Food Control xxx (2010) 1–4

Contents lists available at ScienceDirect

Food Control

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

# Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating *Vibrio parahaemolyticus*

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ARTICLE INFO

*Article history:*
Received 21 January 2010
Received in revised form
28 May 2010
Accepted 8 June 2010

ABSTRACT

Considering its widespread distribution in marine environments, its fast replication times and low infectious doses and the rapid spread of its strains in recent years, intensive and continuous monitoring of potentially pathogenic *Vibrio parahaemolyticus* is strongly recommended in order to assess the human health risk arising from shellfish consumption. The lack of epidemiological data points to the need to develop specific methods for detecting *V. parahaemolyticus*. In this note, the authors compare two plating media currently available for isolating *V. parahaemolyticus* in shellfish. Both approaches involve pre-enrichment of *V. parahaemolyticus*. One uses thiosulphate-citrate-bile salt sucrose (TCBS) as the isolation medium, while the other uses a chromogenic medium (CHROMagar Vibrio). Next, biochemical identification of isolates was performed with API 20E, followed by PCR assay aimed at the *tox*R gene to confirm the cultural and biochemical identification. Comparison of the two methods highlighted that CHROMagar Vibrio is more accurate and specific than TCBS. The analysis of data from 160 shellfish samples showed an accuracy and specificity of just 51% and 71% for TCBS compared with 88% and 95% for CAV.


## 1. Introduction

*Vibrio* species account for a significant proportion of human infections associated with the consumption of raw or undercooked shellfish. One of the members of genus *Vibrio*, i.e. *Vibrio parahaemolyticus*, is a foodborne pathogen causing worldwide health problems. It is a common cause of seafood-borne illness in many Asian countries. *V. parahaemolyticus* infections have been reported to the Centers for Disease Control and Prevention (CDC) (Daniels et al., 2000). In European countries, outbreaks have been reported in Spain and France (Su & Liu, 2007).

Among conventional culture-based methods, selective enrichment with alkaline peptone water (APW) followed by plating onto thiosulphate-citrate-bile salt sucrose (TCBS) agar are common media used for isolating *Vibrio* species, which are facultative anaerobic and grow best under alkaline conditions and in the presence of relatively high levels of bile salts. The identification of suspect isolates is performed using the rapid diagnostic kit API 20E.

However, *V. parahaemolyticus* colonies on TCBS agar are difficult to distinguish visually from other bacterial colonies, since they can be covered by a yellow color produced by sucrose-fermenting bacteria (Hara-Kudo et al., 2001). Indeed, the fermentation of sucrose by *Vibrio alginolyticus* interfered with the detection of *V. parahaemolyticus* colonies because the latter appeared yellowish and similar to colonies of *V. alginolyticus* on TCBS agar (Kourany, 1983). In direct plating of seawater and other specimens from the marine environment, a medium containing tryptone soy agar added to sodium chloride, sucrose, bile salts and triphenyltetrazolium chloride (TSAT) improved differentiation of *V. parahaemolyticus* from *V. alginolyticus* (Kourany, 1983). Several presumptive positive colonies need to be analyzed with biochemical tests to confirm the presence of *V. parahaemolyticus*, all of which increases analysis costs and timeframes. Moreover, the results are often unreliable (Croci et al., 2007).

Several alternative DNA-based methods have been described for detecting *V. parahaemolyticus* from shellfish (Di Pinto, Ciccarese, De Corato, Novello, & Terio, 2008; Su & Liu, 2007). However, epidemiological studies require the availability of accurate culture methods for isolating *V. parahaemolyticus* in foods to establish effective control measures, as well as to ensure seafood safety according to Reg. EC 2073/2005.

Considering the important public health concerns, this paper compares two plating media currently available for isolating *V. parahaemolyticus* in shellfish. The approaches consisted of sample preparations by pre-enrichment in APW, followed by isolation

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doi:10.1016/j.foodcont.2010.06.013

using TCBS and CHROMagar Vibrio (CAV). Biochemical identification of isolates was then performed with API 20E. A PCR assay aimed at the *tox*R gene was carried out to confirm the cultural and biochemical identification.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*V. parahaemolyticus* ATCC 33845 and a laboratory collection *V. parahaemolyticus* strain were used as positive controls, with *V. alginolyticus* ATCC 33839 as negative control. The reference strains were grown on TSA (Tryptone Soy Agar) (Oxoid, Hampshire, UK) supplemented with 3% (w/v) NaCl and incubated at 37 °C for 24 h.

