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Development of a more sensitive and specific chromogenic agar medium for the detection of Vibrio parahaemolyticus and other Vibrio species

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Abstract:	Foodborne infections in the US caused by Vibrio species have shown an upward trend. In the genus Vibrio, V. parahaemolyticus is responsible for the majority of Vibrio-associated infections. Thus, accurate differentiation among Vibrio spp and detection of V. parahaemolyticus is critically important to ensure the safety of our food supply. Although molecular techniques are increasingly common, culture-depending methods are still routinely done and they are considered standard methods in certain circumstances. Hence, a novel chromogenic agar medium was tested with the goal of providing a better method for isolation and differentiation of clinically relevant Vibrio spp. The protocol compared the sensitivity, specificity and detection limit for the detection of V. parahaemolyticus between the new chromogenic medium and a conventional medium. Various V. parahaemolyticus strains (n=22) representing diverse serotypes and source of origins were used. They were previously identified by Food and Drug Administration (FDA) and Centers for Disease Control and Prevention (CDC), and further verified in our laboratory by tlh-PCR. In at least four separate trials, these strains were inoculated on the chromogenic agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which is the recommended medium for culturing this species, followed by incubation at 35-37 oC for 24-96 hr. Three V. parahaemolyticus strains (13.6%) did not grow optimally on TCBS, nonetheless exhibited green colonies if there was growth. Two strains (9.1%) did not yield the expected cyan colonies on the chromogenic agar. Non-V. parahaemolyticus strains (n=32) were also tested to determine the specificity of the chromogenic agar. Among these strains, 31 did not grow or exhibited other colony morphologies. The mean recovery of V. parahaemolyticus on the chromogenic agar was ~96.4% relative to tryptic soy agar supplemented with 2% NaCl. In conclusion, the new chromogenic agar is an effective medium to detect V. parahaemolyticus and to differentiate it from other vibrios.
Author Comments:	

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Question	**Response**
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Cover Letter

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Jan 11, 2016

Dear Sir/Madam,

I am excited to share with you my latest manuscript entitled "Development of a more sensitive and specific chromogenic agar for the isolation and differentiation of *Vibrio* species". Unlike my previous manuscripts focusing on the virulence of *Vibrio parahaemolyticus*, a common foodborne pathogen found in marine environment, this study is dedicated to comparing the performance of different media for the detection, identification and isolation of this pathogen and other related *Vibrio* spp. The research process entails many manual microbiological techniques, in conjunction with color observation and interpretation. All of these strongly suggest that communicating the study results to the public would be much more effective via the JoVE's unique multimedia format.

At the time of this study, Thorsen was an undergraduate student in our Biological Sciences Department at California Polytechnic State University, San Luis Obispo. He spent at least one year working in this study. Together with a few student helpers, they generated all the data. I am the faculty in the department who oversee the project including project design, data analysis and result interpretation.

Alison Hamlin saw our poster during ASM 2015 and invited me to submit a manuscript. She has been very courteous and dedicated in following up. I would not have thought about JoVE without her invitation.

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TITLE:
Development of a more sensitive and specific chromogenic agar medium for the detection of *Vibrio parahaemolyticus* and other *Vibrio* species

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SHORT ABSTRACT:
Detection and isolation of clinically relevant *Vibrio* species require selective and differential culture media. This study evaluated the ability of a new chromogenic medium to detect and identify *V. parahaemolyticus* and other related species. The new medium was found to have better sensitivity and specificity than the conventional medium.

LONG ABSTRACT:
Foodborne infections in the US caused by *Vibrio* species have shown an upward trend. In the genus *Vibrio, V. parahaemolyticus* is responsible for the majority of *Vibrio*-associated infections. Thus, accurate differentiation among *Vibrio* spp and detection of *V. parahaemolyticus* is critically important to ensure the safety of our food supply. Although molecular techniques are increasingly common, culture-depending methods are still routinely done and they are considered standard methods in certain circumstances. Hence, a novel chromogenic agar

medium was tested with the goal of providing a better method for isolation and differentiation of clinically relevant *Vibrio* spp. The protocol compared the sensitivity, specificity and detection limit for the detection of *V. parahaemolyticus* between the new chromogenic medium and a conventional medium. Various *V. parahaemolyticus* strains (*n=22*) representing diverse serotypes and source of origins were used. They were previously identified by Food and Drug Administration (FDA) and Centers for Disease Control and Prevention (CDC), and further verified in our laboratory by *tlh*-PCR. In at least four separate trials, these strains were inoculated on the chromogenic agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which is the recommended medium for culturing this species, followed by incubation at 35-37 ^{o}C for 24-96 hr. Three *V. parahaemolyticus* strains (13.6%) did not grow optimally on TCBS, nonetheless exhibited green colonies if there was growth. Two strains (9.1%) did not yield the expected cyan colonies on the chromogenic agar. Non-*V. parahaemolyticus* strains (*n=32*) were also tested to determine the specificity of the chromogenic agar. Among these strains, 31 did not grow or exhibited other colony morphologies. The mean recovery of *V. parahaemolyticus* on the chromogenic agar was ~96.4% relative to tryptic soy agar supplemented with 2% NaCl. In conclusion, the new chromogenic agar is an effective medium to detect *V. parahaemolyticus* and to differentiate it from other vibrios.

INTRODUCTION:

As a member of the *Vibrio* genus, *V. parahaemolyticus* is a Gram-negative, non-sporeforming, curved, rod-shaped bacterium. It exhibits high motility in both liquid and semi-solid environments. Most *V. parahaemolyticus* strains are non-pathogenic to humans, yet the pathogenic subtypes have caused epidemics and pandemics, hence this species is considered to be an important foodborne pathogen in many countries[1,2]. The incidence of *Vibrio* infection in the US has shown an upward trend since 2000[3]. Among *Vibrio* spp, *V. parahaemolyticus* is the most frequently reported species causing illnesses in the US[4,5]. Other clinically relevant species include *V. alginolyticus*, *V. vulnificus*, *V. cholerae*, etc. A small percentage of the illnesses is caused by multiple species simultaneously.

