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Assessment of Chromogenic Media in Bacterial Fish Pathogens

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ABSTRACT

Accurate and quick identification of bacterial fish disease has great importance for effective disease control and prevent economic loss in aquaculture facilities. Generally used agar media allow the growth of wide-range bacteria but new chromogenic media results in colored colonies that lead to presumptive identification without requiring biochemical or serological tests. In this study, the recently commercialized chromogenic media; chromID[®] CPS[®] Elite, chromID[®] CPS[®] Elite / Columbia CNA +5% sheep blood agar, chromID[®] Vibrio, chromID[®] MRSA Smart and chromID[®] Staphylococcus aureus Elite (BioMérieux, France) were tested with growth performance and colony coloration of bacterial fish pathogens. chromID[®] CPS[®] Elite and chromID[®] CPS[®] Elite / Columbia CNA +5% sheep blood agar were detected the most suitable culture media to grow for all five isolated fish pathogens (i.e., *Aeromonas hydrophila*, *Lactococcus garvieae*, *Pseudomonas fluorescens*, *Yersinia ruckeri* and *Vibrio anguillarum*). Of which, *L. garvieae* was the only one observed with green colonies. chromID[®] Vibrio designed for *V. parahaemolyticus* and *V. cholerae* was lead dark pink/purple colonies by *V. anguillarum*. The chromID[®] MRSA Smart and chromID[®] S. aureus Elite were detected not effective for incubation of bacterial fish pathogens.

Keywords: Chromogenic media, fish diseases, fish pathogens, isolation

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Bakteriyel Balık Patojenleri için Kromojenik Besiyerlerinin Değerlendirilmesi

Öz: Akuakültür işletmelerinde etkin hastalık kontrolü ve ekonomik kayıpların önlenmesi için bakteriyel balık hastalıklarının doğru ve hızlı tanımlanması büyük önem taşımaktadır. Genel olarak kullanılan agar besiyerlerinde çok çeşitli bakteri türünün üremesi gözlenirken, yeni kromojenik besiyerleri, biyokimyasal ve serolojik test gerektirmeden renkli koloni görünümüyle tahmini ön tanımlama imkânı sunmaktadır. Bu çalışmada yakın zamanda ticarileşen kromojenik besiyerlerinde (chromID[®] CPS[®] Elite, chromID[®] CPS[®] Elite / Columbia CNA +5% sheep blood agar, chromID[®] Vibrio, chromID[®] MRSA Smart ve chromID[®] Staphylococcus aureus Elite (BioMérieux, France)) bakteriyel balık patojenlerinin üremesi ve koloni renklemeleri tespit edilmiştir. ChromID[®] CPS[®] Elite ve chromID[®] CPS[®] Elite / Columbia CNA +5% sheep blood agarın test edilen 5 farklı bakteriyel balık patojeni (*Aeromonas hydrophila*, *Lactococcus garvieae*, *Pseudomonas fluorescens*, *Yersinia ruckeri* ve *Vibrio anguillarum*) için en uygun besiyeri olduğu, ancak sadece *L. garvieae*'nin yeşil koloni görünümü ile ürettiği belirlenmiştir. *Vibrio parahaemolyticus* ve *Vibrio cholerae*'nin hızlı teşhisi için geliştirilen chromID[®] Vibrio besiyerinde *V. anguillarum* kolonileri koyu pembe/mor renkli olarak gözlenmiş, chromID[®] MRSA Smart ve chromID[®] S. aureus Elite besiyerlerinin bakteriyel balık patojenlerinin üremesi için uygun olmadığı belirlenmiştir.

Anahtar kelimeler: Kromojenik besiyeri, balık hastalıkları, balık patojenleri, izolasyon

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Introduction

Along with the fastest growing food industry, aquaculture, and the increasing production rates, it is important to identify the fish diseases accurately and quickly that these diseases can cause great economic loss during the production cycle. Proper and quick

identification is the most important step for diagnosis of the fish diseases.

The use of chromogenic media engenders the inhibition of nontarget organisms with differential and selective culture characteristics and target pathogens grow as colored colonies. The system of

these chromogenic media depends on the metabolism of pathogens or enzyme substrates (Perry 2017). chromID® CPS® Elite and chromID® CPS® Elite / Columbia CNA +5% sheep blood are designed for isolation of urine specimens, *Escherichia coli*, *Proteaeae* and *Enterococcus*; chromID® *Vibrio* is for presumptive identification of *Vibrio parahaemolyticus* and *Vibrio cholerae*; chromID® MRSA Smart and chromID® *Staphylococcus aureus* Elite for the direct screening of methicillin-resistant *S. aureus* (MRSA) and *S. aureus* in clinical microbiology. It is important to utilize these media for quick presumptive identification of the bacterial fish disease by easier prediagnosis in order to prevent economic loss in aquaculture facilities.

