Diagnostic Microbiology and Infectious Disease 87 (2017) 95–99

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Bacteriology

Evaluation of the *SHIGA TOXIN QUIK CHEK* and *ImmunoCard* STAT! EHEC as screening tools for the detection of Shiga toxin in fecal specimens

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 19 February 2016
Accepted 7 March 2016
Available online 10 March 2016

Keywords:
Shiga toxin
Shiga toxigenic *Escherichia coli*
Escherichia coli
Immunoassay
Rapid

ABSTRACT

In this study we evaluated the performance of the *SHIGA TOXIN QUIK CHEK* (Techlab®, Blacksburg, VA) and the *ImmunoCard* STAT! Enterohaemorrhagic *E. coli* (EHEC) (Meridian BioScience, Cincinnati, OH, USA) assays as methods for qualitatively detecting the presence of Shiga toxin in human fecal specimens. A multiplex PCR for the detection of *stx1* and *stx2* was used as the standard for comparison. The *SHIGA TOXIN QUIK CHEK* detected all known Shiga toxin subtypes with the exception of Stx2f, while the *ImmunoCard* STAT! EHEC was unable to identify four of the seven Stx2 subtypes, including Stx2b and Stx2d. When compared to multiplex PCR based on Shiga toxin gene presence alone both assays demonstrated 100% specificity, and gave sensitivity values of 50.0% and 41.2% respectively. Correlation between each assay and the multiplex PCR was calculated by the use of kappa, with both assays exhibiting a moderate level of agreement.

1. Introduction

Shiga toxigenic *Escherichia coli* (STEC) are an important cause of bacterial enteric infections in Australia, and can present sporadically or in outbreak situations (Johnson et al., 2006). Infections with STEC usually cause acute and often bloody diarrhea but can be responsible for a wide spectrum of disease ranging from mild non-bloody diarrhoea to hemorrhagic colitis and hemolytic uraemic syndrome (Karmali, 1989). The major pathogenicity factors of STEC are Shiga toxins, which exist in two major antigenic forms, Stx1 and Stx2, and can be further divided into subtypes (Stx1a, Stx1c, and Stx1d, and Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g).

In 2009, the Centers for Disease Control and Prevention (CDC) made recommendations for clinical laboratories to test all feces specimens from patients with acute community-acquired diarrhea for STEC (CDC, 2009), including routine culturing for *E. coli* O157 in addition to testing for non-O157s by toxin detection assays or the presence of *stx* genes. Detection of all STEC serotypes is a requirement of testing as many serotypes, not just O157, have been implicated in human disease, most memorably the recent O104 German outbreak (Rasko et al., 2011). However, in the clinical microbiology laboratory, routine testing of STEC requires a practical, rapid, sensitive, and cost efficient method. PCR assay for *stx1* and/or *stx2* target genes can be tested on fecal enrichment broth cultures or on direct feces and are effective at detecting all STEC. Additionally, a number of immunoassays which detect for Shiga toxin are on the market such as RIDA®QUICK Verotoxin/O157 (R-Biopharm, Darmstadt, Germany), Duopath Verotoxin test (Merck KgaA, Darmstadt, Germany) and *ImmunoCard* STAT! EHEC (Meridian BioScience, Cincinnati, OH, USA) and offer rapid results, however these are all recommended only for use on feces culture enrichments and not on direct feces. The Meridian Premier EHEC EIA assay (Meridian BioScience, Cincinnati, OH, USA) product insert states that it is able to detect Shiga toxins 1 and 2 in both stool specimens and broth cultures however a previous evaluation found it provided low sensitivity and gave many false positive results when used on direct fecal specimens (Staples et al., 2012) and use on feces culture enrichments is preferred. Such enrichment-based methods are inadequate for use when a feces specimen is unable to be cultured.