V. parahaemolyticus is a natural inhabitant of marine water and therefore widely distributed in marine waters throughout the world including the estuaries. The species was discovered in 1950 following an outbreak of food poisoning in Japan. In the US, the species was first isolated in seawater, sediments, and shellfish in the Puget Sound region[6,7]. Filter feeders in marine habitats, such as bivalve shellfish, can harbor *V. parahaemolyticus* as part of their natural flora[8]. As such, *V. parahaemolyticus* infections in human are often linked to the consumption of contaminated seafood, especially raw or undercooked shellfish. A less common route of entry occurs when open wound is exposed to seawater, leading to skin infection. Most *V. parahaemolyticus* strains do not cause human disease, yet certain subtypes harboring virulence factors such as thermostable direct hemolysin (TDH) are pathogenic. The most prevalent symptoms of foodborne *V. parahaemolyticus* infection are diarrhea and abdominal pain, followed by nausea, vomiting, and fever. Headache and chills are also reported. The median incubation period is 15 hr, but can be up to 96 hr after consumption of sufficient amount of pathogenic strains[9]. The illness lasts from two to three days. The gastroenteritis symptoms caused by *V. parahaemolyticus* are largely self-limiting and therefore special treatment is not necessary. Mild cases of

gastroenteritis can be effectively treated by oral rehydration. More severe illnesses can be treated by antibiotics such as tetracycline or ciprofloxacin[10]. Mortality rate is about 2% for gastroenteritis cases, but may be as high as 29% for those who develop bloodstream infection or septicemia. Any person who consumes seafood or has open wound exposed to seawater is at risk of *V. parahaemolyticus* infection. The more severe form of illnesses, life-threatening septicemia, is more common in a subpopulation with underlying medical conditions[11], which include alcoholism, liver disease, diabetes, renal disease, malignancy, and other conditions leading to a weaken immune response. Notably, this group of individuals is also at a higher risk for contracting severe illnesses caused by *V. vulnificus*, which can be found in natural habitats similar to *V. parahaemolyticus*.

V. parahaemolyticus is routinely isolated using thiosulfate-citrate-bile salts-sucrose (TCBS) agar as a selective and differential medium. Enrichment in alkaline peptone water may precede isolation on TCBS agar. Presumptive colonies on TCBS are then further tested in an array of biochemical tests and/or molecular assays targeting the presence of species-specific genes. PCR-based methods are often used to confirm the identities of *V. parahaemolyticus* by amplifying the thermolabile hemolysin gene, *tlh*[12].

Regardless of the choice of confirmation methods, it is important to have an effective medium to isolate and differentiate *V. parahaemolyticus* from other marine vibrios in the first place. TCBS has routinely been used to differentiate species within the *Vibrio* genus according to their abilities to ferment sucrose[12]. Positive fermentation reaction is accompanied by a color change of the pH indicator Bromothymol blue. *V. parahaemolyticus* colonies are fairly distinctive on TCBS, exhibiting blue to green color. However, this medium cannot easily differentiate *V. alginolyticus* and *V. cholerae.* Sucrose-fermenting *Proteus* species may produce yellow colonies resembling *V. cholerae* or *V. alginolyticus*[13]. On initial isolation on TCBS, *V. parahaemolyticus* may also be misidentified as *Aeromonas hydrophila, Plesiomonas shigelloides*, and *Pseudomonas* spp[14]. Strains with delayed sucrose fermentation may be confused with other sucrose nonfermenting *Vibrio*[13], which include *V. parahaemolyticus*. TCBS was found to be not sensitive against *Escherichia coli*, *Pseudomonas putrefaciens*, among others. Several other species yield green to gray colonies which are potentially confused with *V. parahaemolyticus* or *V. vulnificus*[15]. As a result, it is desirable to develop alternative culture media with better sensitivity and specificity toward detecting and isolating *V. parahaemolyticus* and other closely related species.

Several media alternatives have been recently developed. In addition to the inclusion of selective agents, most incorporate chromogenic substrates to differentiate species based on their differential enzymatic activities. For example, indoxyl-β-glucoside and indoxyl-β-galactoside have been used as the chromogenic substrates to differentiate *V. parahaemolyticus* colonies (which appear bluish-green) from those of *V. cholerae* (purple) due to their differential abilities to produce β-glucosidase and β-galactosidase[16]. Different formulations of chromogenic agar developed by several groups have been evaluated and were reported to perform comparably to or better than TCBS[17,18,19]. An advantage of using a chromogenic medium is that the coloring of the surrounding medium is minimal thereby facilitating the isolation of particular colonies. In this study, we evaluated the ability of a newly formulated chromogenic medium to detect and isolate

V. cholerae, *V. parahaemolyticus*, and *V. vulnificus*; with a special focus on its ability to differentiate *V. parahaemolyticus* from other species.

PROTOCOL:

1. Media and Culturing of Microbial strains

Note: Use aseptic techniques in all experiments. Use sterile materials. Sterilize all containers, tools and reagent prior to use. Autoclave all waste materials prior to disposal because they are considered biohazardous. Autoclave temperature and time combination is ≥121 ^{o}C x ≥15 min for all of the following procedures.

1.1) To make ~1-L tryptic soy agar (TSA), first add 1 L deionized water in a 2-L Erlenmeyer flask containing a magnetic stir bar. Use a flask that is at least two times larger than the final volume. Add 30 g of tryptic soy broth (TSB) powder and 20 g agar granules into the flask.

1.1.1) Mix thoroughly by turning on the stirrer. While stirring, turn on the heat to boil the mixture. Remove the flask from the heater as soon as the mixture begins to boil. Loosely cover the flask with a tin foil. Tape the foil to secure it to the flask and autoclave.

Note: Use 2% agar instead of 1.5% to limit swarming of some *Vibrio* spp.

1.1.2) To make tryptic soy broth (TSB), omit the agar from the recipe in step 1.1.

Note: May use bottles instead of Erlenmeyer flask.

1.1.3) To make tryptic soy agar supplemented with 2% sodium chloride or NaCl (TSAS), add 20 g of NaCl in the mixture prior to stirring and heating. To make tryptic soy broth supplemented with 2% NaCl (TSBS), omit the agar, add 20 g of NaCl in the mixture prior to stirring and heating.

1.2) To make brain heart infusion (BHI) agar, suspend 37 g of BHI powder and 15 g agar granules in 1 L of purified water. Heat with frequent agitation to dissolve the powder. Autoclave. Omit the agar to make BHI broth.

1.3) To make TCBS agar, suspend 89 g of the TCBS powder in 1 L of purified water. Heat with frequent agitation and boil for 1 min to completely dissolve the powder. Do not autoclave.

1.4) For all agar media, cool the hot agar to 45-50 ^{o}C in a water bath. Arrange empty petri plates in stacks of five to six plates. Starting from the bottom of the stack, pour the molten agar into each petri plate to reach about half full. Close the petri plate lid after pouring. Allow the agar to solidify by letting the plates sit at room temperature.

1.4.1) Use the agar plates the next day or after 12 hr. Store unused plates in a refrigerator for up to two weeks. Before use, remove plates from the refrigerator and equilibrate them at room

temperature for at least 15 min.

Note: One-liter agar makes ~45 agar plates. Allow the agar plates to dry sufficiently on the day of preparation, and equilibrate them to room temperature after cold storage to effectively reduce the spreading of the colonies.

1.5) Obtain chocolate and chromogenic agar plates and equilibrate them at room temperature before each experiment.

1.6) Subculture all 54 microbial strains shown in Table 1 every few days.

1.6.1) Use a sterile inoculating loop to transfer cultures from a frozen stock or a previous batch to nonselective media such as BHI, TSB/TSA or chocolate agar. Grow halophilic *Vibrio* spp on TSBS/TSAS.

1.6.2) To check the purity of the culture, streak all strains in a pattern that would allow for observation of isolated colonies. For example, use a three-phase streaking pattern to dilute a large amount of bacteria to smaller amount, eventually yielding isolated colonies.

