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Performance of the Check-Direct ESBL Screen for BD MAX™ for detection of asymptomatic faecal carriage of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Objectives: Accurte diagnostic methods are crucial for the detection of extended-spectrum β-lactamase-producing Enterobacterales (ESBL-E). Besides culture-based gold-standard methods, new molecular gene detection tests are reaching the market. The aim of this study was to investigate the performance of the direct quantitative PCR (qPCR)-based methods Check-Direct ESBL and CPE Screen for BD MAX™ in relation to traditional culture-based methods for detection of ESBL-E faecal carriage.
Methods: Faecal samples were collected from healthy adult volunteers. Samples were cultured on chromogenic ESBL agar plates and were screened for ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*. Confirmed ESBL- and AmpC-producing isolates were further analysed using whole-genome sequencing. In addition, faecal samples were analysed using Check-Direct ESBL and CPE Screen for BD MAX™ and the results were compared with the gold-standard culture-based method.
Results: Of 176 faecal samples examined, 11 (6.3%) grew ESBL-producing *E. coli* or *K. pneumoniae* isolates. Among 173 analysed samples, Check-Direct ESBL Screen for BD MAX™ detected 22 (12.7%) ESBL-positive samples. No carbapenemase-producing isolates were detected. Two culture-positive samples remained negative with Check-Direct ESBL Screen for BD MAX™. Culture-negative but qPCR-positive discrepancy was observed in 12 samples (6.9%). Altogether, concordant results were obtained for 158 samples (91.3%; 9 positive and 149 negative).
Conclusion: Check-Direct ESBL Screen for BD MAX™ is a fast screening method for ESBL carriage. However, several discrepant results were observed, which hinders interpretation. More clinical samples should be tested in combination with culture to evaluate the true benefits of this method.

1. Introduction

Antimicrobial resistance is an increasingly recognised global problem posing a serious threat to human health [1,2]. During the last decade, the prevalence of extended-spectrum β-lactamase-producing Enterobacterales (ESBL-E) has shown a steady increase in healthcare settings as well as in the community. Rising trends of asymptomatic intestinal carriage of ESBL-E are a cause for concern [3,4]. Several studies have identified overseas travel and previous use of antibiotics as risk factors for colonisation with ESBL-E [3,5,6]. The current increase in ESBL-E prevalence has been linked especially to strains carrying the CTX-M gene family [7]. CTX-M ESBL genes are mainly located on transferrable plasmids, allowing the efficient spread of these genes within and between bacterial species, which may have promoted their global dissemination [4,8].

High ESBL-E carriage rates create a challenge in healthcare settings. Infection control measures needed to prevent ESBL-E spread are laborious and costly, and treatment options for infections caused by ESBL-E are limited. Thus, effective, accurate and rapid diagnostic methods would be of great advantage. Besides the traditional, culture-based, two-step protocol for ESBL screening, new molecular gene detection tests are available to identify resistance genes from bacterial colonies or directly from patient samples. Molecular tests aim to shorten the diagnostic turnaround time compared with the two-step culture-based method, which is currently considered the gold-standard [9].

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In this study, the performance of the direct quantitative PCR (qPCR)-based methods Check-Direct ESBL and CPE Screen for BD MAX™ was investigated in relation to traditional culture-based methods for detection of ESBL-E faecal carriage in Finnish healthy adults. In addition, whole-genome sequencing (WGS) was performed for identification of the genomic content of the ESBL-E isolated during the study.

2. Methods

2.1. Study design

During 2016, a prospective, clinical study was conducted to investigate asymptomatic faecal carriage of ESBL-E (*Escherichia coli* and *Klebsiella pneumoniae*) in healthy adults in Southwest Finland as part of the Northern Dimension Antibiotic Resistance Study (NoDARS) [10]. The current study is an extension of the original NoDARS study. Fig. 1 describes the laboratory workflow followed.

2.2. Recruitment of study subjects and sample collection

Volunteers were recruited among students from four universities in Southern Finland as well as among elective surgery patients at two outpatient clinics in Southwest Finland at Turku University Hospital from February to December 2016 [10].

Each study subject provided only one faecal sample in an ESwab™ collection tube (Copan Diagnostics Inc., Murrieta, CA, USA) for analysis at the University of Turku.

