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RESEARCH ARTICLE

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Identification of *Vibrio* spp. (other than *V. vulnificus*) recovered on CPC agar from marine natural samples

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Summary Two hundred and eighty four presumptive but not confirmed *Vibrio vulnificus* isolates grown on cellobiose-polymixin B-colistin agar (CPC) at 40°C, recovered from sea water samples from Valencia, Spain, during a microbiological survey for *V. vulnificus*, were phenotypically identified. Most of the isolates (91%) corresponded to *Vibrio* species. *V. harveyi* (24%) and *V. splendidus*(19%) were the most abundant species identified, followed by *V. navarrensis* (13%), *V. alginolyticus* (8%) and *V. parahaemolyticus* (5%). The ability to grow on CPC agar and ferment cellobiose of several *V. vulnificus* strains from different origins and serovars, including reference strains, was tested. Most serovar E isolates and 25% of non-serovar E isolates could not grow on CPC agar.

Key words *Vibrio* spp. · *Vibrio vulnificus* · Marine bacteria · Phenotypic characterization · CPC agar

Introduction

Among the different selective media frequently used to recover *V. vulnificus*, cellobiose-polymixin B-colistin agar (CPC) has been especially recommended for the isolation of *V. vulnificus* from environmental samples, either with its original composition [1, 7, 10, 12, 15, 16] or with a reduced concentration of colistin [8, 17, 18]. Direct streaking on CPC agar has also been employed to estimate densities of *V. vulnificus* in sea water and in shellfish samples [6].

In a recent survey on *V. vulnificus*, Arias et al. used CPC agar and thiosulfate-citrate-bile-sucrose agar (TCBS) to recover this pathogenic species in sea water and shellfish from the Spanish Mediterranean coast, combining a culture-based approach with a DNA-based technique (PCR) with specific primers to carry out a rapid and reliable identification of the presumptive isolates [2]. In that study, *V. vulnificus* was detected for the first time in the Spanish Mediterranean coast, but only 7.6% of the colonies grown showing the typical morphology of *V. vulnificus* were confirmed by specific PCR, and no attempt was made to identify those colonies that were not confirmed by PCR. Therefore, we performed the identification of a representative number of those strains recovered on CPC in the mentioned study [2] that had the same appearance as *V. vulnificus*, but had been not further confirmed. We aimed to check if that low percentage of recovery was due to poor accuracy of the PCR method used or to the presence of other competing bacteria able to yield the typical morphology of *V. vulnificus* on CPC.

For this purpose, we randomly selected 284 strains corresponding to presumptive but not confirmed *V. vulnificus* obtained in the previous study on CPC agar [2], and identified them by previously described phenotypic procedures [4]. Ten *Vibrio* species could be identified (Table 1): *V. harveyi*, *V. splendidus*, *V. navarrensis*, *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. anguillarum*, *V. tubiashii*, *V. pelagius* and *V. proteolyticus*. None of the PCR-negative isolates tested was ascribed to *V. vulnificus*. Fifty-seven isolates remained as unidentified *Vibrio* spp. These unidentified strains were positive for arginine dehydrolase test and only a few of them were decarboxylase negative. In addition, a low percentage of *Enterobacteriaceae* were identified by API 20E and a few non fermenters remained unidentified. The most abundant species was *V. harveyi*, which accounted for 24% of the isolates, followed by *V. splendidus*, *V. navarrensis*, *V. alginolyticus*, *V. parahaemolyticus*. The rest of species accounted each one for less than 1% of the isolates. *V. harveyi* and *V. splendidus*

are the most abundant species in the studied environment during the warm and cold seasons respectively [4], and can competitively displace other species with lower salinity requirements, such as *V. vulnificus* and *V. parahaemolyticus*. This is coherent with the low incidence of these two species in the mentioned environment [4, 13, 14]. It seems, therefore, that Mediterranean high salinity values are more favorable for the development of other *Vibrio* species, so that the numbers of *V. vulnificus* remain nearly in the threshold limit of detection by the techniques employed [2]. *V. alginolyticus*, the fourth most abundant species, has also been frequently isolated from this environment [13, 14]. On the contrary, the presence of *V. navarrensis* was unexpected, since only a few strains from freshwater environments have been described so far [20]. A few *Enterobacteriaceae* were identified as well, *Serratia* sp., *Citrobacter* sp., and *Proteus* sp. being the predominant. These isolates that grew on CPC agar came from one sea water sample with unusually high levels of fecal coliforms. Note that two of the identified species, *V. alginolyticus* and *V. parahaemolyticus*, are unable to use cellobiose as sole carbon and energy source [5]. This excess of other competing *Vibrio* species that include cellobiose-positive strains obviously reduces the ability of CPC agar to select and properly differentiate the few isolates corresponding to *V. vulnificus*, and constitutes a shortcoming of this medium for environmental samples where this species is minoritary.