1.7) Incubate the plates up-side down at 35-37 ^{o}C for up to 48 hr. For *Campylobacter* spp, incubate tubes or plates in a closed-lid jar containing a gas pouch to produce a microaerophilic environment. Observe colony morphology after incubation. Pure cultures should yield colonies that exhibit similar colony morphology.

Note: Incubate all plates upside-down to prevent condensed water droplets formed on the underside of the lid from falling on the colonies.

2. Species determination by PCR

2.1) Conduct *tlh*-PCR to confirm the identity of *V. parahaemolyticus* strains. Use primers *tlh*-F (5' AAA GCG GAT TAT GCA GAA GCA CTG 3') and *tlh*-R (5' GCT ACT TTC TAG CAT TTT CTC TGC 3') to amplify a 450-bp fragment of the thermolabile hemolysin gene[20].

2.1.1) Use a sterile inoculating loop to transfer a few isolated colonies of each *V. parahaemolyticus* strain from TSAS to 5 ml of TSBS. Incubate at 35-37 ^{o}C for 16-24 hr.

2.1.2) Centrifuge cultures at 14,000 x g for 1 min. Remove supernatant and wash the pellet twice with phosphate buffered saline (PBS). Boil the suspension for 3 min to yield cell lysate.

Note: *V. parahaemolyticus* is easy to be lysed. Therefore lysis reagent is not required. It is also possible to use a bit of colony directly as the template.

2.1.3) Perform PCR in a 25-µl reaction volume. Prepare a reaction mixture containing a final concentration of 1X PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 1 µM of each primer, 1 U

Taq Polymerase and 1 µL of cell lysate. After preincubation at 94 °C for 5 min, run 35 amplification cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 60 sec[21].

2.1.4) Load aliquots of 5-µL amplicon in 1.5% agarose gels. Turn on power supply to start electrophoresis. Visualize the presence or absence of amplicons under UV illumination after ethidium bromide staining.

3. Growth on selective and differential media

3.1) Two to four days before the experiment, streak all microbial strains shown in Table 1 on nonselective medium (TSAS, BHI or chocolate agar) for colony isolation. Incubate plates at 35-37 °C for 48 hr. Check the purity of the cultures by observing colony morphology after incubation. Pure cultures should yield colonies that exhibit similar colony morphology.

3.2) Transfer a few isolated colonies from Step 3.1 into 5 ml of broth. Incubate tubes at 35-37 °C for 16-24 hr.

Note: Use young colonies, which are less than four days old, to prepare overnight cultures in all experiments.

3.3) Streak a loopful of overnight cultures on selective and differential media (TCBS and chromogenic agar) for colony isolation. Incubate plates at 35-37 °C for up to 96 hr.

3.4) Record the overall growth of all strains by examining both the culture density on the plate and the size of isolated colonies. Record the color of colonies under ambient and/or UV light. Note other characteristics of isolated colony such as the elevation, margin and form.

4. Recovery assay

4.1) Select a representative subset of *V. parahaemolyticus* strains (*n*=14) that encompass different serotypes and origins of isolation[22]. Inoculate young cultures from the plates into 5 ml of TSBS. Incubate the tubes at 35-37 °C for 16-24 hr.

4.2) Conduct a Standard Plate Count method of the overnight cultures as described below.

4.2.1) Vortex to mix the overnight cultures well. Make a 10-fold or 10^{-1} dilution by transferring 100 µL of the overnight culture into a tube containing 900 µL PBS. Vortex to mix well.

4.2.2) Use a new pipet tip to transfer 100 µL from the 10^{-1} dilution tube to another tube containing 900 µL PBS. Vortex to mix well. This constitutes 10^{-2} dilution. Repeat the process sequentially to obtain 10^{-7} dilution.

4.2.3) Using the 10^{-4} to 10^{-7} dilution tubes, plate 100 µL each on the chromogenic, TCBS and TSAS agar plates.

Note: The dilution factor (*df*) on the plate becomes 10^{-5} to 10^{-8}, respectively.

4.2.4) Spread the aliquots evenly on the agar surface.

Note: It is fine to use the same spreader per strain on the same medium, as long as the most diluted aliquot is spread first (i.e., from 10^{-8} to 10^{-5}). Do not use the same spreader for different media.

4.2.5) Incubate plates at 35-37 °C for up to 96 hr. Count colonies on the plates. Ignore plates bearing colonies that are too numerous to count (tntc) or less than 25. Calculate CFU/ml according to the following:

$$N = \frac{C}{\left[\left(1 \times n_1\right) + \left(0.1 \times n_2\right)\right] \times df}$$

Where
N = the number of cells in the undiluted tube, expressed as CFU/ml or CFU/g

C = the total number of colonies counted on plates that bear 25-300 colonies

n_1 = the number of plate(s) where counted colonies are from the lower *df*

n_2 = the number of plate(s) where counted colonies are from the subsequent 10-fold dilution

df = the lower dilution factor (i.e., more concentrated dilution)

4.3) Compare CFU/ml among different media. Use CFU/ml on TSAS as 100%, calculate the % recovery of *V. parahaemolyticus* grown on the chromogenic and TCBS agar.

5. Competition assay

5.1) Choose a subset of strains that exhibit different colony morphologies on the chromogenic and TCBS agar.

Note: This way, it will be possible to count colonies originated from *V. parahaemolyticus* only, despite the presence of other species in the inoculum.

5.1.1) Choose a *V. parahaemolyticus* strain that yields the expected turquoise and cyan colonies on TCBS and the chromogenic agar, respectively.

5.1.2) Choose a non-*V. parahaemolyticus* and a non-*Vibrio* species that do not grow on either of these media, or exhibit different colony color.

Note: For example, *V. metschnikovii* grows very weakly on TCBS and did not grow on the chromogenic agar. *Shigella sonnei* does not grow on TCBS but yields magenta colonies on the chromogenic agar.

5.2) After selection of the strains above, prepare overnight broth cultures using isolated colonies grown on nonselective media.

5.2.1) Make overnight cultures of *V. parahaemolyticus* and *V. metschnikovii* by transferring a few isolated colonies from TSAS to 5 ml of TSBS. Incubate at 35-37 ^{o}C for 16-24 hr.

5.2.2) Make overnight cultures of *Shigella sonnei* by transferring a few isolated colonies from BHI agar to 5 ml of BHI broth. Incubate at 35-37 ^{o}C for 16-24 hr.

5.3) For each strain, perform a dilution series similar to Steps 4.2.1 and 4.2.2. Plate appropriate dilutions on TSAS or BHI to determine CFU/ml of the overnight culture, using the equation shown in Step 4.2.5.

Note: Typically, 100 µL from the 10^{-5} to 10^{-7} dilution tubes works for most cultures. Use the CFU/ml values to back calculate the exact amount of cells used in next step. The calculated CFU/ml values are obtained after incubation, although the following steps are performed on the same date as Step 5.3.