2.3. Culture-based methods

Upon arrival of the samples at the laboratory, 100 μL each of the ESwab™ liquid sample suspension was inoculated onto two different chromogenic culture plates, namely chromID® ESBL and chromID® OXA-48 (bioMérieux, Marcy-L'Étoile, France) to search for growth of ESBL-producing *E. coli* and *K. pneumoniae*. In addition, 10 μL of the sample was inoculated on chromogenic agar (chromID CPSE agar plate; bioMérieux) to isolate possible additional *E. coli* and *K. pneumoniae* isolates not growing on the abovementioned selective chromogenic culture plates.

All plates were incubated at 35 °C and were screened for *E. coli* and *K. pneumoniae* colonies according to the manufacturer's instructions. Antimicrobial susceptibility testing was performed for *E. coli* and *K. pneumoniae* isolates from chromID ESBL or chromID OXA-48 agar plates. In addition, if detected, antimicrobial susceptibility testing was performed for one *E. coli* and *K. pneumoniae* isolate from the chromID CPSE agar plate. Antimicrobial susceptibility testing was performed by the disk diffusion method and using minimum inhibitory concentration (MIC) test strips for colistin and fosfomycin according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v.6.0 (http://www.eucast.org/clinical_breakpoints/). Isolates with reduced susceptibility to third-generation cephalosporins were tested for ESBL and AmpC production by the combination disk test according to EUCAST recommendations. For phenotypically confirmed ESBL- and AmpC-producing strains, the species was confirmed with matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) instrumentation (Bruker).

2.4. Block-based PCR

Multiplex, block-based PCR was used to identify $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} genes from the phenotypically confirmed ESBL- or AmpC-producing *E. coli* and *K. pneumoniae* isolates. PCR was performed as described previously [11]. A loopful of overnight-cultured bacteria was suspended in 100 μL of molecular-grade water. The suspension was heated at 100 °C for 5 min, was centrifuged (13 000 rpm, 5 min) and the supernatant was used as a PCR template.

2.5. qPCR-based method

Check-Direct ESBL Screen for BD MAX™ and Check-Direct CPE Screen for BD MAX™ (Check-Points B.V., Wageningen, The Netherlands) were used for the direct detection of ESBL and carbapenemase (CPE) genes from the ESwab™ liquid sample

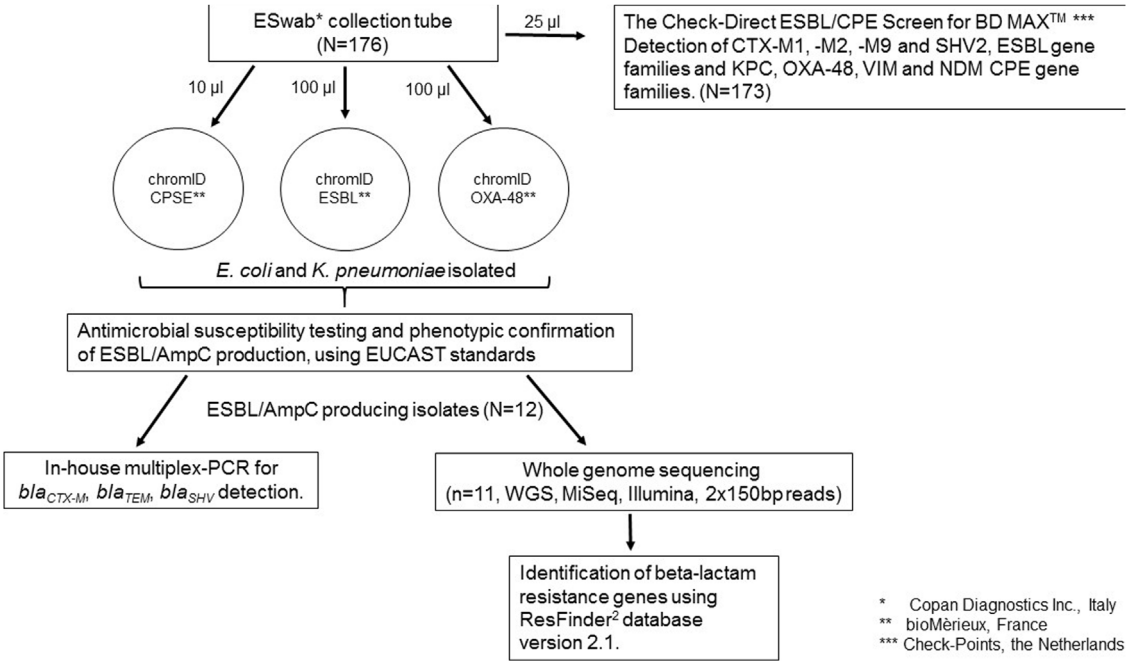

Fig. 1. Laboratory workflow used in the study. ESBL, extended-spectrum β-lactamase; CPE, carbapenemase; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

