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CHROMagar Yersinia, a New Chromogenic Agar for Screening of Potentially Pathogenic *Yersinia enterocolitica* Isolates in Stools

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CHROMagar Yersinia (CAY) is a new chromogenic medium for the presumptive detection of virulent *Yersinia enterocolitica* in stools. Based on a comparative analysis of 1,494 consecutive stools from hospitalized patients, CAY was found to be just as sensitive as the reference medium (cefsulodin-irgasan-novobiocin agar) but was significantly more specific and had a very low false-positive rate. CAY reduces the workload (and thus costs) for stool analysis and can therefore be recommended for routine laboratory use.

In temperate and cold areas of the world, *Yersinia enterocolitica* is the third most common human bacterial enteropathogen (1). Although it mainly causes sporadic cases of gastroenteritis, outbreaks have been reported (2, 3). The species is subdivided into six biovars (1A, 1B, 2, 3, 4, and 5) and can also be separated into serogroups on the basis of the somatic (O) antigens, some of which are common to two or more biovars (2). However, the pathogenicity of the various serogroups of *Y. enterocolitica* is heterogeneous. When considering the 30 O serogroups defined by Wauters and coworkers (4, 5), most of the strains isolated from humans with diarrhea come from serogroups O:3, O:5,27, O:8 and O:9 (2). Despite the publication of a controversial report by Australian microbiologists 10 years ago (6), biovar 1A strains are generally considered to be nonpathogenic because they lack (in contrast to biovars 1B and 2 to 5) the pYV plasmid that is essential for bacterial virulence (2). This 70-kb extrachromosomal genetic element encodes (i) the Ysc type III secretion system that injects the Yop protein effectors that interfere with the eukaryotic host's immune signaling pathways (7) and (ii) the YadA adhesion/invasion protein that facilitates bacterial colonization of the intestinal mucosa (8).

Isolation of *Y. enterocolitica* from stools is fastidious since it is frequently outgrown by other *Enterobacteriaceae*. Partially selective media containing antimicrobial cocktails have been proposed in order to facilitate *Y. enterocolitica* recovery from fecal specimens, and the semiselective cefsulodin-irgasan-novobiocin (CIN) agar developed by Schiemann more than 30 years ago (9) is still the medium most widely used by medical microbiologists for this purpose. However, observation of the colony morphology (a red "bull's-eye" surrounded by a clear border, unfortunately not strictly specific for *Y. enterocolitica*) does not enable one to distinguish between pathogenic and nonpathogenic *Y. enterocolitica* isolates. This discrimination requires characterization of the biochemical properties associated with avirulence (i.e., esculin hydrolysis and pyrazinamidase activity, which are features of biovar 1A [10], or colony-PCR detection of pYV-encoded proteins [11]). With a view to selecting virulent *Y. enterocolitica* (VYE) colonies, Fukushima (12) developed VYE agar by adding esculin and ferric citrate to CIN. However, it was found that black esculin hydrolysis products produced by non-*Yersinia* colonies diffused within the medium and masked potentially virulent, esculin-negative *Y. enterocolitica* colonies (13).

Culture media incorporating enzyme substrates linked to heat-stable, water-soluble, indolyl chromophores (14) have become widely used in clinical laboratories. The cleavage of the substrate by specific enzymes in the target microorganism releases the chromophore and thus colors the growing microbial colonies. Hence, the use of chromogenic agars can eliminate the need for subcultures and can provide presumptive identification of a chosen microorganism. This approach shortens the turnaround time for microbial diagnosis, reduces the analytical workload, and generates cost savings relative to conventional methods for the isolation and identification of bacterial pathogens. A *Yersinia enterocolitica* chromogenic medium (YeCM) was reported by Weagant (13) in 2008 for quality control in the food industry. In fact, YeCM was shown to be just as sensitive as CIN (13) and was well suited for rapid detection of virulent *Y. enterocolitica* in the tonsils of slaughtered pigs (15), the main animal reservoir of strains that are pathogenic in humans (1, 2). However, to the best of our knowledge, YeCM has not been evaluated in a clinical microbiology setting in humans. CHROMagar Yersinia (CAY) is a new, selective, chromogenic medium for the presumptive identification of pathogenic *Y. enterocolitica* colonies. The latter are colored mauve after 48 h of incubation at 28°C (the species' optimal growth temperature), whereas nonpathogenic strains of the species and occasional isolates of other *Enterobacteriaceae* grow as metallic blue colonies after as little as 24 h of incubation at the same temperature. In the present study, we evaluated the performance of CAY relative to the CIN reference medium for the routine detection of pathogenic *Y. enterocolitica* in stools from patients with diarrhea.