5.4) Using the overnight cultures and dilution tubes in Steps 5.2 and 5.3, mix different amounts of a *V. parahaemolyticus* strain and a non-*V. parahaemolyticus* species. For example, mix 500 µL of the 10^{-5} dilution tube of *V. parahaemolyticus* with 500 µL of the overnight cultures of *V. metschnikovii*.

Note: This mixture simulates high microflora background. To simulate a low microflora background, mix 500 µL each of the 10^{-5} dilution tube from both species.

5.5) Spread 100 µL of the mixture each on the chromogenic, TCBS and TSAS agar plates.

5.6) After incubation at 35-37 ^{o}C for up to 96 hr, count colonies of *V. parahaemolyticus* and the non-*V. parahaemolyticus* species based on their difference in growth and colony morphology on the chromogenic and TCBS agar.

Note: For example, if the non-*V. parahaemolyticus* species does not grow on the chromogenic and TCBS agar, all colonies will be of *V. parahaemolyticus*. If the non-*V. parahaemolyticus* species grows on both media, only the turquoise colonies on TCBS and cyan colonies on chromogenic agar will be of *V. parahaemolyticus*. The non-*V. parahaemolyticus* species may or may not exhibit similar colony morphology to *V. parahaemolyticus* on TSAS.

5.6.1) If the non-*V. parahaemolyticus* species grows similarly to *V. parahaemolyticus* on this

nonselective medium, divide the colony count by two to obtain numbers for *V. parahaemolyticus* only. Compare the actual colony count with the expected count derived from Step 5.3.

6. Effects of oyster homogenates

6.1) Weigh ≥50 g oyster meat from ≥12 molluscan shellfish including meat and liquor.

6.1.1) Add equal amount of PBS to the oyster meat and liquor. Blend the mixture at high speed for 90 sec. This constitutes 2^{-1} diluted oyster homogenate.

6.1.2) Add 100 g of 2^{-1} diluted oyster homogenate to 400 g of PBS. Use a scale to measure the weight, not volume. Blend the mixture at 7,000 x g for 1 min. Autoclave the oyster homogenate.

Note: This will be the oyster homogenate used for spiking.

6.2) Repeat the Recovery assay (Step 4) in the presence of oyster homogenate.

6.2.1) After the 500 g-oyster homogenate cools down, add 100 µL of *V. parahaemolyticus* overnight cultures grown in TSBS to it. Determine the actual amount of *V. parahaemolyticus* cells in the inoculum by conducting the Standard Plate Count procedures described in Step 4.2.

6.3) Repeat the Competition assay (Step 5) in the presence of oyster homogenate.

6.3.1) After the 500 g-oyster homogenate cools down, add 100 µL each of overnight cultures of *V. parahaemolyticus* and non-*V. parahaemolyticus* to it. Determine the actual amount of bacterial cells in the inoculum by conducting the Standard Plate Count procedures described in Step 4.2

6.4) Mix the bacterial cells with oyster homogenate well by using a homogenizer.

Note: After mixing, the oyster homogenate containing the intentionally added cells is called spiked oyster homogenate.

6.5) Make dilutions of the spiked oyster homogenate to obtain 10^{-1} to 10^{-3} dilution tubes according to the procedures described in Step 4.2.2. Spread 100 µL of each dilution onto the chromogenic, TCBS and TSAS agar. Incubate plates at 35-37 °C for up to 96 hr.

6.6) Compare the actual colony count on chromogenic and TCBS agar with the expected colony count deduced from Steps 6.2.1 and 6.3.1.

Note: For example, if a tube of *V. parahaemolyticus* overnight culture contains 10^8 CFU/ml, an inoculum of 100 µL means that 10^7 cells are added to the 500-g of oyster homogenate, yielding 5 x 10^4 cells/g. After dilution and plating, the plate having *df* = 10^{-2} should yield 500 colonies; while that having *df* = 10^{-3} should yield 50 colonies. These are the expected colony counts.

REPRESENTATIVE RESULTS:

In this study, 54 microbial strains were assembled, which included 22 strains within the *V. parahaemolyticus* species, 19 other *Vibrio* species, and 13 non-*Vibrio* species (Table 1). Most *V. parahaemolyticus* strains were either received from FDA, CDC or other state health departments. They represent diverse serotypes and isolation sources. These strains were previously identified by the regulatory agencies. We further confirmed the identities of these *V. parahaemolyticus* by conducting a *tlh*-PCR[21,22].

[Place Table 1 here]

Four separate trials were conducted to determine the growth and colony morphology of these strains on the selective and differential media – TCBS and the chromogenic agar. TCBS is the conventional medium used for the isolation of some *Vibrio* species, including *V. cholerae* and *V. parahaemolyticus*[12]. Color variation of colonies grown on TCBS was yellow, turquoise (green) or clear (Figure 1).

[Place Figure 1 here]

The ability of a new chromogenic medium to select for clinically relevant *Vibrio* species from food and environmental samples was tested. Additionally, the ability of this medium to simultaneously distinguish these species from each other was evaluated. Colony color observed on the chromogenic agar were cobalt, cyan, magenta, yellow or clear (Figure 2). As shown in Table 1, both TCBS and the chromogenic agar exhibited certain degrees of selectivity against non-*Vibrio* microorganisms.

[Place Figure 2 here]

Following the Standard Plate Count method on overnight cultures, colonies of *V. parahaemolyticus* were observed on the chromogenic agar plates receiving the highest dilution factor (i.e., $df = 10^{-8}$). These results were comparable to those on a nonselective medium (TSAS). This suggests that the detection limit of the chromogenic medium is similar to nonselective media, which is approximately 10 cells or lower in the absence of food matrix. The detection capacity for other *Vibrio* species such as *V. alginolyticus, V. fluvialis* and *V. damsel* was also decent compared to the nonselective medium. However, some strains of *V. cholerae*, *V. vulnificus* and *V. mimicus* yielded 10- to 100-fold less CFU on the chromogenic agar. The selective agents used in the chromogenic or other selective media inevitably inhibit some cells. Injured cells, for instance, could not recover in selective media. Nevertheless, the slightly poorer detection ability is a non-issue in most routine procedures that employ an enrichment step. Small amount of microorganisms in the food or environmental sample would multiply to a high level in the enrichment broth, surpassing the detection limit. Enrichment in alkaline peptone water is often done to determine the prevalence of *Vibrio* species in environmental samples[12].

In the presence of oyster homogenate, the chromogenic agar continued to display a good degree

of recovery. In other words, a large proportion (>70%) of *V. parahaemolyticus* cells were able to grow on the chromogenic agar, unaffected by the presence of oyster matrix (Figure 3a). The growth and recovery of *V. parahaemolyticus* cells was also not affected by the presence of another *Vibrio* species (Figure 3b). This is an important attribute because environmental samples are bound to contain different *Vibrio* species, which are at high numbers especially after an enrichment procedure.