### 2.2. Sampling

One hundred and sixty shellfish samples from different purification centers in Apulia (SE Italy) were collected between July 2008 and June 2009. Each sample consisting of 10 mussels (*Mytilus galloprovincialis*) was stored at 6 °C, transported to the microbiology laboratory and processed immediately.

### 2.3. Isolation and biochemical identification of V. parahaemolyticus

#### 2.3.1. Preparation of test sample

Shellfish specimens were scrubbed, washed in water containing 50 ppm active $Cl_2$ and rinsed with sterile distilled water. Analytical portions (25 g) of shellfish bodies and intervalve water were aseptically removed and collected in a sterile bag with 225 ml of alkaline saline peptone water (ASPW) pH 8.6 (Oxoid, Hampshire, UK). According to the recent ISO/TS 21872-1:2007 (E) indications for fresh products, the samples were homogenized using a stomacher (PBI International, Milan, Italy) at 11000 rev $min^{-1}$ for 3 min and incubated at 42 °C for 6 h. A further enrichment was performed employing 1 ml of the first enrichment and 9 ml of ASPW. This broth culture was incubated at 42 °C for 18 h.

#### 2.3.2. TCBS isolation and biochemical identification of V. parahaemolyticus

The enrichment cultures from incubation, one at 42 °C for 6 h and the other at 42 °C for 18 h were plated onto thiosulphate-citrate-bile salt sucrose (TCBS) (Oxoid, Hampshire, UK) agar and incubated at 37 °C for 24 h. Five green, blue–green or yellow–green colonies, 2–3 mm in diameter on TCBS agar plates, presumptively selected as *V. parahaemolyticus* colonies, were transferred onto Nutrient Agar plates (Oxoid, Hampshire, UK) added to 5 g/l NaCl to bring it to a final concentration of 1% and incubated at 37 °C for 24 h according to ISO/TS 21872-1:2007 (E).

#### 2.3.3. Vibrio CHROMagar isolation

Two separate platings of enrichment culture onto Vibrio CHROMagar plates (PBI International, Milan, Italy) were carried out and the plates were incubated at 37 °C for 24 h. Five mauve colonies, 2–3 mm in diameter on CHROMagar plates, presumptively selected as *V. parahaemolyticus* colonies, were transferred to Nutrient Agar plates (Oxoid, Hampshire, UK) added to 5 g/l NaCl to bring it to a final concentration of 1% and incubated at 37 °C for 24 h according to ISO/TS 21872-1:2007 (E).

#### 2.3.4. Biochemical identification of V. parahaemolyticus

After incubation at 37 °C for 24 h, the isolates were subjected to the Gram stain, the oxidase test using Oxidase Sticks (Oxoid, Hampshire, UK), Triple-Sugar-Iron (TSI) (Oxoid, Hampshire, UK) and biochemical identification with API 20E (bioMérieux, Marcy l'Étoile, France) according to Di Pinto et al., 2008. The identification profiles were obtained by the APIweb software (bioMérieux, Marcy l'Étoile, France).

### 2.4. Biomolecular analysis

#### 2.4.1. Identification of V. parahaemolyticus isolates by toxR-PCR

The colony identified through the biochemical system as *V. parahaemolyticus* was transferred onto Nutrient Agar (Oxoid, Hampshire, England) and incubated at 37 °C for 24 h. Next, they were transferred into Tryptone Soya Broth (TSB) (Oxoid, Hampshire, England) with 1% NaCl incubated at 37 °C for 24 h in order to obtain the culture-broths, which were subjected to DNA extraction and purification with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Finally, the DNA was eluted with 80 μl of AE Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and purity were established by evaluating the ratio $A_{260\ nm}/A_{280\ nm}$ using a Beckman DU-640B Spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The oligonucleotide primers used, 5′-GTCTTCTGACG-CAATCGTTG-3′ (forward) and 5′-ATACGAGTGGTTGCTGTCATG-3′ (reverse) described by Kim et al. (1999) amplify a 368 bp amplicon of toxR gene. The primers were synthesized by PRIMM Srl (Milan, Italy). The PCR reactions were performed in a final volume of 25 μl, using 12.5 μl of HotStarTaq Master Mix 2× (QIAGEN, Hilden, Germany), containing 2.5 units of HotStarTaq DNA Polymerase, 1.5 mM of $MgCl_2$ and 200 μl of each dNTP, 1 μM of each oligonucleotide primer and 3 μl of DNA. The amplification profile involved an initial denaturation step at 95 °C for 15 min, followed by 30 cycles consisting of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C. The PCR reactions were processed in a Mastercycler Personal (Eppendorf, Milan, Italy). All reactions were performed in triplicate.