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MATERIALS AND METHODS

Culture media. The proprietary product CAY was provided for evaluation by CHROMagar Microbiology (Paris, France) as prepared and in-

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TABLE 1 *Yersinia* stock isolates used in the present study

Species	Strains[a]	No. of strains	Colonial aspect on CAY[b]	
			Color	Size (mm)
Y. enterocolitica				
Biovar 1A	NEM7, LUMC3192, LUMC3847, RDH25383, RDH30963, RUMC646896, RUMC648126, RUMC663603	8	Metallic blue	1–3
Biovar 1B	8081, NEM6, WA, LUMC1497, LUMC 3100	5	Mauve	1–2
Biovar 2	NEM3, NEM5, LUMC1484, LUMC1503, LUMC1681, LUMC1682	6	Mauve	1–2
Biovar 3	IP383, IP864, LUMC1135, RDH29135, RDH30976	5	Mauve	1–2
Biovar 4	LUMC992, NEM1, NEM2, NEM4, LUMC4173, LUMC1491, LUMC1683, LUMC1996, LUMC4035, RDH25203, RDH28460, RDH29088, RDH30205	13	Mauve	1–2
Biovar 5	LUMC2491, LUMC1502, LUMC1680	3	Mauve	1–2
Y. pseudotuberculosis	IP1553, IP2775, IP2790, IP2926, YPIII, KM, ST, LUMC1136, LUMC1498, LUMC1499, LUMC1500, LUMC1501, LUMC1504, LUMC1505, LUMC1506, LUMC1507, LUMC2085, LUMC2149, RDH28967	19	No growth	No growth
Y. kristensenii	IP7577, IP7117, IP24418, LUMC1573, LUMC2147	5	No growth	No growth
Y. aldovae	LUMC2151, LUMC2152, LUMC2153, LUMC2219	4	No growth	No growth
Y. bercovieri	IP24495, IP24511, IP25595, RUMC521167	4	Mauve	1–2
Y. frederiksenii	IP14523, IP24548, IP24551	3	Metallic blue	1–3
Y. intermedia	IP24596, IP24604, IP24617	3	No growth	No growth
Y. mollaretii	IP24077, IP24084, IP24538	3	No growth	No growth

[a] IP, Institut Pasteur, Paris, France; LUMC, Lille University Medical Center, Lille, France; NEM, Necker Children's Hospital; RDH, Robert Debré Hospital, Paris, France; RUMC, Rennes University Medical Center, Rennes, France.
[b] After 48 h of incubation in air at 28°C.

cluded 85-mm plates containing 20 ml of colorless, transparent medium. As recommended by the manufacturer, CAY plates were stored at 4°C in the dark and used within 8 weeks. CIN and Mueller-Hinton (MH) media were purchased as commercially prepared plates (Oxoid, Dardilly, France). All plates were incubated in air at 28°C in the dark. Tryptic soy broth (Oxoid) was used for liquid cultures.

Stock isolates. Eighty-one stock *Yersinia* strains (representing 8 species) were used in this study (Table 1). There were 40 *Y. enterocolitica* strains.

Medium fertility. The capacity of CAY and CIN to grow *Y. enterocolitica* stock isolates was compared to that of nonselective MH medium. For this purpose, 100 μl of bacterial suspensions containing ca. 200 CFU, prepared from each of the 40 isolates, was plated simultaneously on the CAY, CIN, and MH media and then incubated at 28°C for 48 h before colonies were counted. The percentage of growth on CAY and CIN compared to MH was calculated as the ratio (×100) of the number of colonies on CAY or CIN to the number of colonies on MH.

Clinical samples. Between November 2011 and March 2012, we analyzed all diarrheic stools from inpatients and outpatients (one sample per patient) at Lille University Medical Center. A standardized sample volume (50 μl of liquid stool or stool liquefied in saline solution) was streaked onto CAY and CIN plates. All plates were incubated in the dark at 28°C in air for 48 h. All suspect colonies (i.e., mauve colonies measuring 1 to 2 mm in diameter on CAY and red "bull's-eye" colonies on CIN) were identified.

Bacterial identification. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was performed on a Microflex system (Brüker Daltonics, Wissembourg, France), as previously described (16) for the identification of bacterial colonies. When MALDI-TOF MS did not provide sufficient interspecies discrimination, identification was performed by colony-PCR amplification and sequencing of the 16S rRNA genes using the fD1/rp2 primers designed by Weisburg et al. (17). All clinical isolates of *Y. enterocolitica* were screened for the *yadA* gene, using the PCR assay described by Blais and Phillippe (18).

A protocol reported by Robins-Browne was adapted for *Y. enterocolitica* biotyping (19). Briefly, we studied esculin and Tween 80 hydrolysis, nitrate reductase and pyrazinamidase activities, indole production from tryptophan, and D-xylose use. Pathogenic (*yadA*-positive) *Y. enterocolitica* isolates were serotyped by slide agglutination using O:3, O:5, O:8, O:9, and O:27 (SIFIN, Berlin, Germany).

