particular stx_{2a} or stx_{2c}, and samples categorized in PT group I + II (LEE-positive), while the presence of the stx_1 gene was associated with samples categorized in PT group III. Similar to a previous study performed in Belgium, stx_{1a} was the most detected subtype (41). Furthermore, the detection frequency of the stx_{2f} gene in our study (12.3%) was comparable with previous studies (41, 42). Stx_{2f} was, together with stx_{2b}, the first-most detected stx_2 subtype among samples that were serogroup O157 PCR negative in this study. Similar to previous studies, all stx_{2f} PCR positive samples also contained the *escV* gene (LEE-positive) (41-43). With exception of stx_{2b}, the detection frequencies of subtypes stx_{1c} (9%) and stx_{2e} (3%) that are associated with milder disease or asymptomatic carriage were similar to the incidence detected in Belgium (41).

Furthermore, cluster analysis of VF clearly showed a separation into an *escV*-negative (PT group III) and *escV*-positive cluster (PT group I + II) with a significant difference in the number of “accessory” virulence factors (VF) present between these PT groups. Furthermore, VF that play an important role in toxin production, and attachment to host cells, were highly associated with PT group I + II or PT group I alone, while other VF were associated with PT group III. Previous studies also reported that

the number of VF present in STEC isolates increases the pathogenic potential of STEC and the strong association of certain "accessory" VF and severe illness and outbreaks (12, 17, 22, 27, 33, 34, 36, 44). Interestingly, the accessory virulence gene content of both the stx_{2f} STEC positive isolates, that clustered in a distinct branch with two *stx*-negative EPEC isolates, was lower compared to the other STEC isolates categorized in PT group II. Others have also reported that stx_{2f} STEC form a distinct group within STEC with regard to virulence genes and their association with a relatively mild disease (41, 42).

Our findings with respect to the main clinical features of STEC infection are consistent with those of others (9, 40). Patients with STEC infections categorized in PT group I presented significantly more often with (bloody) diarrhea, suggesting that the pathogenic potential of STEC in this group is higher compared to STEC categorized in PT group II + III, as was confirmed by *stx* subtyping and genetic characterization. Although there was no clear association between patient age and PT groups in our study, the age distribution of patients in PT group I was considerably lower in comparison to patients categorized in PT group II and PT group III. Others did also report a close relation between patient's young age and infection with more virulent (LEE-positive) STEC strains (40). Furthermore, this study revealed a high number (45%) of other enteric pathogens detected in individual *stx*-PCR positive samples (data not shown). However, the clinical relevance of these mixed infections was beyond the scope of this study.

Although *stx* subtyping and genetic characterization confirmed the validity of the PT classification, categorization with this molecular-based PT approach should be regarded as presumptive. Additional subtyping of *stx* genes, genetic characterization and O:H-serotyping of STEC isolates will provide a clearer assessment of the potential public health risk. Hence, a high culture yield remains important for facilitating these laboratory procedures.

An important step in the diagnostic algorithm is the use of an enrichment step, which was performed on the initial *stx* PCR positive stool samples. Performing this step has several advantages. First, confirmation by performing PCR on the enrichment broth increases the positive predictive value for detection of STEC; 89% of the initial *stx* PCR positive results could be confirmed. In the majority of the samples (85%) the *stx* Ct values were lower after enrichment, which suggests the presence of viable STEC. In a part of the samples (11%) no *stx* genes could be detected after enrichment (PT group IV). The *stx* Ct values for direct qPCR were relatively high in all these samples, suggestive for presence of low loads of non-viable STEC or free *stx* DNA. Another possibility would be detection of free *stx* phages in the stool of these patients, which has been described previously in stool of healthy individuals (45).

Second, *stx* subtyping was performed directly from the DNA isolate obtained from the enriched broth. To our knowledge, subtyping of *stx* genes is only being performed on STEC isolates, which will take additional time for obtaining final subtyping results. Third, although not statistically proven, culture yield will improve using enrichment; in the majority of the initial *stx* PCR positive samples the "*stx* gene" load increased after enrichment, suggestive for the presence of viable STEC. Higher amounts of STEC bacteria in the background of intestinal flora will increase the odds of isolation by culture. The culture yield in our study (38%) was lower compared to other studies (24, 41). However, the amount of colonies screened with PCR (maximum of 10) in our study was considerably lower compared to those studies. Hence, increasing the total amount of colonies screened, and routinely screening DNA isolated from a loopful of bacteria growth from the first streaking area of culture plates (as was performed for two samples in this study) would increase the probability of obtaining an isolate or at least confirm the growth of STEC.

Furthermore, due to more easily identification of suspicious colonies the CHROMagar STEC medium proved to be an effective supplemental medium for isolation of especially more virulent (LEE-positive) STEC serotypes as described previously by others (46-48). The medium also supported the growth of EAEC (O104:H4) and EPEC (O88:H25, and ONT:H31), suggesting that it could also be an useful tool for support of EAEC and EPEC isolation as described previously (49).