suspension. Check-Direct ESBL and CPE Screen for BD MAX™ detect the clinically most common ESBL gene families, namely CTX-M-1, CTX-M-2, CTX-M-9 and SHV-2, and the CPE gene families, namely KPC, OXA-48, VIM and NDM, respectively. The assay is a combination of a DNA extraction procedure (BD MAX™ ExK™ DNA-1) followed by multiplex real-time PCR. The tests were performed according to the manufacturer's instructions. The manufacturer's instructions were used for determining positive samples. Sample was considered qPCR-positive for ESBL or CPE if a Ct value was observed for the sample and the Ct value for the sample processing control of the same sample was in a predefined range.

2.6. Whole-genome sequencing

All phenotypically confirmed ESBL/AmpC-producing *E. coli* and *K. pneumoniae* isolates were analysed by WGS. DNA isolation for WGS analysis was performed using a MagAttract HMW DNA Kit (QIAGEN). DNA libraries were generated using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. WGS was performed withIllumina MiSeq instrument (Illumina Inc.) generating 2 × 150-bp paired-end reads. Trimmomatic software [12] was used for quality trimming of the reads. The resistance genes and sequence type (ST) were identified using ResFinder v.2.1 [13] and MLSTcheck software [14,15]. Reads of the *E. coli and K. pneumoniae* isolates included in the WGS analysis are deposited in the NCBI's Sequence Read Archive (SRA) database with Bioproject accession number PRJNA628849.

2.7. Ethics

This study was conducted in accordance with Good Clinical Practice Guidelines and with the current revision of the Declaration of Helsinki. The study protocol was approved by the ethical committee at the Hospital District of Southwest Finland. Written informed consent was obtained from the study subjects prior to enrolment.

Table 1
Summary of the results of antimicrobial susceptibility testing (AST), block-based PCR, whole-genome sequencing (WGS) and Check-Direct ESBL Screen for BD MAX™, respectively, of *Escherichia coli* and *Klebsiella pneumoniae* isolates. In each column, a positive samples or isolate for the respective test method is shaded in grey.

	Isolate (*E. coli* /*K. pneumoniae*)								ESwab™				
Sample no	Species (sequence type)	Resistance panel*	combination disk		block-based PCR			ß-lactame resistance genes (WGS) **	Check-Direct ESBL Screen for BD MAX™				
			ESBL	AmpC	blaCTX-M	blaTEM	blaSHV		Test result	Gene group detected			
										CTX-M1	CTX-M2	CTX-M9	SHV2
1	*E. coli* (131)	W, SXT, AMP, AMC, CTX, CAZ, CN						**$bla_{CTX-M15}$**, bla_{TEM-1B}	positive				
2	*E. coli* (10)	AMP, CTX, CAZ						**bla_{SHV-12}**	positive				
3	*E. coli* (131)	AMP, AMC, CTX, CAZ						**$bla_{CTX-M-15}$**	positive				
4	*E. coli* (1312)	W, SXT, AMP, CTX, CAZ						**$bla_{CTX-M15}$**, bla_{TEM-1B}	positive				
5	*E.coli* (73)	AMP, AMC, CTX, CAZ						Not included	positive				
6	*E. coli* (SLV 206)	AMP, CTX, CAZ						**$bla_{CTX-M-132}$**	positive				
7	*E. coli* (131)	W, SXT, AMP, AMC, CIP, CTX, CAZ, CN						**$bla_{CTX-M15}$**, bla_{TEM-1C}, bla_{OXA-1}	positive				
8	*E. coli* (58)	AMP, CTX, CAZ						**$bla_{CTX-M-55}$**	positive				
9	*E.coli* (93)	W, SXT, AMP, AMC, FOX, CTX, CAZ, colistin						bla_{TEM-1B}, bla_{CMY-2}, *mcr-1*	negative				
10	*K. pneumoniae* (1715)	AMP, CTX, CAZ						**$bla_{CTX-M-15}$**, bla_{SHV-1}	positive				
11	*K. pneumoniae* (513)	W, SXT, AMP, AMC, CTX, CAZ						**$bla_{CTX-M-15}$**, bla_{TEM-1B}, bla_{SHV-1}	negative				
12	*E. coli* (38)	W, SXT, AMP, AMC, CIP, FOX, CTX, CAZ						bla_{OXA-1}, bla_{CMY-2}	positive				
13									positive				
14									positive				
15									positive				
16									positive				
17									positive				
18									positive				
19									positive				
20									positive				
21									positive				
22									positive				
23									positive				
24									positive				

ST, sequence type; ESBL, extended-spectrum β-lactamase; SLV, single-locus variant.
TMP, trimethoprim; SXT, trimethoprim/sulfamethoxazole; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; FOX, cefoxitin; COL, colistin.
[a]ESBL genes are marked in bold.

