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Differentiation of *Yersinia enterocolitica* biotype 1A from pathogenic *Yersinia enterocolitica* biotypes by detection of β-glucosidase activity: comparison of two chromogenic culture media and Vitek2

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Aesculin hydrolysis (ESC) is one of the key reactions in differentiating pathogenic *Yersinia enterocolitica* biotypes 1B, 2, 3, 4 and 5 from the less-pathogenic biotype 1A. Because the ESC reaction is caused by β-glucosidase (βGLU) activity of the bacteria, we studied whether two commonly used methods (BBL CHROMagar Orientation and Vitek2 Gram-negative identification card) could be used in assessing βGLU activity of 74 *Yersinia* strains. Both methods were sensitive (100 % and 97 %) and specific (100 % and 100 %) in differentiating βGLU-positive YE BT1A from βGLU-negative *Y. enterocolitica* biotypes. For a subset of strains (n=69), a new selective CHROMagar Yersinia showed excellent agreement with the strains' βGLU activity. Thus all the methods evaluated in this study may be used to differentiate between YE BT1A and other *Y. enterocolitica* biotypes.

INTRODUCTION

The role of *Yersinia enterocolitica* biotype 1A (YE BT1A) in human disease is controversial. YE BT1A has classically been considered apathogenic or less pathogenic than other *Y. enterocolitica* biotypes (YE BT1B, -2, -3, -4 and -5). It does not usually harbour major chromosomal virulence genes or the 70 kb *Yersinia* virulence plasmid pYV, which are important in the development of pathogenicity of the strain. However, in some studies YE BT1A has been shown to cause human disease similar to pathogenic *Y. enterocolitica* biotypes (Bhagat & Virdi, 2011). The gastrointestinal disease caused by YE BT1A may be less severe than that caused by non-1A biotypes and complications such as reactive arthritis are rare in YE BT1A infection (Huovinen *et al.*, 2010). It remains to be resolved whether enigmatic YE BT1A should be divided into two or more groups according to their pathogenic potential as suggested recently (Stephan *et al.*, 2011; Sihvonen *et al.*, 2012). About two-thirds of all *Yersinia* spp. isolated from human stool samples are YE BT1A in Finland (Sihvonen *et al.*, 2009). Thus, accurate identification of YE BT1A is important assuming that it may be clinically significant.

Commercial bacterial identification systems such as API20E (bioMérieux) are not able to differentiate YE BT1A from pathogenic *Y. enterocolitica* biotypes (Linde *et al.*, 1999). The current version of the Gram-negative identification (GN-ID) card of Vitek2 (bioMérieux) identifies only the *Y. enterocolitica* group, which (according to the Vitek2 database) includes *Yersinia aldovae*, *Y. frederiksenii*, *Y. enterocolitica*, *Y. intermedia* and *Y. kristensenii*. Identification of the different *Y. enterocolitica* bio/serotypes requires several methods such as biotyping and O-serotyping (Wauters *et al.*, 1987; Sihvonen *et al.*, 2009).

Recently, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was used in rapid identification and biotyping of *Y. enterocolitica* (Stephan *et al.*, 2011). In the future, MALDI-TOF may change *Y. enterocolitica* biotyping remarkably. Currently identification of *Y. enterocolitica* is unfortunately still laborious and time-consuming. Moreover, identification of *Y. enterocolitica*-like species is sometimes impossible without DNA sequence-based analysis. Testing for aesculin hydrolysis (ESC) is an important method used to differentiate pathogenic (ESC-negative) *Y. enterocolitica* biotypes (YE BT1B, -2, -3, -4 and -5) from the less-pathogenic (ESC-positive) YE BT1A biotype (Wauters *et al.*, 1987; Sihvonen *et al.*, 2009). The ESC reaction is derived from β-glucosidase (βGLU) activity of the bacteria (Edberg & Bell, 1985). This provides alternative routes to rapidly identify the most frequently occurring biotype (YE BT1A) as there is a wide range of commercially available chromogenic media containing substrates which are targeted by βGLU activity of the bacteria. These media are commonly used in identification of *Escherichia coli* and other members of the family *Enterobacteriaceae*. Thus, detecting βGLU activity of *Yersinia* spp. with these chromogenic media might be of some value in a routine microbiology laboratory setting.

Abbreviations: βGLU, β-glucosidase; CAY, CHROMagar Yersinia; CIN, cefsulodin–irgasan–novobiocin; ESC, aesculin hydrolysis; GN-ID, Gram-negative identification card: MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; YE BT, *Yersinia enterocolitica* biotype.