#### 2.4.2. Detection of V. parahaemolyticus from enrichment broth

One milliliter of ASPW enrichment broth from each shellfish sample was centrifuged with an Eppendorf 5415R (Eppendorf, Milan, Italy) at 13000 g for 5 min at room temperature. The cell pellets were subjected to DNA extraction as described above. A 3 μl aliquot of each sample was used for PCR amplification. All reactions were performed in triplicate.

#### 2.4.3. Detection of amplified products

PCR amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1× TBE buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with ethidium bromide (0.5 μg/ml). A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf, Milan, Italy).

### 2.5. Data analysis

Data analysis was performed according to Duan and Su (2005). Results from TCBS agar and CAV were compared with *tox*R-PCR results from enrichment broth considered as standard method for detection. The accuracy and specificity were then established respectively as Accuracy (%) = 100 × [True positive/(True positive + False positive + False negative)], Specificity (%) = 100 × [True negative/(True negative + False positive + False negative)].

## 3. Results

### 3.1. TCBS isolation and biochemical identification of V. parahaemolyticus

TCBS plating showed 2–3 mm green, blue–green or yellow–green colonies in 10/160 (6.3%) shellfish samples. The fifty suspect

Please cite this article in press as: Di Pinto, A., et al., Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating *Vibrio parahaemolyticus*..., Food Control (2010), doi:10.1016/j.foodcont.2010.06.013

**Table 1**
Data analysis.

| Agar medium | Presumptive positive samples (colonies) with culture method | API20E-positive shellfish samples (isolated colonies) | *tox*R-PCR-positive shellfish samples (isolated colonies) | False-positive samples | False-negative samples | Accuracy | Specificity |
|---|---|---|---|---|---|---|---|
| TCBS | 10 (50) | 6 (14) | 4 (4) | 2 | 43 | 51% | 71% |
| CAV | 43 (215) | 41 (201) | 41 (194) | 0 | 6 | 88% | 95% |

colonies (five from each sample) were subjected to Gram stain, oxidase test, TSI test and biochemical identification with API 20E. Then, 14 of the 50 isolates resulted *V. parahaemolyticus* positive, corresponding to 6/160 (3.8%) shellfish samples. Biochemical analysis gave identification profiles with an identification percentage of between 52.2% and 99.9%. The remaining 36 colonies resulted *V. alginolyticus* positive. Subsequently, *tox*R-PCR carried out on the 14 isolates identified as *V. parahaemolyticus* confirmed positivity in 4/14 isolates, corresponding to 4/160 (2.5%) shellfish samples only, while the other colonies were *tox*R-PCR negative. Also the other 36 isolates identified *V. alginolyticus* resulted *tox*R-negative. This indicates that two false-positive shellfish samples were detected using TCBS plating.

### 3.2. Vibrio CHROMagar isolation and biochemical identification of V. parahaemolyticus

The mauve colonies on CHROMagar Vibrio plates were isolated in 43/160 (26.9%) shellfish samples. The Gram stain, oxidase test, TSI test and biochemical identification with API 20E detected *V. parahaemolyticus* in 201/215 isolates with an identification percentage of between 75.5% and 99.9%, corresponding to 41 samples (25.6%). The other isolates were identified as *Aeromonas salmonicida*, *Aeromonas hydrophyla* and *Vibrio fluvialis*. In the next step, *tox*R-PCR resulted positive in 194/201 isolates, corresponding to 41/160 (25.6%) shellfish samples, confirming the results obtained with CHROMagar Vibrio plating and API 20E detection. This indicates that no false-positive shellfish samples were detected by CHROMagar Vibrio. The other 14 isolates identified as *A. salmonicida*, *A. hydrophyla* and *V. fluvialis* resulted *tox*R-negative.