Table 1 Species, other than *Vibrio vulnificus*, recovered on CPC agar from natural marine samples

Species	No. strains	% total
Vibrio spp.:		
V. harveyi	67	24
V. splendidus	54	19
V. navarrensis	38	13
V. alginolyticus	22	8
V. parahaemolyticus	14	5
V. fluvialis	2	0.7
V. anguillarum	2	0.7
V. tubiashii	2	0.7
V. pelagius	1	0.3
V. proteolyticus	1	0.3
Unidentified *Vibrio spp.*	57	20
Non-*Vibrio*:		
Non- fermenters	4	1.5
Enterobacteria	20	7
TOTAL	284	100

CPC agar was described by Massad and Oliver [10] as a highly selective and differential medium for *V. cholerae* and *V. vulnificus*. They tested its selectivity with 19 *Vibrio* species and some other genera, and found that, when incubated at 40°C, only one strain out of nine of *V. parahaemolyticus* could grow. In later field studies, several authors have used CPC agar or a modified CPC medium, with a reduced concentration of colistin methanesulfonate (mCPC), for the isolation and/or enumeration of *V. vulnificus* from sea water and shellfish samples in different geographical areas [8, 12, 15–18]. In some of these studies, cellobiose-positive colonies were hybridized with a DNA probe for the cytotoxin-hemolysin gene previously described for the identification of *V. vulnificus* by Morris et al. [11], and only 28.7% of the colonies were positive [12]. Morris et al. found that some cellobiose positive strains on CPC could be *V. fluvialis*/*V. harveyi*. In a recent study, Høi et al. [9] reported the use of an improved selective medium, cellobiose-colistin (CC) agar, which makes polymyxin B unnecessary, since this antibiotic and colistin have the same antimicrobial activities. The colistin concentration was the same than in mCPC agar. They found that this modified new medium (CC) improved significantly the isolation rates of *V. vulnificus* from water and sediment samples with respect to mCPC agar. Nevertheless, the reduced concentration of antibiotics in CC agar does not inhibit completely the competing and interfering *Vibrio* species.

We observed that some *V. vulnificus* strains, mostly isolates from diseased eels, were unable to grow on CPC. This could cause an underestimation of the presence/incidence of this species when CPC is used. Therefore, we tested the ability of 125 confirmed *V. vulnificus* strains from our own collection [3], including reference and clinical strains, to grow on this selective medium. All *V. vulnificus* isolates were grown in marine broth (MB) at 28°C for 24 h and were spot-inoculated by a multipoint inoculator onto CPC and incubated at 40°C for 24 h. Any growth as typical cellobiose positive, was considered positive, even those due to a few colonies on the spot. The test was performed in duplicate, using two batches of CPC agar prepared independently. Most diseased eel isolates (serovar E) and 25% of environmental and clinical isolates (non serovar E) failed to grow (Table 2). The lack of growth of *V. vulnificus* from diseased eels could be explained by the high incubation temperature (40°C), close to the maximal temperature of these strains [19]. This finding reveals that the ability to grow on CPC agar is not shared by all *V. vulnificus* strains, as had already been reported by Høi et al. [9], who found that several eel pathogenic strains and a few clinical ones showed MICs identical to or below the concentration of colistin in CPC agar.

Table 2 Growth of *Vibrio vulnificus* strains on CPC agar

Vibrio vulnificus[a]	No. strains tested	Show typical growth (%)
Non-serogroup E isolates		
Clinical isolates	6	4 (67%)
Environmental strains	85	64 (75%)
Serogroup E isolates	34	3 (9%)
TOTAL	125	71 (57%)

[a]Strains are listed in reference [3].

From the results obtained in the present study and in previous ones on *V. vulnificus* in the Mediterranean coast we can conclude: (i) The specificity of CPC agar is low when the samples contain large amounts of other cellobiose-positive *Vibrio* spp. such as *V. harveyi* and *V. splendidus*; this can also

happen with minoritary species, such as *V. navarrensis*, that are cellobiose positive. (ii) Some strains of *Vibrio* species which are not able to use cellobiose as sole carbon and energy source (*V. parahaemolyticus* and *V. alginolyticus*) can grow with the typical morphology of *V. vulnificus* on directly inoculated CPC plates, which suggests the low accuracy of this medium and its low discriminatory capability. (iii) Not all *V. vulnificus* strains tested can grow on CPC agar. Our results have shown that the confirmation of *V. vulnificus* by PCR using specific primers directed against 23S rRNA sequences, as described previously [2], was a reliable technique since in the present study none of the PCR-negative isolates corresponded to *V. vulnificus*. Therefore, the low selectivity of CPC agar for *V. vulnificus* isolated from samples of the Spanish Mediterranean coast can be explained by the presence of high numbers of other competing *Vibrio* species better adapted to this particular environment, which are not successfully inhibited. Note that, not until a combination of CPC agar followed by specific confirmation by PCR was used, was it possible to detect the presence of this pathogenic species at the Mediterranean Sea, and to test the efficiency of different molecular typing techniques [2, 3].

Our overall data stress the need of carrying out some reliable confirmation of the presumptive *V. vulnificus* colonies grown on CPC agar before assuming the presence and/or abundance of this species in a sample. Furthermore, the absence of growth on CPC agar should not be interpreted as a safety guarantee, especially when the presence of eel pathogenic strains is suspected or searched for.

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