3. Results

Altogether 176 samples were included in the analysis. Of these, in 11 samples (11/176; 6.3%) grew isolates that were phenotypically confirmed to be ESBL-producers (9 *E. coli* and 2 *K. pneumoniae*), of which 3 *E. coli* isolates were also phenotypically AmpC-producers. In addition, one *E. coli* isolate was phenotypically confirmed to be only an AmpC-producer. Eleven of the isolates were isolated from chromID ESBL agar plates and one from a CPSE agar plate (Table 1, sample no. 9). No CPE-producing isolates were found by culture (Table 1).

Phenotypically confirmed ESBL-producing isolates were first analysed by block-based multiplex PCR to detect the three common ESBL/β-lactamase gene groups, namely $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. $bla_{\text{CTX-M}}$ was the most commonly detected gene group (9/11; 81.8%), followed by bla_{TEM} (6/11; 54.5%) and bla_{SHV} (3/11; 27.3%) (Table 1). A combination of at least two genes was detected in six isolates.

Check-Direct ESBL Screen for BD MAX™ (called Check-Direct qPCR hereafter in the text) was successfully performed for 173 samples. Check-Direct qPCR detected 22 (12.7%) ESBL-positive samples. When comparing the Check-Direct qPCR results with those obtained by the culture-based method (Table 1), nine of the abovementioned samples that grew a phenotypically confirmed ESBL-E isolate were also positive by Check-Direct qPCR screening. Two culture-positive samples (sample nos. 9 and 11) remained negative by Check-Direct qPCR screening. One of these isolates contained only a non-ESBL β-lactamase gene not detectable by the test ($bla_{\text{TEM-1B}}$, sample no. 9). There was a culture-negative but Check-Direct qPCR-positive discrepancy in 12 samples (6.9%). In addition, one sample was qPCR-positive but only an AmpC-producing *E. coli* was identified from the sample by the culture-based method (sample no. 12). This isolate did not contain any ESBL genes.

In total, concordant results were obtained from 158 samples (91.3%; 9 positive and 149 negative). Check-direct qPCR showed a sensitivity of 81.8%, a specificity of 92.0%, a positive predictive value of 40.9 % and a negative predictive value of 98.7%.

All samples were negative for Check-Direct CPE Screen for BD MAX™.

3.1. Molecular characteristics of the Check-Direct qPCR screening-positive samples

The most prevalent ESBL gene family detected by Check-Direct qPCR screening was CTX-M-1 (10/22; 45.5%) (Table 1). Of these, seven were also identified by block-based multiplex PCR. Eight samples (8/22; 36.4%) were positive for CTX-M-2 group. One of these grew only an AmpC-producing *E. coli* (sample no. 12). This isolate was negative for $bla_{\text{CTX-M}}$ by block-based PCR and WGS. Seven CTX-M-2 group-positive samples remained negative by the culture-based method. In two of these, the signal level was low and Ct values were relatively high (Ct values of 40.8 and 40.7). Ct values of the remaining CTX-M-2-positive samples were between 20 and 30 cycles (data not shown).

CTX-M-9 family was detected in one sample (4.5%), which was also confirmed by the culture-based method and block-based PCR (sample no. 8).

The SHV gene family was detected in four samples (18.2% of 22 positive samples), of which one was positive by the culture-based method and was confirmed by block-based PCR (sample no. 2).