We studied the growth characteristics of *Yersinia* spp. on one of these chromogenic media, a non-selective BBL CHROMagar Orientation (Orientation; Becton Dickinson) and compared it with a novel selective CHROMagar Yersinia (CAY; CHROMagar Microbiology). Additionally, we evaluated whether the automated bacterial identification system Vitek2 could be used in the identification of YE BT1A because the βGLU reaction is included in the GN-ID card of Vitek2, although the Vitek2 database does not utilize the βGLU result.

METHODS

Eighty-four *Yersinia* spp. strains were isolated from 18 477 human stool samples in Eastern Finland (population 570 000) between March 2010 and February 2013 (3 years). Stool samples were routinely cultured on cefsulodin–irgasan–novobiocin (CIN) agar (Oxoid) and incubated for 2 days in an aerobic atmosphere at 30 °C. Cold-enrichment was not used. Identification of typical colonies with a bull's eye appearance was carried out using API20E. *Y. enterocolitica* biotyping with β-xylosidase, pyrazinamidase, ESC and trehalose was carried out with Diatabs (Rosco). Additionally indole production and lipase activity were determined (Wauters *et al.*, 1987; Sihvonen *et al.*, 2009). Lipase activity was studied with egg yolk agar plates at 30 °C. When a *Yersinia* species other than *Y. enterocolitica* was suspected and the ESC reaction was negative, the strain was additionally sent to the Finnish *Yersinia* reference laboratory at the National Institute of Health and Welfare for further identification. Since March 2011 all pathogenic *Y. enterocolitica* biotypes were also serotyped with O : 3 and O : 9 antisera (Statens Serum Institut) (data not shown).

Of 84 *Yersinia* strains 74 (88 %) were available for βGLU detection with Orientation agar and Vitek2. Briefly, strains were subcultured onto Orientation and incubated for 48 h in an aerobic atmosphere at 30 °C. The plates were examined after 24 and 48 h. The colour and size of the colonies were observed. Each isolate was also tested with Vitek2 using the GN-ID card. During the preparation of this manuscript a new CAY was introduced. Growth characteristics of a subset of 69 *Yersinia* strains still available to us were studied with this *Y. enterocolitic*-selective medium. Incubation and examination of CAY were carried out as with CIN agar.

RESULTS

Growth characteristics of *Yersinia* spp. on two chromogenic media, as well as βGLU activity according to Vitek2 are shown in Table 1. As a blue-green colony colour on Orientation indicates βGLU activity of the strain, the sensitivity and specificity of Orientation to detect βGLU activity of *Y. enterocolitica* biotypes were both 100 %. After 48 h of incubation, differences in colony colours were even more distinctive (Fig. 1).

All *Yersinia* strains (n=74) were identified as '*Y. enterocolitica* group' with Vitek2, and βGLU reaction was positive for 33/34 YE BT1A strains (sensitivity 97 %) and negative for all pathogenic *Y. enterocolitica* biotype strains (specificity 100 %). In all cases, including *Y. enterocolitica*-like species, pathogenic *Y. enterocolitica* biotypes could be ruled out with a positive βGLU Vitek2 result.

Twelve apathogenic *Y. enterocolitica*-like strains were studied with Orientation and Vitek2 (Table 1). Interestingly, some subcultures of *Yersinia bercovieri* on Orientation agar at 30 °C showed a pale green tinge. Consequently, we cultured *Y. bercovieri* and other βGLU-negative *Yersinia* strains on Orientation at 25 and 37 °C. All strains produced purple colonies at 25 °C and blue-green colonies at 37 °C.

All *Yersinia* strains studied (n=69), including *Y. enterocolitica*-like species, grew on CAY after overnight incubation; however, the distinctive mauve colour appeared in four pathogenic *Y. enterocolitica* biotype strains only after 48 h incubation. All YE BT1A strains studied (n=34) produced metallic-blue colonies and all pathogenic *Y. enterocolitica* biotype strains (n=24) mauve colonies. *Y. bercovieri* (n=4), *Y. kristensenii* (n=2) and *Yersinia mollaretii* (n=1), which are ESC-negative, also grew as mauve colonies similar to pathogenic *Y. enterocolitica* biotypes. ESC-positive *Y. frederiksenii* (n=4) produced metallic-blue colonies similar to YE BT1A.

Pathogenic YE BT1B and YE BT5 are very rare in Finland (Sihvonen *et al.*, 2009). Because no YE BT1B strain was found in our patient samples, we studied the YE BT1B type strain (ATCC 23715), which is ESC-negative. It produced purple colonies on Orientation and mauve on CAY, and was βGLU-negative with Vitek2 as it should be. Unfortunately, no YE BT5 strain was available to us.