Statistical analyses. Intermedium differences in fertility and specificity were evaluated with a Wilcoxon test and a Yates-corrected χ^2 test, respectively. The threshold for statistical significance was set to $P < 0.05$.

RESULTS AND DISCUSSION

We first evaluated CAY's ability to support the growth of *Yersinia* spp. All 40 *Y. enterocolitica* strains grew on CAY; the 32 pathogenic strains (biovars 1B and 2 to 5) produced colorless colonies after 24 h of incubation and mauve colonies (1 to 2 mm in diameter) after 48 h (Fig. 1A). In contrast, the 8 nonpathogenic (biovar 1A) strains grew as metallic blue colonies (1 to 3 mm in diameter) after both 24 and 48 h of incubation (Fig. 1B). Next, we compared the growth of the 32 non-1A strains on CAY and CIN, using nonselective MH agar as a reference (100% growth). Table 2 shows that CAY was slightly less inhibitory ($P = 0.06$) than CIN for *in vitro* growth of pathogenic *Y. enterocolitica*.

The genus *Yersinia* includes another enteropathogenic species (*Y. pseudotuberculosis*) and other species seldom isolated from human stools but which are generally regarded as nonpathogenic (*Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. mollaretii*, *Y. aldovae*,

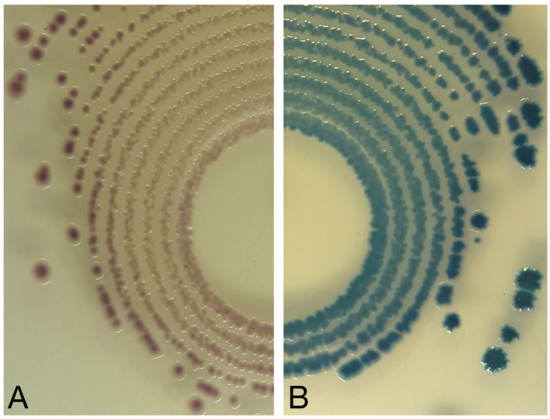

FIG 1 Colonies of *Y. enterocolitica* on CHROMagar Yersinia after a 48-h incubation at 28°C. Note the growth of both a pathogenic isolate (A; mauve colonies) and a nonpathogenic isolate (B; metallic blue colonies).

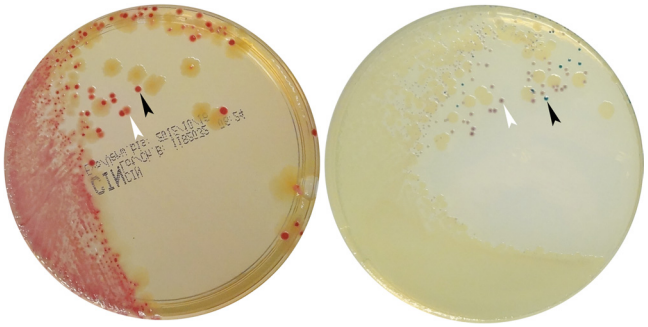

FIG 2 Colonies plated on cefsulodin-irgasan-novobiocin (CIN) agar (left) and CHROMagar *Yersinia* (CAY; right) from a stool specimen containing *Citrobacter freundii*, *Morganella morganii*, and pathogenic *Yersinia enterocolitica*. On CAY, mauve colonies of pathogenic *Y. enterocolitica* (white arrowheads) can be readily distinguished from blue-green colonies of *C. freundii* (black arrowheads) after 48 h of incubation at 28°C.

and *Y. bercovieri*). None of the 19 tested *Y. pseudotuberculosis* strains grew on CAY. Similarly, *Y. kristensenii* ($n = 5$), *Y. aldovae* ($n = 4$), *Y. intermedia* ($n = 3$), and *Y. mollaretii* ($n = 3$) did not grow on CAY. In contrast, *Y. frederiksenii* ($n = 3$) and *Y. bercovieri* ($n = 4$) yielded colonies that looked the same as nonpathogenic (metallic blue) and pathogenic (mauve) *Y. enterocolitica* colonies, respectively (Table 1).