[Place Figure 3 here]

To further compare the chromogenic and TCBS agar, the sensitivity and specificity were calculated. Table 2 shows the ability of these media to accurately identify *V. parahaemolyticus*. Sensitivity is related to the percentage of true positive, which means the *V. parahaemolyticus* strains yielded the expected colony morphology on the media. Specificity is related to the percentage of true negative, which means non-*V. parahaemolyticus* strains should exhibit poor to no growth, or a different colony morphology than *V. parahaemolyticus*. The sensitivity and specificity for TCBS to identify *V. parahaemolyticus* are 86.4% and 71.8%, respectively. In comparison, the sensitivity and specificity of the chromogenic medium are 90.9% and 96.9%, respectively.

[Place Table 2 here]

Figure Legends:

Figure 1: Colony morphology of *Vibrio* spp on TCBS agar. *V. parahaemolyticus* (turquoise), *V. cholerae* (yellow), and a mixed inoculation of the two species (a). Colonies of *V. parahaemolyticus* appear turquoise, with a circular, entire, and convex morphology (b).

Figure 2: Colony morphology of *Vibrio* spp on the newly developed chromogenic agar. *V. parahaemolyticus* (cyan), *V. cholerae* (magenta), and a mixed inoculation of the two species (a). Colonies of *V. parahaemolyticus* appear cyan, with a circular, entire, and convex morphology (b).

Figure 3: Percent recovery of a *V. parahaemolyticus* strain in oyster homogenate with and without a bacterial competitor. Recovery (mean ± SD) was calculated according to the observed vs the expected CFU count on the agar plates. Expected CFU count was calculated based on the bacterial load in the inoculum. High recovery of a *V. parahaemolyticus* strain was observed on all media without competition (a). Similar level of recovery was observed when high levels of *V. metshnikovii* cells was present (b).

Table 1: Microbial species used in this study and their growth characteristics on the selective and differential media. Color calling was based on a color wheel. Cyan is similar to teal; magenta is similar to pinkish lavender, turquoise is similar to green, yellow encompasses olive and brown.

Table 2: Sensitivity and specificity of TCBS (a) and chromogenic (b) agar in the detection of *V. parahaemolyticus*. The identities of *V. parahaemolyticus* were determined previously by

biochemical test and *tlh*-PCR.

DISCUSSION:

This study focuses on culture media development and evaluation. Conventionally, TCBS is the selective and differential medium used for isolating and detecting *V. parahaemolyticus, V. cholerae* and *V. vulnificus*[12]. However, limitations have been reported for this medium, such as the inability to differentiate *V. cholerae* from other *Vibrio* species. Sucrose and pH indicator are the differentiation agents of TCBS. Thus, acid production by sucrose fermenter causes color change of the medium. The coloring of the medium is a drawback of TCBS because it may obscure observation of colony morphology. The newly developed chromogenic medium utilizes high pH and salinity to suppress the growth of non-*Vibrio* species found in many marine samples. The new chromogenic medium has a final pH of 8.6±0.2. Per liter of deionized water, it consists of 10 g of peptone, 10 g of sea salts mixture, 10 g of oxbile, 10 g of sodium thiosulfate, 5 g of yeast extract, 5 g of sodium citrate, 2.2 g of sodium carbonate, 2 g of lactose, 0.5 g of sodium pyruvate, 0.3 g of a chromogenic mix and 15 g of agar. The chromogenic mix allows the differentiation among *Vibrio* spp according to their differential abilities to produce certain enzymes. Instead of altering the color of the TCBS agar medium, different *Vibrio* spp would exhibit different colony color on the new chromogenic medium. In comparison, a commercially available chromogenic medium contains 15 g of agar, 8 g of peptone and yeast extract, 51.4 g of salts, 0.3 g of a chromogenic mix and a final pH of 9.0±0.2.

The robustness of an assay evaluation depends on the sample size. Since this study emphasizes on the effectiveness of the new chromogenic medium to isolate and detect *V. parahaemolyticus*, it is important to amass many diverse *V. parahaemolyticus* strains. Previous studies comparing TCBS and new culture media often involved environmental or food samples containing unknown types and quantity of microorganisms[23,24,25,26]. Multiple colonies per sample were isolated yet the clonal nature of the isolates was often undetermined. Ideally, different strains should be tested in assay development otherwise the accuracy of the assay would be inflated. Strain identity can be established by conventional subtyping methods such as pulsed-field gel electrophoresis (PFGE) or multi-locus sequencing. *V. parahaemolyticus* strains in this study were previously characterized by ribotyping and PFGE[19].

During assay development, sensitivity, specificity and detection limit are among the key factors to be investigated. Sensitivity, also known as accuracy or diagnostic sensitivity, is the positive percent agreement between the reference and test methods. Specificity, also known as precision or diagnostic specificity, is the negative percent agreement between the reference and test methods. In this study, we used PCR-based method as the reference method because the results were verified among different groups. TCBS and the chromogenic medium were the test methods. To determine sensitivity, results from the *V. parahaemolyticus* strains were used; i.e., Sensitivity = 100% x True Positive / (True Positive + False Negative). On the other hand, results from non-*V. parahaemolyticus* strains were used to determine specificity; and hence, Specificity = 100% x True Negative / (True Negative + False Positive). To provide more reliable specificity results, non-*V. parahaemolyticus* strains should be isolated from an environment where sampling will be conducted. Our sensitivity and specificity calculations are based on documents written by

government[27], academia[28] and scientific organization[29] on a topic related to assay development and validation.

Based on our results, the chromogenic medium showed better performance than TCBS in the identification of *V. parahaemolyticus*. The overall percent agreement between the reference method and chromogenic medium is 94.4%, compared to 77.8% between the reference method and TCBS. The sensitivity and specificity of the chromogenic medium are 90.9% and 96.9% respectively, which are greater than those of TCBS (86.4% and 71.9%, respectively). Previous studies evaluating other chromogenic media for the detection of *V. parahaemolyticus* found that sensitivities ranged from 85 to 88% whereas specificities ranged from 94 to 95%[23,24,25]. However, these studies did not use the same calculation method as our study. Further, they sometimes used a different reference method or they combined results from both biochemical analyses and chromogenic medium as one test method. As a result, it is difficult to directly compare our results with these previous studies.

In addition to exhibiting a better performance in detecting *V. parahaemolyticus*, the chromogenic agar used in this study could also differentiate more *Vibrio* species than TCBS due to the inclusion of a chromogenic mix in the medium formula, yielding multiple colors. Although it was not the focus of this study to detect *V. cholerae* and *V. vulnificus*, it is worth to note that the magenta colonies exhibited by these species can be further differentiated by fluorescence under UV. A limitation to use the chromogenic agar for the detection of *V. cholerae* and *V. vulnificus* is its apparent low recovery for these species. To circumvent this issue, an enrichment step must be included. Another possible limitation of the chromogenic agar is its shorter shelf life than TCBS. Ongoing optimization of this new medium is required to maintain pH during storage.