Unfortunately, our diagnostic algorithm only includes direct molecular screening for the *stx* genes, rendering the detection of shiga toxin lost (STL) EHEC impossible (25, 26, 50). Another limitation of this study was the concise amount of clinical information that was available on request forms that may have influenced clinical associations. Furthermore, the number of STEC isolates that were characterized was limited.

In conclusion, the proposed diagnostic algorithm for risk categorization of STEC infection offers a rapid testing format that could be easily implemented in laboratories that already perform qPCR-based detection of STEC. It enables *stx*-PCR positive stool samples to be categorized for the potential risk to public health. This risk assessment may provide valuable information to aid community health services in estimating the level of action required (with regard to source/contact tracing, and intervention measures to minimize secondary transmission) to address the potential threat, as well as a useful tool for public health surveillance. However, the proposed risk categorization should be regarded as presumptive and interpreted with care as infections with STEC serotypes categorized in PT group III, such as O117:H7, can still pose a public health concern as has been shown recently (51). Currently, a multicenter prospective cohort study is being conducted that will verify the performance of the proposed molecular-based pathotyping approach on a larger scale, in order to justify its

application in case of STEC infections for determining if swift action by community health services is warranted or not.

ACKNOWLEDGEMENTS

The authors would like to thank Alex W. Friedrich (Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands) for critically reviewing this manuscript and his helpful suggestions. We also thank Evert van Zanten (Certe Laboratory for Infectious Disease, Department of Research & Development, Groningen, The Netherlands) for help with construction of dendrograms for cluster analysis of VF.

This study formed part of a larger multidisciplinary research initiative called STEC-ID-net.

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Supplementary Materials

Supplementary Table S1. Primer and probe sequences used for five real-time (multiplex) qPCR reactions.

Target	Multiplex reaction	Sequence name	Sequence (5' – 3')[a, b]	Tm (°C)	Conc (nM)	Reference
stx$_1$	A	stx1F934_mod	TGG CAT TAA TAC TGA ATT GTC ATC ATC	59.2	300	
		stx1F934F_mod1d	TGG CAT TAA TAT TAA ATT GCC ATC AT	58.7	300	
		stx1R1042_G	GCG TAA TCC CAC GGA CTC TTC	59.6	300	
		stx1R1042_modC	GCG TAA TCC CAC GCA CTC TT	58.8	300	
		stx1R1042_mod1d	GAG TAA TCC CAC GCC CAC TTC	59.4	300	
		stx1P990_mod_MGB	**FAM**-TTC CTT CTA TGT GTC CGG CAG-NFQMGB	69.0	100	(1, 2)
		stx1P990_mod1c_MGB	**FAM**-CCT TCT ATG TGC CCG GTA G-NFQMGB	69.0	100	
		stx1P990_mod1d_MGB	**FAM**-TCC TTC TAT GTG CCC GAC AG-NFQMGB	69.0	100	
stx$_2$	A	stx2F_LvI	CCG GAA TGC AAA TCA GTC GT	59.5	300	
		stx2R_G_LvI	ACC ACT GAA CTC CAT TAA CGC C	59.0	300	
		stx2R_A_LvI	TAC CAC TAA ACT CCA TTA ACG CCA	58.7	300	(2)
		stx2P_LvI_MGB	**VIC**-ACT CAC TGG TTT CAT CAT A-NFQMGB	68.9	100	
stx$_{2f}$	A	stx2F_mod2f_LvI	GGA ACG TAC AGG GAT GCA GAT T	59.0	300	
		stx2R_mod2f_LvI	CGT CCT CTG AAC TCC ATT AAA TCC	59.0	300	(1)
		stx2P_mod2f_LvI	**VIC**-ATG AAC CAA CCA GTG AAT-NFQMGB	69.0	100	
escV	A	escV_F	GCG TCA TT**Y** TGA CCG CTT TAG	56.4	300	
		escV_R1	TCC TGA AAA GAG AGC ACG GG	59.0	300	
		escV_R2	TCC TGA AAA GAA AGC ACA GGG	58.0	300	(3)
		escV-TM	**CY5**- ACT GAC GGG AAC GAA CCT TCA ATC ATT TTC -BBQ	68.9	200	
wzx$_{O103}$	B	Ec_wzxO103_F	CGT TGT TAT CTA TGG TGG GCT TAG T	58.6	300	
		Ec_wzxO103_R	CAC CTG CAA CCG CAT TAT TTA A	58.7	300	This study
		Ec_wzxO103_PTM	**FAM**-TTG GCC TCA AAG GCG CAT TAG TGT CT-BHQ1	68.2	75	
wzx$_{O121}$	B	Ec_wzxO121_F	CAT GGC GGG ACA ATG ACA	58.4	400	
		Ec_wzxO121_R	CGA TAG TGA AGA ACA AAA TAT GAA GAG TTC	59.2	400	(4)
		Ec_wzxO121_PMGB	**VIC**-TGC TGG ACT ACA GAA AA-NFQMGB	69.0	100	
rfbE$_{O157}$	B	Ec_rfbEO157_F	CGA TGA GTT TAT CTG CAA GGT GAT	58.3	600	
		Ec_rfbEO157_R	TTT CAC ACT TAT TGG ATG GTC TCA A	58.6	600	(5)
		Ec_rfbEO157_PTM	**CY5** -CCT TAA TTC CTC TCT TTC CTC TGC GGT CCT -BBQ	68.5	150	
wzx$_{O26}$	C	Ec_wzxO26_F	CGC GAC GGC AGA GAA AAT T	59.9	400	
		Ec_wzxO26_R	AGC AGG CTT TTA TAT TCT CCA ACT TT	58.2	400	(5)
		Ec_wzxO26_PMGB	**VIC**-CCG TTA AAT CAA TAC TAT TTC ACG A-NFQMGB	68.0	100	adapted
ihp1$_{O145}$	C	Ec_ihp1O145_F	CGA TAA TAT TTA CCC CAC CAG TAC AG	58.0	400	
		Ec_ihp1O145_R	GCC GCC GCA ATG CTT	59.0	400	(6)
		Ec_ihp1O145_PMGB	**FAM**-CGA TAT TGT GTG CAT TCT-NFQMGB	68.0	100	adapted