DISCUSSION

In this study we have shown that βGLU activity of *Yersinia* spp. can be reliably detected with CHROMagar Orientation. To our knowledge, this has not been reported earlier, although a single *Y. enterocolitica* strain was reported to produce blue colonies on CHROMagar Orientation in a study which focused on other *Enterobacteriaceae* (Merlino *et al.*, 1996). Initially, we also tested the performance of CPS ID (bioMérieux) and UTI Clearance (Oxoid) to detect βGLU activity with refrigerated *Y. enterocolitica* strains. These media were as capable as Orientation of differentiating YE BT1A from pathogenic *Y. enterocolitica* biotypes (data not shown). However, the colours on Orientation were estimated by three individual readers to be most informative. This may be due to the fact that Orientation was in routine use in our laboratory and the personnel were more familiar with it.

Earlier, a *Yersinia*-selective chromogenic medium (YeCM) was shown to detect potentially virulent *Y. enterocolitica* by targeting βGLU (Weagant, 2008) and a selective chromogenic *Yersinia* plate (YECA; AES Chemunex) was used to differentiate YE BT1A from pathogenic *Y. enterocolitica* biotypes from pig tonsils (Denis *et al.*, 2011). However, these media are not commercially available, nor are they used in clinical microbiology laboratories, at least not in Finland. Recently, CAY was recommended for routine use in faecal cultures to screen pathogenic *Y. enterocolitica* (Renaud *et al.*, 2013). The composition of CAY has not

Table 1. Colony colour of *Yersinia* spp. on BBL CHROMagar Orientation and CAY, and βGLU activity of *Yersinia* spp. according to Vitek2 GN-ID

	ESC	BBL CHROMagar Orientation			Vitek2 GN-ID card			CAY		
		n	Colour		*n*	βGLU reaction		*n**	Colour	
			Purple	Blue-green		−	+		Mauve	Metallic-blue
YE BT1A	+	34	0	34	34	1	33	34	0	34
YE BT2, -3, -4	−	28	28	0	28	28	0	24	24	0
Y. bercovieri	−	5	5†	0	5	5	0	4	4	0
Y. frederiksenii	+	4	0	4	4	0	4	4	0	4
Y. kristensenii	−	2	2	0	2	2	0	2	2	0
Y. mollaretii	−	1	1	0	1	1	0	1	1	0
Total		74			74			69		

Data are presented as number of strains.
*Five of 74 *Yersinia* strains were not available for culturing on CAY.
†Four of five *Y. bercovieri* strains grew as pale green at 30 °C.

been published. Renaud *et al.* indicated that the chromogenic reaction on CAY is targeted by chromosomally encoded *Y. enterocolitica* enzymes, but they did not specify these enzymes more precisely. As shown in Table 1, the growth patterns of *Yersinia* spp. on CAY are in 100 % accordance with βGLU production and ESC of *Yersinia* spp. This suggests that at least one of the potential chromogenic substrates on CAY is targeted by βGLU. CAY should be inhibitory to some apathogenic *Y. enterocolitica*-like strains. However, all *Y. enterocolitica*-like species grew well on CAY in our study.

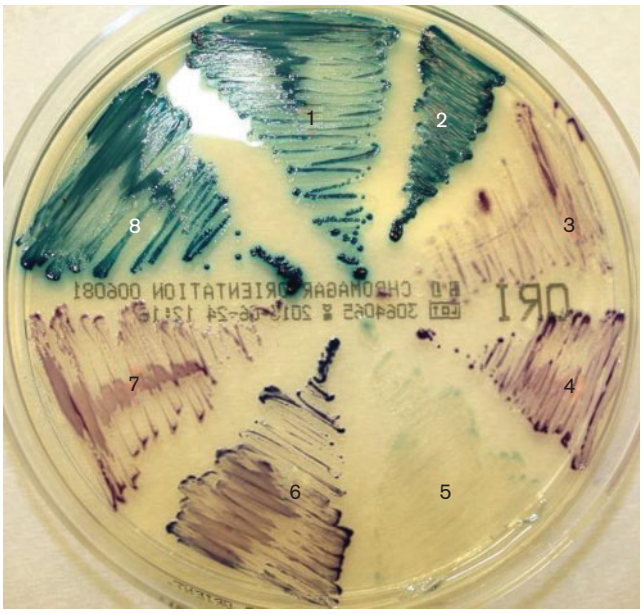

Fig. 1. *Yersinia* strains isolated from stool specimens on BBL CHROMagar Orientation after 48 h incubation at 30 °C. 1, *Y. enterocolitica* biotype 1A; 2, *Y. frederiksenii*; 3, *Y. kristensenii*; 4, *Y. bercovieri*; 5, *Y. bercovieri*; 6, *Y. enterocolitica* bio/serotype 2/O : 9; 7, *Y. enterocolitica* bio/serotype 4/O : 3; 8, *Y. enterocolitica* biotype 1A.