Despite the popularity of molecular techniques, culture-depending methods are still valuable as they are often less costly and have a better detection limit. These culturing methods can be used as a screening tool. Conversely, they can be used to confirm the identity and viability of microorganisms following molecular analyses. To enumerate bacteria via a culture-depending approach, Standard Plate Count is recognized as the standard method. The equation shown in Step 4.2.5 is a more accurate way to determine CFU/g or CFU/ml than averaging CFUs from different dilution factors. It is important to note that all diluents and media throughout the procedures must contain sufficient salt to maintain the viability of halophilic bacteria such as *V. parahaemolyticus*. Incubation temperature and environment must be optimal for the bacterial growth.

The chromogenic medium is designed to detect *Vibrio* species that are important human pathogens. Therefore, further studies must be conducted to determine its effectiveness to detect other environmental *Vibrio* species that are not medically relevant. In future applications, the new chromogenic agar can be incorporated into routine testing of environmental samples for *V. parahaemolyticus*. This can be done by direct streaking of samples on the chromogenic agar to detect for the presence of presumptive *V. parahaemolyticus*. The chromogenic agar can also be used to enumerate *V. parahaemolyticus* following dilutions. Quantification can also be estimated by carrying out a Most Probable Number method using enrichment broth, followed by streaking

on the chromogenic agar for confirmation.

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DISCLOSURES:
Some media were generously provided by Hardy Diagnostics, Santa Maria, CA. Thorsen conducted this study while a student at California Polytechnic State University. He is currently an employee of Hardy Diagnostics.

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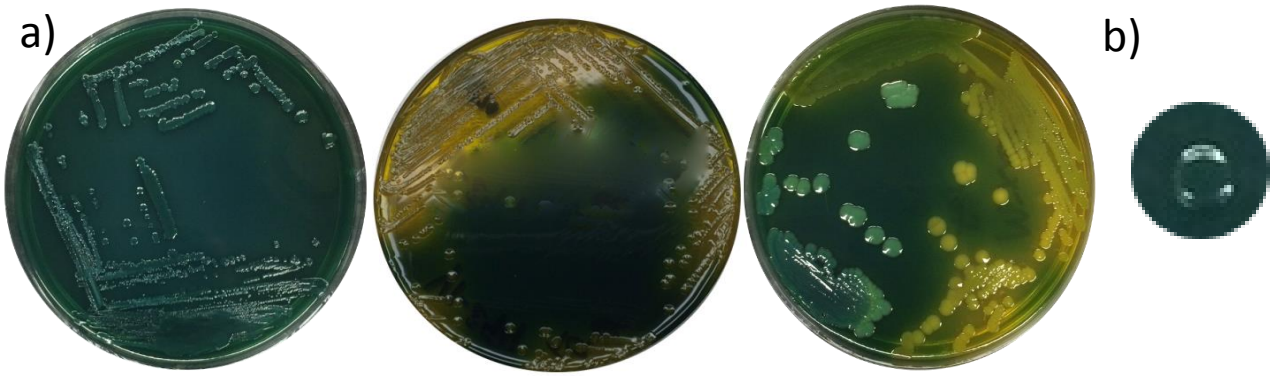
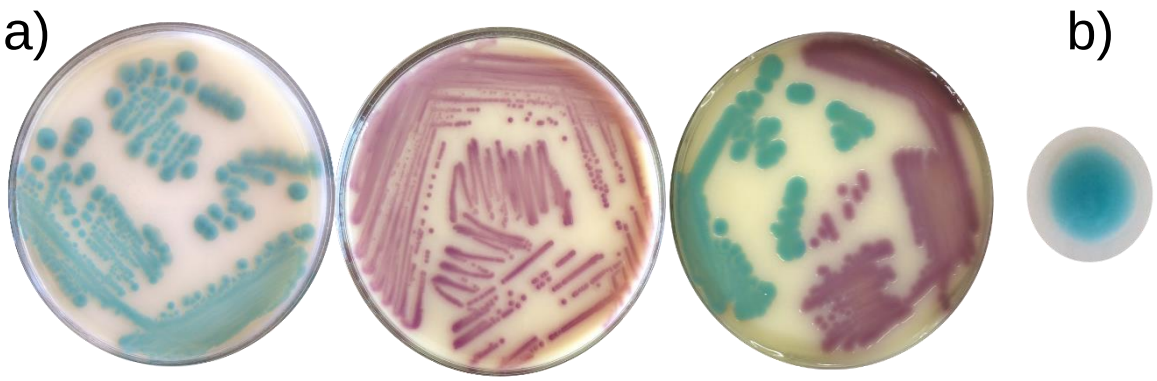
Thorsen and Yeung, Fig 1

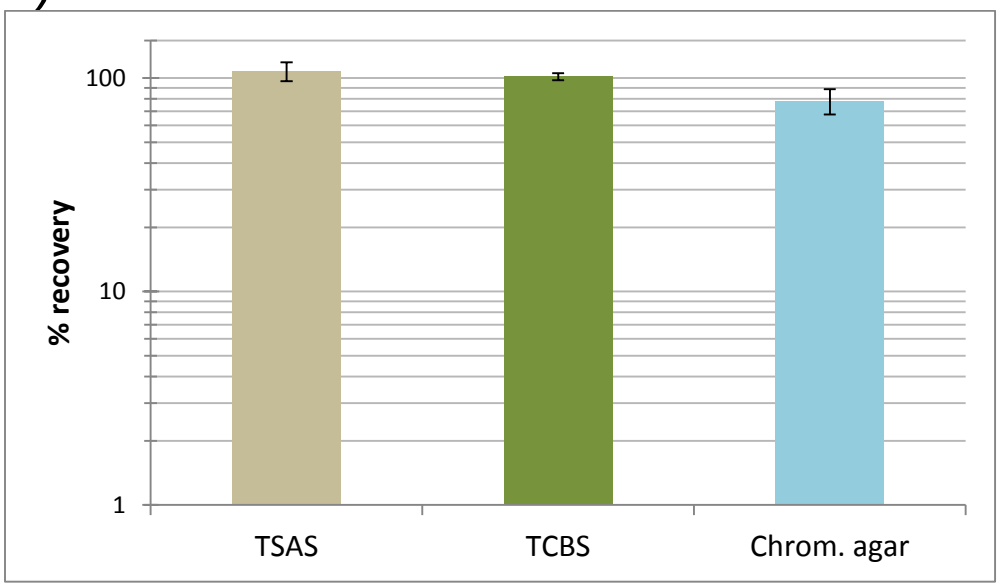


Thorsen and Yeung, Fig 2



Thorsen and Yeung, Figure 3

a)