3.2. Whole-genome sequencing analysis of the phenotypically confirmed isolates

Altogether, 11 isolates that were phenotypically confirmed to be either ESBL (n = 10) and/or AmpC (n = 4) producers were analysed by WGS. ResFinder and MLSTcheck software were used to search for β-lactam resistance genes and STs from the sequence data (Table 1). The most prevalent ST in *E. coli* isolates was ST131 (n = 3). The most common ESBL gene was $bla_{\text{CTX-M-15}}$, which was identified in six isolates. One isolate contained $bla_{\text{CTX-M-55}}$ and another contained $bla_{\text{CTX-M-132}}$. $bla_{\text{SHV-12}}$ was identified from one isolate. These WGS findings confirm the results from block-based PCR and Check-Direct qPCR screening from the respective samples. In addition to these, other non-ESBL β-lactamase genes were also detected (Table 1). In one phenotypic ESBL isolate (sample no. 9), only the bla_{TEM} gene was identified by block-based PCR, and the only β-lactam resistance gene identified by WGS was $bla_{\text{TEM-1B}}$, which does not confer resistance to third-generation cephalosporins. In addition, this isolate carried a plasmid-mediated colistin resistance gene *mcr-1* [16]. Check-Direct qPCR screening remained negative for this isolate as bla_{TEM} is not identified by this method. The $bla_{\text{CMY-2}}$ gene was detected in two of four phenotypically AmpC-producing *E. coli* isolates.

4. Discussion

This study was an extension of a collaborative study on asymptomatic faecal carriage of ESBL-E (*E. coli* and *K. pneumoniae*) conducted in six countries from the Baltic Sea region [10]. Additional molecular characterisations were performed for the isolated Finnish ESBL-E strains. In addition, the performance of Check-Direct ESBL and CPE Screen for BD MAX™ screening system was evaluated in relation to the traditional culture-based method.

In total, 6.3% of study subjects were found to carry an ESBL-producing *E. coli* or *K. pneumoniae* when evaluated by the two-step culture-based method. WGS analysis of these phenotypically confirmed isolates showed that $bla_{\text{CTX-M-15}}$ was the most frequently identified ESBL gene (54.5% of isolates). Both of these findings were more or less expected as similar rates have been recently reported elsewhere [4]. CTX-M is currently the largest ESBL group and is spreading extensively on all continents [8].

A total of 173 samples were successfully tested with Check-Direct qPCR screening. Overall sensitivity of the Check-Direct ESBL screening was 81.8% (9 qPCR-positive of 11 phenotypically confirmed samples). Two samples showed a discrepant result, being positive in the culture-based ESBL combination disk assay but qPCR screening negative. One of these was an *E. coli* isolate carrying a resistance gene $bla_{\text{TEM-1B}}$, which is not detected by Check-Direct ESBL Screen for BD MAX™. This isolate was originally considered as an ESBL-producing *E. coli* based on the phenotypic confirmation, but after WGS analysis the isolate should be considered as a false-positive by the culture-based method. This discrepancy was solved only with the more detailed WGS analysis performed. In addition, this isolate was found to also carry an *mcr-1* gene leading to colistin resistance [16]. The other culture-based method-positive but qPCR screening-negative isolate was an ESBL-producing *K. pneumoniae* that remained negative with Check-Direct ESBL Screen for BD MAX™ system. This isolate had the $bla_{\text{CTX-M-15}}$ ESBL gene in its genome.

Culture-based method-negative but qPCR screening-positive discrepancies were detected more frequently, altogether in 12 samples (6.9%). From these, three samples were positive for the SHV gene family and one sample was positive for the CTX-M-1 gene family with Check-Direct qPCR screening. The remaining discrepancies considered especially the CTX-M-2 gene family. Eight samples were positive for CTX-M-2 by Check-Direct ESBL Screen for BD MAX™. We did not perform any culture enrichments to increase the sensitivity of the culture method. It is known that pre-enrichment might increase the positivity rate of ESBL but it is not routinely used in clinical settings because of the limited benefit as opposed to prolonged

turnaround time [17]. Instead, these samples were re-cultured on chromID ESBL agar plates but no colonies were detected. Another more robust method for confirmation was tried. bla_{CTX-M} PCR was performed from the whole bacterial suspension of these samples collected from the blood agar. We were able to detect a bla_{CTX-M} signal from five CTX-M-2-positive samples. However, the bacterial species could not be confirmed. As the original NoDARS study set-up was designed mainly to search for *E. coli* and *K. pneumoniae* isolates from the samples, it is of course also possible that the positive ESBL gene signal derives from some other bacterial species. We were able to show that one of these samples carried an ESBL-producing *Enterobacter cloacae*. However, the possible ESBL genes of this isolate were not analysed. The Ct value of these qPCR-positive/culture-negative samples were relatively early (Ct 20–30), which is proportional to high template concentration.