The *SHIGA TOXIN QUIK CHEK* (Techlab®, Blacksburg, VA, USA) is a rapid membrane enzyme immunoassay, which claims to be suitable for use with direct fecal specimens and offers simultaneous and qualitative detection and differentiation of Shiga toxins 1 and 2. In this study, the *SHIGA TOXIN QUIK CHEK* was evaluated on its ability to detect all known subtypes of Shiga toxin and its suitability for testing on direct fecal specimens. Additionally the performance of the *ImmunoCard* STAT! EHEC was also tested as an alternative Shiga toxin detection assay. Both assays were evaluated using multiplex PCR for Shiga toxin target genes as the standard.

2. Materials and methods

2.1. Control isolates used for detection of Shiga toxin subtypes

Thirteen *E. coli* isolates known to collectively express all known Shiga toxin subtypes were resuscitated from the −80 °C storage in the

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http://dx.doi.org/10.1016/j.diagmicrobio.2016.03.011
0732-8893/

Public Health Microbiology Laboratory strain collection. Ten of these were strains that were typed, analyzed, and selected by The Danish WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*, checked using accredited procedures according to DANAK accreditation No. 397, standard DS/EN ISO/IEC17025, and supplied by the Department of Microbiology and Infection Control, Statens Serum Institut, Denmark. The remaining three *E. coli* were isolated in the Public Health Microbiology Laboratory and characterized molecularly as per Sections 2.2 and 2.3. All 13 isolates were tested by each of the *SHIGA TOXIN QUIK CHEK* and *ImmunoCard* STAT! EHEC assays as detailed in the 'Plate method' sections of each respective manufacturer's instructions.

2.2. Fecal specimens for Shiga toxin assay evaluation

The study period occurred between May 2013 and May 2014 using frozen patient fecal specimens received by the Public Health Microbiology Laboratory for routine STEC testing from clinical laboratories and hospitals. Specimen sources included symptomatic individuals in addition to asymptomatic contacts of previous STEC cases. Upon receipt, specimens were thawed and a small pea-sized sample was inoculated into 5 ml of sterile *Escherichia coli* (EC) broth (BD Difco, Franklin Lakes, NJ) and incubated at 36 °C ± 1 °C for 24–48 hours. The remaining specimen was stored at −20 °C. Enrichment broths that had evident coliform growth after 24 or 48 hours were tested for the presence of Shiga toxin target genes as per Section 2.3. Fecal specimens that were unable to be cultured in EC broth were subjected to bacterial DNA extraction using the QIAGEN QIAamp® DNA Stool Mini Kit as per the manufacturer's instructions (QIAGEN Pty Ltd, Valencia, CA) prior to molecular analysis as per Section 2.3. Immediately upon detection of a positive STEC result, the positive feces and two STEC negative specimens tested in the same multiplex PCR run were processed for testing by both the *SHIGA TOXIN QUIK CHEK* and *ImmunoCard* STAT! EHEC assays. Altogether, 20 STEC positive and 40 STEC negative specimens were tested by both toxin detection assays. Duplicate patient specimens were excluded from the analysis.

2.3. PCR for detection of Shiga toxin genes

Detection of Shiga toxin target genes (*stx1*, *stx2*) was performed on control isolates, fecal enrichments, and DNA extractions by multiplex PCR assay using the method described by Paton and Paton (2002). Amplification products were separated on 2% agarose gels with ethidium bromide incorporated for visualization under ultra-violet light. Specimens that amplified *stx1* and/or *stx2* were considered positive for the presence of STEC. Positive (possessing both *stx1* and *stx2* genes), negative, and no template controls were included for each reaction. A separate 16S rDNA PCR was also run concurrently with the multiplex to ensure all samples had sufficient bacterial DNA present and no PCR inhibition occurred (Holland et al., 2000).

Real-time PCR was also conducted on any STEC-positive feces specimens for which Shiga toxin was undetected by the immunoassays using primers for *stx1* (Grys et al., 2009), *stx2* (Imamovic et al., 2010) and 16S (Greisen et al., 1994) which offered different binding regions and an improved limit of detection for the STEC target genes when compared to the multiplex PCR.