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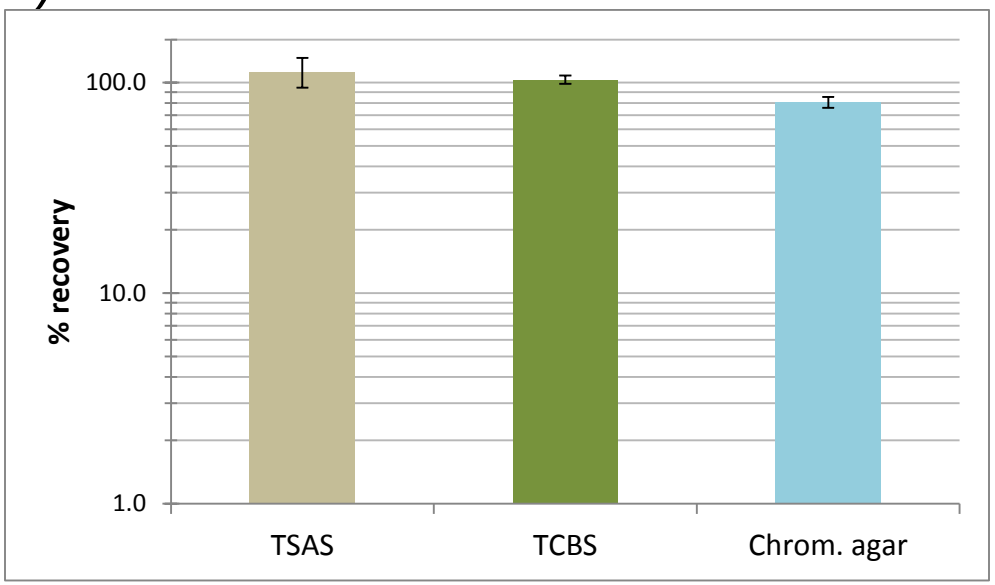

Table 1

Species	No. of strains tested	Growth and c
		TCBS
Aeromonas hydrophila	2	No growth
Candida albicans	1	Weak growth, yellow
Campylobacter jejuni	1	No growth
Escherichia coli	1	No growth
Proteus mirabilis	1	No growth
Pseudomonas aeruginosa	2	Turquoise
Staphylococcus aureus	1	Weak growth, yellow
Salmonella Choleraesuis	1	No growth
Shigella boydii	1	No growth
Shigella flexneri	1	No growth
Shigella sonnei	1	No growth
Vibrio alginolyticus	3	Yellow or turquoise
V. cholerae	4	Yellow or turquoise
V. damsela	1	Turquoise
V. fisherii	1	Weak growth, yellow
V. fluvialis	1	Turquoise
V. furnissii	1	Yellow
V. hollisae	1	Turquoise
V. metschnikovii	1	No growth
V. mimicus	1	Turquoise
V. parahaemolyticus	22	Most strains turquoise
V. proteolyticus	1	Turquoise
V. vulnificus	4	Turquoise or clear

:olor of colonies
Chromogenic medium
1 strain no growth;
1 strain magenta color
No growth
No growth
Weak growth, magenta
No growth
Yellow
No growth
No growth
No growth
No growth
Weak growth, magenta
Yellow
Magenta
Cobalt
No growth
Cobalt
Yellow
Yellow
No growth
Magenta
Most strains cyan; few clear
Cobalt
Magenta

Table 2

a)

TCBS agar	*V. parahaemolyticus*
Good growth and turquoise colonies	19
Poor growth or non-turquoise colonies	3

b)

Chromogenic agar	*V. parahaemolyticus*
Good growth and cyan colonies	20
Poor growth or non-cyan colonies	2

Non-V. parahaemolyticus
9
23

Non-V. parahaemolyticus
1
31

Excel Spreadsheet- Table of Materials/Equipment

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Reagent/Equipment	Company	Catalog #	Comments
Agar	Fisher Scientific	DF0140-15-4	may use other brands
Autoclave	Any		
BHI powder	Fisher Scientific	DF0418-17-7	may use other brands
Blender	Any		to blend oyster meat
CampyGen gas generator	Hardy Diagnostics	CN035A	to provide a microaerophilic atmosphere; may use other brands
Chocolate agar plates	Hardy Diagnostics	E14	may use other brands
Common PCR reagents (dNTPs, $MgCl_2$, *Taq* Polymerase)	Any		or use PCR beads (Fisher Sci 46-001-014)
Culture tubes	Fisher Scientific	S50712	may use other brands
Eppendorf tubes	Fisher Scientific	S348903	may use other brands
Gel doc	Any		
HardyChrom Vibrio agar plates	Hardy Diagnostics	G319	This study evaluates this medium
Incubator	Any		
Inoculating loops	Fisher Scientific	22-363-606	10 microliter-size was used in this study
NaCl	Fisher Scientific	BP358-212	may use other brands
Oysters	Any		
PBS	Fisher Scientific	R23701	may use other brands
Petri dish	Fisher Scientific	FB0875713	may use other brands
Pipette and tips	Any		Sterilized tips
Primers for *tlh*	IDT DNA		
Scale	Any		
Spreader	Fisher Scientific	08-100-11	Beads may be used instead
Stomacher blender	Stomacher	400	Samples were homogenized at 200 rpm for 30 sec. Other homogenizer can be used.
Sterile filter bags for blenders	Fisher Scientific	01-812-5	
TCBS powder	Hardy Diagnostics	265020	This study evaluates this medium
Thermocycler	Any		
TSB powder	Fisher Scientific	DF0370-07-5	may use other brands
UV viewing cabinet	Any		Emit long-wave UV light
Water bath	Any		

Species	Provider or source
Aeromonas hydrophila	ATCC
Candida albicans	ATCC
Campylobacter jejuni	ATCC
Escherichia coli	ATCC
Proteus mirabilis	ATCC
Pseudomonas aeruginosa	ATCC
Staphylococcus aureus	ATCC
Salmonella Choleraesuis	ATCC
Shigella boydii	ATCC
Shigella flexneri	ATCC
Shigella sonnei	ATCC
Vibrio alginolyticus	ATCC
V. cholerae	FDA, ATCC (O139, O1, non O1, El Tor biovars)
V. damsela	FDA
V. fisherii	Environment
V. fluvialis	CDC
V. furnissii	CDC
V. hollisae	FDA
V. metschnikovii	ATCC
V. mimicus	FDA
V. parahaemolyticus	ATCC, FDA, CDC, environment (serotypes include O3:K6, O1:K56, O4:K8, O5:K15, O8, etc)
V. proteolyticus	FDA
V. vulnificus	FDA

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Editorial comments:

• Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript. **Please use the updated manuscript located in your Editorial Manager account (under "File Inventory") for all subsequent revisions**.

The instruction was followed.

• Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

The manuscript was revised and proofread.

• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Reference section is revised an include DOIs whenever possible.

•Formatting:

-Please use "hr" as the abbreviation for "hour(s)".

The format was revised as suggested.

-All centrifuge speeds should be listed in terms of centrifugal g force rather than rpm.

The manuscript was revised to use RCF instead of RPM.

•Grammar: "bromthymol" should be "bromothymol".

According to PubChem, bromthymol blue and bromthymol blue are MeSH synonyms. The term was revised as suggested.

•Additional detail is required:

-What is the composition of the chromogenic agar?

The composition of the agar is now included in the Discussion.

-3.4 – Is any equipment used to analyze the colonies?