### 3.3. Detection of V. parahaemolyticus from ASPW enrichment broth

The PCRs performed on enrichment broth from each shellfish sample gave positive results for *V. parahaemolyticus* in 47/160 shellfish samples (29.4%).

### 3.4. Data analysis

Given the assumption that the toxR-PCR from ASPW enrichment broth considered as standard method for *V. parahaemolyticus* detection, the analysis of data performed according to Duan and Su (2005) showed that CAV is more accurate and specific than TCBS agar, as reported in Table 1.

## 4. Discussion

Among *Vibrio* species, *V. parahaemolyticus* is known to be the cause of much foodborne disease in the world (Su & Liu, 2007). Several outbreaks of *V. parahaemolyticus* infection associated with shellfish consumption have highlighted the need to enforce guidelines, in order to guarantee public health. Considering its widespread distribution in marine environments, its fast replication times, and the rapid spread of its strains in recent years, intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* is strongly recommended in order to assess the human health risk arising from shellfish consumption. According to Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs, there is a need to develop reliable methods for detecting *V. parahaemolyticus* in order both to establish specific microbiological criteria in live bivalve shellfish and to implement a specific health plan. Stringent sanitary control measures require accurate and specific methods for the detection of *V. parahaemolyticus* in shellfish, ensuring efficient control and prevention planning.

Conventional culture methods based on plating and isolating *V. parahaemolyticus* are time-consuming, require intensive workloads and are rather insensitive due to numerous interfering species which inhibit *V. parahaemolyticus* detection. The most common culture method for detecting *V. parahaemolyticus* in foods uses TCBS. However, it does not provide a good growth medium, nor does it effectively isolate *V. parahaemolyticus* (Croci et al., 2007). In fact, it cannot differentiate *V. parahaemolyticus* from some strains of *Vibrio vulnificus* or *Vibrio mimicus* (Su & Liu, 2007). Compared with TCBS agar, CAV provided higher specificity and accuracy of approximately 95% and 88% respectively.

In fact, the CAV medium easily differentiates *V. parahaemolyticus* from *V. vulnificus*, *Vibrio cholerae* and other *Vibrios* directly at the isolation step on the basis of colony color, with greater sensitivity than conventional TCBS agar. No *V. alginolyticus* was isolated in Vibrio CHROMagar from the samples, it being easy to differentiate the mauve *V. parahaemolyticus* colonies chromatically from the more colorless *V. alginolyticus* colonies. On CAV, *V. parahaemolyticus* colonies are mauve, *V. vulnificus* and *V. cholerae* colonies appear blue, while *V. alginolyticus* colonies are colorless. Moreover, CAV displayed the colonies better, highlighting a higher number of *V. parahaemolyticus* colonies than TCBS plates.

Confirming previous findings (Croci et al., 2007; Popovic, Coz-Rakovac, & Strunjak-Perovic, 2007), this study highlights the fact that biochemical tests used to facilitate identification of suspect isolates are of limited importance for identifying and differentiating some bacterial species and give only presumptive data. In particular, the API 20E system leads to several misidentifications, with only few reaction profiles being accepted. Although these kits are fairly easy to use and can quickly identify *V. parahaemolyticus*, they need to be compared with the PCR-based methods to confirm the data and/or exclude an erroneous biochemical identification.

Additionally, unlike the TCBS medium, the CAV medium may accurately and reliably isolate *V. parahaemolyticus* for DNA-based typing analysis, a good approach to overcome the deficiencies in current knowledge regarding the incidence/frequency of pathogenic *V. parahaemolyticus* in water and in shellfish, in order to carry out risk assessment for pathogenic *V. parahaemolyticus.*

## Acknowledgments

The authors would like to thank Anthony Green for his valuable assistance in correcting the manuscript.

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Please cite this article in press as: Di Pinto, A., et al., Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating *Vibrio parahaemolyticus*..., Food Control (2010), doi:10.1016/j.foodcont.2010.06.013