There are only a limited number of reports about the appearance of the CTX-M-2 gene family in Europe and other continents. The CTX-M-2 gene family was the main genotype in South America in early 2000, after which it was replaced by other CTX-M genotypes [18]. Other reports on the high prevalence of CTX-M-2 are from Japan [19] and Israel [20]. At least in Israel, the frequency of CTX-M-2 has declined with the clonal expansion of $bla_{CTX-M-15}$ and other genotypes [20]. From Finland, besides the current study, there is only one other relatively recent study on asymptomatic carriage of ESBL-E, in which Kantele et al. investigated the effect of international travel on ESBL carriage in healthy Finnish adults [5]. They identified only a 1.2% carriage rate in pre-travel samples of 430 subjects. However, the ESBL genes were not investigated from pre-travel samples [5].

To our knowledge, there are only two previous publications evaluating Check-Direct ESBL Screen for BD MAX™ [21,22]. These studies used rectal swabs from patients, whereas samples in the current study were faecal samples provided by healthy volunteers. The sensitivity of the method in these two Dutch studies was 87.7% and 95.2%, respectively. Discrepant culture-negative/qPCR-positive samples were detected in both studies. Souverein et al. reported 3.4% (12/352) discordant results [22]. In their study, none of the qPCR-positive samples were for the CTX-M-2 gene family. The other study reported 3.1% (18/573) qPCR-positive/culture-negative samples, of which 2 were for the CTX-M-2 gene family [21]. The authors state that the interpretation of these discrepant results was challenging as confirmation of the results was not possible.

There might be several explanations for the discrepant results. First, it is acknowledged that the culture-based method is relatively insensitive compared with qPCR-based methods. Samples used in this study were stool samples collected by the study volunteers themselves, causing variation in the amount of stool in the ESwab™ collection tube. This might affect the sensitivity of both methods used. Part of the ESwab™ samples were frozen at −80 °C before performing the qPCR screening. However, we were able to obtain a qPCR-positive result also from the frozen samples that were positive by the culture-based method.

According to the manufacturer's manual, Check-Direct ESBL Screen for BD MAX™ detects the respective gene families from Enterobacterales. Our culture-based method focused only on *E. coli* and *K. pneumoniae*. We cannot rule out the possibility that the qPCR result might be obtained from non-viable bacteria. A weakness of the Check-Direct qPCR screening is that it does not confirm the species. Before the clinical study, the Check-Direct qPCR screening system was evaluated with samples initially sent for ESBL screening to the clinical microbiology laboratory. In this evaluation, all 20 ESBL culture-positive samples tested were also ESBL-positive by Check-Direct qPCR screening method. Two samples were culture method-negative but Check-Direct qPCR screening-positive. However, as clinical microbiology samples are not routinely screened for ESBL genes, we do not have further information on ESBL gene frequencies in such a setting in Finland.

Limitations in this study should be acknowledged. First, the number of analysed samples was rather low. The frequency of ESBL-E is low in Finland and the number of positive samples remained low in the studied cohort. Second, initial pre-enrichment of the samples was not performed to increase the sensitivity.

In this study, WGS was used to identify all β-lactamase resistance genes and to confirm the findings obtained by block-based PCR and Check-Direct qPCR screening.

5. Conclusions

Check-Direct ESBL Screen for BD MAX™ is a fast screening method that could be beneficial for screening of risk-group patients. Current Finnish guidelines as well as Nordic and British guidelines [23–25] for control of multidrug-resistant microbes recommend culture confirmation of positive molecular results. According to the results of the current study, a substantial part of PCR-positive results would remain unconfirmed, which would result in difficulties in interpreting their significance and the required infection control measures. More clinical samples should be tested in combination with culture to evaluate the true benefits of this method. As suggested by Souverein et al. [22], this test might be suitable for outbreak investigation when there is a special need for a fast result and where false-positive results are less meaningful, and the surveillance could be focused on an ESBL type covered by this assay.

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Competing interests

None declared.

Ethical approval

This study was conducted in accordance with Good Clinical Practice Guidelines and with the current revision of the Declaration of Helsinki. The study protocol was approved by the ethical committee at the Hospital District of Southwest Finland [15.12.2015, decision: 157/1801/2015]. Written informed consent was obtained from the study subjects prior to enrolment.

Availability of data and material

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request. The reads of the *E. coli and K. pneumoniae* isolates included in the WGS analysis are deposited in NCBI SRA database (BioProject accession number PRJNA628849) .

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