Equipment is not required to analyze the colonies. Colony morphology observed by naked eyes under ambient light is sufficient to differentiate *Vibrio parahaemolyticus* from other species. It is an advantage of this chromogenic medium because it allows the user to rapidly differentiate *Vibrio* species without the need of expensive equipment and sophisticated analyses. The medium is designed to be used as a screening tool primarily and therefore cost per test must be comparable to existing screening methods. Our study shows that the chromogenic medium demonstrate better sensitivity and specificity.

-6.2.1, 6.3.1 – How much oyster homogenate is used?

These steps follow 6.1.2. Therefore 500 g diluted oyster homogenate was used for Steps 6.2.1 and 6.3.1. The amount of the oyster homogenate is now clarified in these steps.

•Results: Figure 1 and Figure 2 are cited incorrectly in the results text.

We acknowledge this error. Thanks for pointing it out. The figures are now cited correctly in the results.

Reviewers' comments:

Reviewer #1:

Editorial Note: Please note that this reviewer has raised some significant concerns regarding your method and your manuscript.

Manuscript Summary:

This paper is about a more sensitive and specific chromogenic agar medium for the isolation and differentiation of Vibrio species.

(1)The object of this study is to evaluate the ability of a newly formulated chromogenic medium to differentiate V. parahaemolyticus from other species. Authors used 54 microbial strains including Vibrio, E. coli, Pseudomonas and so on (Table 1). However, ATCC standard strain is not included in the experimental strains, all microbial strains are isolated bacteria. Such a design is not scientific because the stability of the isolated strains is not as good as the standard strains.

Thanks for the comments. ATCC strains were used in this study along with environmental and clinical isolates primarily provided by FDA and CDC. Since the new medium is developed with

a purpose to test environmental samples, we feel that it is important to include environmental isolates to determine its effectiveness. The purity of all ATCC strains and isolates was checked periodically by streaking for colony isolation. In addition, PCR with species-specific primers was done to confirm the identity of the isolates. The identity of the isolates were determined by multiple laboratories, such as CDC and FDA. All strains and isolates were subjected to standard operating procedure, including frozen stock preparation. All strains and isolates were subcultured at least twice prior to each experiment. This is done to ensure all cells are not in injured state which may cause aberrant results.

In addition, the source of experimental strains and their serotypes are not clear in Table 1.

The Table is revised to include source of strains. Serotypes info, which is relatively more important for *V. cholerae* and *V. parahaemolyticus*, is also included.

(2)Academic rigor of the paper is poor. There are several writing mistakes in the paper. Example 1: in Abstract, authors tell us V. parahaemolyticus was incubated at 37 oC for 24-96 h. But in the part of Protocol, the culture temperature of V. parahaemolyticus is 35 oC (lines 209-210; lines 239-240).

Thanks for the comments. We had a digital thermometer placed in the incubator throughout the experiments which monitored the temperature fluctuation due to door opening and closing. The minimum and maximum temp recorded by the digital thermometer was 35 and 37^{o}C respectively. We revised the manuscript to show the range of incubation temp.

Example 2: according to the description of lines 409-413, Fig.1 should be the result of TCBS, Fig.2 is the results of new chromogenic medium. However, Figure legends show the opposite description (lines 462-468).

We acknowledge this error. The Figures are now cited correctly in the results.

In addition, single colonies in Figure 1 and 2 are seldom, which is not conducive to isolate the target bacteria.

The streak plate technique was used to observe isolated colonies. It is common to observe isolated colonies in the last section. Differentiation of *Vibrio* species on TCBS or the chromogenic medium is based on the morphology of isolated colonies. Color of the colony is the most important attribute in the differentiation decision for both media. Figures 1 and 2 show the color of the cultures as well as isolated colonies. These are representative images from four replicate experiments.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The manuscript described the development of a more sensitive and specific chromogenic agar for isolation and differentiation of Vibrio species. The manuscript is well written.

Major Concerns:

A major concern for the writing is the insufficient information provided for the new chromogenic agar. It is unclear what is the component of this agar, including the pH and NaCl concentration. I think authors need to provide this information and highlight the detail of how different is this new agar (the significance of these differences) with the existing TCBS or existing Chrom agar available in the market.

Information about the exact amount and composition of commercially available chromogenic agar media is considered proprietary information by the companies. A brief comparison between our medium with a commercial medium is now included in the Discussion. This study highlights the procedure to evaluate a newly developed growth medium before commercialization. The general ingredients such as carbon and nitrogen source, minerals are similar to other complex media. We discussed levels of pH and salt must be tested to select for *Vibrio* species effectively. The role of chromogenic substrate to differentiate different *Vibrio* species is also emphasized in the manuscript.

Minor Concerns:

These references should be cited in this work:-

1) Letchumanan et al., 2014. doi: 10.3389/fmicb.2014.00705

This reference is a review paper that summarizes the pathogenesis, prevalence and advance molecular identification techniques of *Vibrio parahaemolyticus*. We do not believe this review paper adds to the current manuscript as the pertinent information is similar to the review and book chapter written by our group previously. Therefore we do not wish to include this reference in our manuscript.

2) Raghunanth et al., 2015. doi: 10.3389/fmicb.2014.00805

This is a review article summarizing the roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. Although this is an interesting review article, we do not believe it is relevant to our manuscript. The review article stresses the distribution of *tdh* and *trh* genes in samples collected from different locations using PCR based methods. Our manuscript focuses on the detection of all *V. parahaemolyticus* using culture-depending method which is often the first step carried out by environmental labs. Thereafter, the isolates can be further analyzed such as the presence of *tdh* or *trh*. Owing to the irrelevancy, we do not wish to include this reference in our manuscript.

3) Haendiges et al., 2015. doi: 10.3389/fmicb.2015.00125

This study compared the whole genome sequences between 34 *Vibrio parahaemolyticus* strains, which were isolated from clinical cases in the state of Maryland, with the genomes of 17 strains obtained from US and 15 international strains. The authors had a specific study aim, which was to determine the feasibility of whole genome sequencing as a tool to improve public health surveillance. They built phylogenetic trees to determine relatedness of the strains. They concluded that the Maryland strains were highly diverse yet genetically distinct from other strains. While it is an interesting study about *V. parahaemolyticus*, we do not believe it is relevant to our manuscript. Therefore we do not wish to include this reference.

Additional Comments to Authors:

The method section should be simplify to ease the readers' understanding.

The method section was not simplified due to the instruction given by the publisher as follows:

"**Editorial recommendation**: Please keep JoVE's protocol requirements in mind as you address the above comment - the protocol must contain sufficient details in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text."

Reviewer #3:

Manuscript Summary:

The title of the manuscript is to vague,because the most important species you vocalize much to V. parahamolyticus. Also, this medium destine to Foodborne infections Vibrio (it should be appear in the title)

The title was revised to be more specific.

the results (sensitivity, specificity) of this manuscript should be discussed and compared with the results found in other studies

More previous studies are now included to discuss and compare the results.

we don't see any author in the discussion part.

More previous studies are now included in the discussion.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

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