CAY and CIN agar were then compared prospectively for the routine isolation and presumptive identification of pathogenic *Y. enterocolitica* in a medical laboratory. In all, 1,494 consecutive diarrheic stool samples were plated in parallel on the two media. Cultures were examined by a single operator after 48 h. From the 1,494 stools analyzed, we isolated a total of six pathogenic (*yadA*-positive and esculin- and pyrazinamidase-negative) isolates on CAY, which all grew as typical mauve colonies. These pathogenic isolates variously belonged to bioserovars 4/O:3 ($n = 2$), 2/O:9 ($n = 1$), and 5/O:3 ($n = 3$). There were 14 false positives on CAY, corresponding to colonies of *Stenotrophomonas maltophilia* (number of stool specimens [n] = 9), *Shewanella putrefaciens* ($n = 1$), *Y. bercovieri* ($n = 1$), and *Brevundimonas terrae* (a species previously unknown in this setting; $n = 3$). In contrast, culture on CIN revealed 12 typical *Y. enterocolitica* isolates, of which six were the *yadA*-positive, esculin- and pyrazinamidase-negative isolates also identified on CAY. The remaining six were *yadA* negative, esculin and pyrazinamidase positive, belonged to biovar 1A, and had grown on CAY as blue colonies; they had been isolated in a clinical setting that did not suggest yersiniosis. Besides those six nonpathogenic *Y. enterocolitica* isolates, 137 false-positive results were observed on CIN, which corresponded to colonies of *Citrobacter freundii* ($n = 88$), *Providencia stuartii* ($n = 23$), *Providencia rettgeri* ($n = 12$), *Aeromonas* spp. ($n = 12$), *Acinetobacter johnsonii* ($n = 1$), and *Achromobacter xylosoxidans* ($n = 1$).

TABLE 2 Growth of *Yersinia enterocolitica* stock isolates on CHROMagar Yersinia and CIN agar compared to MH agar

Biovar	No. of strains	% growth relative to MH on[a]: CAY	CIN	*P*
Nonpathogenic				
1A	8	60 ± 26[a]	39 ± 39	NS[b]
Pathogenic				
1B	5	32 ± 34	21 ± 21	NS
2	6	52 ± 37	48 ± 41	NS
3	5	43 ± 24	37 ± 33	NS
4	13	44 ± 38	30 ± 31	NS
5	3	27 ± 23	22 ± 39	NS
All non-1A	32	60 ± 33	32 ± 32	0.06

[a] Values are means ± standard deviations.
[b] NS, not significant.

Here, we found that the new chromogenic medium CAY is well suited to routine laboratory screening for pathogenic *Y. enterocolitica* in stools (Fig. 2). Compared to CIN, CAY did not detect nonpathogenic isolates, thus limiting the risk of inappropriate antibiotic prescription. Although the composition of CAY is a trade secret held by CHROMagar, a pathogenic (pYV-positive) wild-type strain, 8081, and its isogenic, plasmid-cured (i.e., nonpathogenic) derivative both yielded mauve colonies on the medium (data not shown). This finding indicates that the chromogenic reaction targets chromosomally encoded *Y. enterocolitica* enzymes. By studying almost 1,500 consecutive stool samples, we found that CAY's specificity (99%) was significantly greater than that of CIN (90.4%) ($P < 0.001$). Furthermore, CAY had an approximately 10-fold-lower false-positive rate than CIN. Whereas the use of CIN agar requires complementary tests to establish whether *Y. enterocolitica* isolates are pathogenic or not, the CAY medium detects pathogenic *Y. enterocolitica* directly and generates few false positives (since the main species *S. maltophilia* and *Y. bercovieri* are infrequently encountered in clinical stool analysis). Six pathogenic *Y. enterocolitica* isolates (0.4% of all stool specimens) were identified by use of CAY and CIN media. This low incidence most likely reflects the time of year of stool collection (November to March), since the occurrence of human *Y. enterocolitica* infection is known to be higher during summer and fall. The equal number of isolates suggests that CAY and CIN have the same sensitivity. However, our preliminary experiments showed that CAY was slightly less inhibitory than CIN for the growth of pure suspensions of *Y. enterocolitica*.

According to a recent report from the European Union (1), the other enteropathogenic *Yersinia* species (*Y. pseudotuberculosis*) causes approximately 100-fold fewer human infections than *Y.*

enterocolitica does. This disparity might be related not only to epidemiological differences between the two microorganisms but also to less efficient recovery of *Y. pseudotuberculosis* from feces leading to underestimation of the incidence of human pseudotuberculosis. Indeed, it has been shown that the growth of some *Y. pseudotuberculosis* serotypes (particularly II and III) was impaired on CIN agar (20, 21) due to the presence of cefsulodin in this selective culture medium (20). This is also a weakness of CAY, since none of the tested *Y. pseudotuberculosis* strains grew on this new medium. It would be worth reformulating the medium so that it would be selective for both enteropathogenic *Yersinia* species. Such a modification would entail a new evaluation of the medium.

In conclusion, the new chromogenic medium CAY is just as sensitive as CIN agar but is significantly more specific. It can be recommended for the routine screening of pathogenic *Y. enterocolitica* in human diarrheic stools.

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