Target	Multiplex reaction	Sequence name	Sequence (5' – 3')[a, b]	Tm (°C)	Conc (nM)	Reference
wbdI$_{O111}$	C	Ec_wbdIO111_F	CGA GGC AAC ACA TTA TAT AGT GCT TT	58.8	400	
		Ec_wbdIO111_R	TTT TTG AAT AGT TAT GAA CAT CTT GTT TAG C	58.8	400	(5)
		Ec_wbdIO111_PTM	**CY5**-TTG AAT CTC CCA GAT GAT CAA CAT CGT GAA-BBQ	68.7	150	
wzx$_{O104}$	D	Ec_wzxO104_F	TGT CGC GCA AAG AAT TTC AAC	59.8	400	
		Ec_wzxO104_R	ATC CTT TAA ACT ATA CGC CCT AGA AAC C	59.6	400	(7) adapted
		Ec_wzxO104_PMGB	**VIC**-TTT GTA TTA GCA ATA AGT GGT GTC-NFQMGB	68.0	100	
aggR	D	aggR_F	CAA TAA GGA AAA G**R**C TTG AGT CAG A	59.4	300	
		aggR_R1	TCA AGC AAC AGC AAT GCT GC	59.7	300	
		aggR_R2	TTA TCA AGC AAT AGC AAT GCT GCT	59.1	300	(3)
		aggR_P	**FAM**-CCT TAT GCA ATC AAG AAT-NFQMGB	69.0	50	
aat	D	aat_F	GGG CAG TAT ATA AAC AAC AAT CAA TGG	59.8	300	
		aat_R1	GGG CAG TAT ATA AAC AAC AAC CAG TG	58.9	300	
		aat_R2	GCT TCA TAA GCC GAT AGA AGA TTA TAG G	59.2	300	(3)
		aat_PMGB1	**FAM**-TCT CAT CTA TTA CAG ACA GCC-NFQMGB	69.0	25	
		aat_PMGB2	**FAM**-CTC ATC TAT TAC AGA CAG CAA T-NFQMGB	69.0	25	
bfpA	D	bfpA_F1	ATC ACA CCT GCG GTA ACG G	58.0	600	
		bfpA_F2	TCA CAC CGG CGG TAA CG	58.6	600	
		bfpA_R	CGA **R**AA AGG TCT GTC TTT GAT TGA	60.7	600	(3)
		bfpA_PTM	**CY5**-CAG CAA GCG CAA GCA CCA TTG C-BBQ	68.7	200	
PhHV (internal control)	All	PhHV-267s	GGG CGA ATC ACA GAT TGA ATC	58.1	300	
		PhHV-337as	GCG GTT CCA AAC GTA CCA A	58.1	300	(2, 8)
		PhHV-1-MGB	**NED**-CGC CAC CAT CTG GAT-NFQMGB	70.0	100	

[a] Fluorescent dyes used are represented in bold. FAM, 6-carboxyfluorescein; VIC, 6-carboxyrhodamine; BHQ1, black hole quencher-1; NFQ-MGB, non-fluorescent quencher minor groove binder; Cy5, reactive water-soluble fluorescent dye of the cyanine dye family; BBQ, black berry quencher; NED, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein.

[b] **Y**, C or T; **R**, A or G.

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