2.4. Isolation, identification, and characterization of STEC isolates

Isolates were sought from culturable specimens that were positive for STEC genes by PCR. For this, colonies were individually picked from MacConkey agar plates which were subcultured from the EC broth, prepared as crude Tris/EDTA boils and screened by the multiplex PCR for the presence of the target genes until a single STEC colony was recovered. The subtype was determined for each *stx* gene detected, from either pure isolates or the fecal DNA extraction. Identification of *stx1a*, *stx1c*, and *stx1d* was performed using a triplex PCR as previously described (Scheutz et al., 2012). Subtyping for *stx2* was performed using a collaboration of published methods; *stx2a, stx2b, stx2c,* and *stx2d* were identified as per Scheutz et al. (2012), *stx2e* as per Wang et al. (2002), *stx2f* as per Osek (2003) and *stx2g* as per Beutin et al. (2007). Additionally, isolates were subjected to serotyping for somatic (O) and flagella (H) antigens which was performed by the Microbiological Diagnostic Unit, Melbourne.

2.5. SHIGA TOXIN QUIK CHEK

Fecal specimens for toxin analysis were thawed at room temperature and processed for testing by the *SHIGA TOXIN QUIK CHEK* assay as per the manufacturers' instructions (Techlab®, 2012).

2.6. ImmunoCard STAT! EHEC rapid immunoassay

Fifty microliters or a 3–4 mm round pellet of each fecal specimen for analysis was thawed at room temperature and inoculated into 8 mL GN broth (BD Difco, Franklin Lakes, NJ) and incubated at 36 °C ± 1 °C for 24 hours. Only broths exhibiting visually observed growth were used for the testing procedure, which was carried out as per manufacturers' instructions (Meridian BioScience, 2011).

2.7. Statistical analysis

Sensitivity, specificity, positive (PPV), and negative (NPV) predictive values were calculated using previously published formulae (Carpenter, 2007). The level of agreement between the assays and the multiplex PCR was determined by calculating the kappa statistic, with the interpretation previously described (Viera and Garrett, 2005).

3. Results

3.1. Detection of Shiga toxin subtypes using control isolates

Characterized control isolates of all known Shiga toxin subtypes were tested by both assays. The *SHIGA TOXIN QUIK CHEK* successfully detected all three Stx1 subtypes (Stx1a, Stx1c, and Stx1d). It also detected most Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, and Stx2g) but was unable to detect Stx2f (Table 1).

Interestingly, although the *SHIGA TOXIN QUIK CHEK* was able to identify Stx2e, it failed to detect this subtype from an isolate expressing a mucoid capsule. Four isolates producing Stx2e were tested by this assay; three were nonmucoidal and were correctly identified, but the

Table 1
Detection of Shiga toxin subtypes for each assay.

Isolate reference	Genes present	Mucoid colony type	Shiga toxin subtypes	*ImmunoCard* STAT! EHEC	*SHIGA TOXIN QUIK CHEK*
EDL933	*stx1, stx2, ehxA, eaeA*	No	Stx1a, Stx2a	Stx1, Stx2	Stx1, Stx2
EH250	*stx2*	No	Stx2b	ND	Stx2
S1191	*stx2*	Yes	Stx2e	No flow	ND
PHM1	*stx2*	No	Stx2e	ND	Stx2
PHM2	*stx2*	No	Stx2e	ND	Stx2
PHM3	*stx2*	No	Stx2e	ND	Stx2
O31	*stx2*	No	Stx2b, Stx2c	Stx2	Stx2
7v	*stx2, ehxA*	No	Stx2g	Stx2	Stx2
DG131/3	*stx1, stx2*	No	Stx1c, Stx2b	Stx1	Stx1, Stx2
94C	*stx1, stx2, ehxA, saa*	No	Stx1a, Stx2a	Stx1, Stx2	Stx1, Stx2
T4/97	*eaeA*	No	Stx2f	ND	ND
MHI813	*stx1*	No	Stx1d	Stx1	Stx1
C165-02	*stx2*	No	Stx2d	ND	Stx2

ND, no toxin detected.