Subculturing *Yersinia* colonies on chromogenic plates or using CAY in primary cultures may also facilitate the identification of *Yersinia* spp. in mixed-culture cases. In our study, both YE BT1A (βGLU-positive) and *Y. mollaretii* (βGLU-negative) strains were cultured from a single patient sample on CIN agar. These were readily distinguishable from each other after subculturing on Orientation agar. In a large Finnish study, 2 % of *Yersinia*-positive faecal samples were characterized by a co-existence of different *Yersinia* spp. in the same sample (Sihvonen *et al.*, 2009).

Twelve apathogenic *Y. enterocolitica*-like strains were studied with Orientation and Vitek2 and the βGLU results obtained with Orientation and Vitek2 were in accordance with previously published ESC results (Sihvonen *et al.*, 2009). The pale green colour of some *Y. bercovieri* strains on Orientation at 30 °C suggests that *Y. bercovieri* is probably on the threshold of βGLU production and more prone to low-level βGLU production than other βGLU-negative *Yersinia* spp. (including pathogenic *Y. enterocolitica* biotypes) at this temperature. This phenomenon could be used in presumptive identification with four out of five of the *Y. bercovieri* strains presented here. *Y. bercovieri* is the second most common *Y. enterocolitica*-like species (after *Y. frederiksenii*) and the most common ESC/βGLU-negative *Y. enterocolitica*-like species in clinical microbiology laboratories in Finland (Sihvonen *et al.*, 2009). Thus, simply observing a pale green colony colour on Orientation might facilitate interpretations between pathogenic *Y. enterocolitica* biotypes and *Y. bercovieri*. The limitation of our study is that only 12 *Y. enterocolitica*-like strains were included. These belonged to four different *Y. enterocolitica*-like species, which, however, compose over 80 % of all *Y.*

enterocolitica-like isolates in human stool samples in Finland (Sihvonen *et al.*, 2009). It might be useful to study βGLU production of other *Y. enterocolitica*-like species on Orientation and other non-selective chromogenic media at different temperatures. On CAY, colony colours were not affected by incubation temperature, and the colours remained stable from 25 to 37 °C.

Vitek2 is quite commonly used in clinical microbiology laboratories. An earlier version of the Vitek GN-ID card was shown to correctly identify 96 % of *Yersinia* spp. to the genus level. However, the identification was correct for only 57 % of the *Yersinia* strains to the species level (Linde *et al.*, 1999). Here, the current version of the GN-ID card and the database of Vitek2 were shown to work reliably in identification of *Yersinia* spp. as all *Y. enterocolitica* and *Y. enterocolitica*-like strains were identified as '*Y. enterocolitica* group'. Our data suggest that pathogenic *Y. enterocolitica* biotypes may be excluded because of the negative βGLU reaction (number 17) of the GN-ID card, even though the incubation temperature (37 °C) in Vitek2 is suboptimal for identification of *Yersinia* spp. Only one *Y. enterocolitica* BT1A was misclassified as βGLU-negative by Vitek2.

Currently, we routinely also subculture bull's eye colonies from CIN agar onto Orientation, egg yolk and cystine-lactose-electrolyte-deficient (BBL CLED, Becton Dickinson) agars. After overnight incubation, an isolate which is blue-green on Orientation, lipase-positive on egg yolk agar and indole-positive by spot indole test is presumptively identified as *Y. enterocolitica* BT1A. Most importantly, other *Y. enterocolitica* biotypes are rapidly excluded in this way. For definitive identification, we use biochemical tests as shown above. Unfortunately, apathogenic *Y. enterocolitica*-like strains still present significant challenges to *Yersinia* identification. *Y. frederiksenii* especially poses a threat to rapid YE BT1A identification because it is also βGLU-positive in Vitek2 and it grows indistinguishably from YE BT1A on Orientation and CAY agars. As shown here, observing growth characteristics of *Y. enterocolitica*-like species, e.g. *Y. bercovieri,* on non-selective chromogenic agars, may be of assistance in initial identification. Altogether, detection of βGlu activity of *Yersinia* spp. with chromogenic agars and even with the Vitek2 GN-ID card provides a straightforward way for presumptive identification of *Y. enterocolitica* BT1A in a routine clinical microbiology laboratory.

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