There is no single media for isolation of bacterial fish pathogens. Besides the generally used culture media, some selective media are used for eliminating the growth of a wide range of bacteria. Thiosulfate Citrate Bile Sucrose (TCBS) Agar was originally designed for the isolation of *V. parahaemolyticus* and *V. cholerae* but it is also used for the isolation of some *Vibrio* species that are infectious for fish such as *V. alginolyticus*, *V. anguillarum*, *V. fischeri*, *V. harveyi*, *V. ordalii*, *V. splendidus* and *V. pelagus*. Waltman-Shotts medium has been devised in order to isolate another fish pathogen, *Yersinia ruckeri* with precipitation of insoluble calcium salts around the colonies depending on the Tween 80 hydrolysis (Austin and Austin 2007). Similarly, Yersinia CIN agar supplies selective cultivation of Yersinia species, commonly for *Y. pseudotuberculosis* and *Y. enterocolitica*, but also for isolation of infectious fish pathogen, *Y. ruckeri*. GSP agar is a selective agar base for detection of *Pseudomonas* and *Aeromonas* and has been using for isolation of infectious *Aeromonas* and *Pseudomonas* species in fish. The *Pseudomonas* selective agar base, Cetrimide agar has been especially used for the isolation of *Pseudomonas aeruginosa* by yellow/green colonial growth. Likewise, Eosin Methylene-Blue (EMB) agar is selective for *Enterobacteriaceae* family, coliform bacteria and especially for *E. coli* by greenish metallic sheen colored colonies caused by the lactose fermentation.

The aim of this study was to test the growth and the selectivity of five commercially available chromogenic media (chromID® CPS® Elite / Columbia CNA +5% sheep blood, chromID® CPS® Elite, chromID® *S. aureus* Elite, chromID® *Vibrio*, and chromID® MRSA Smart) for the isolation and presumptive identification of *A. hydrophila*, *L. garvieae*, *P. fluorescens*, *V. anguillarum* and *Y. ruckeri* while comparing its

specificity and sensitivity with generally used selective media.

Materials and Methods

Medium

The recently commercialized chromID® CPS® Elite / Columbia CNA +5% sheep blood (product no. 418229), chromID® CPS® Elite (product no. 418284), chromID® *S. aureus* Elite (product no. 419042), chromID® *Vibrio* (product no. 43761) and chromID® MRSA Smart (product no. 413050) were purchased from BioMérieux (France) and compared with routinely employed selective agar media (Thiosulfate Citrate Bile Sucrose (TCBS-Merck, Germany) Agar, Cetrimide Agar (Merck, Germany), Yersinia CIN Agar (Merck, Germany), EMB Agar (Merck, Germany), Glutamate Starch Phenol Red Agar - *Pseudomonas Aeromonas* Selective Agar (GSP, Merck, Germany) and Waltman-Shotts Medium for the isolation of *Y. ruckeri* (WS)) for detection and identification of infectious fish pathogens. In addition, comparison of the growth performance and advantages in isolation and identification were performed between new chromID® media and former media.

Microbial Strains

The bacterial fish pathogens previously isolated and identified from rainbow trout farms were used for all media. *A. hydrophila*, *L. garvieae*, *P. fluorescens*, *V. anguillarum* and *Y. ruckeri* were obtained from İzmir Katip Çelebi University, Faculty of Fisheries Fish Disease and Biotechnology Laboratory.

Inoculation of Isolates onto Culture Media

Each isolate was suspended in 1 ml of saline (0.85 %) until the turbidity reached at 0.5 McFarland standard (approximately 1.5×10^8 cfu/ml) then they were inoculated onto each medium type by using a loop. The petri dishes were incubated at 21°C. The growth was observed every 12, 24 and 48 hours of incubation. All strains were inoculated in duplicate on separate occasions.

Results

The growth of *A. hydrophila* on chromID® CPS® Elite was observed as pale brown colonies but no colonies grew on Columbia CNA +5% sheep blood agar, chromID® MRSA Smart, chromID® *S. aureus* Elite and chromID® *Vibrio* (Figure 1, Table 1). Yellowish/green colonies were observed on GSP agar which is selective for *Pseudomonas/Aeromonas* species. *L. garvieae* grew as green colonies on chromID® CPS® Elite /

Columbia CNA +5% sheep blood and chromID® CPS® Elite but was not recovered from chromID® MRSA Smart, chromID® *S. aureus* Elite and chromID® *Vibrio* (Figure 1, Table 1).

Table 1. Growth of different infectious fish pathogens on various media after 48 h of incubation at 21 °C

Species	Total number of isolates	Growth on chromID® CPS® Elite / Columbia CNA +5% sheep blood	Growth on chromID® CPS® Elite	Growth on chromID® <i>Vibrio</i>	Growth on chromID® MRSA Smart	Growth on chromID® <i>S. aureus</i> Elite
<i>A. hydrophila</i>	2	2	2	0	0	0
<i>L. garvieae</i>	21	21	21	0	0	0
<i>P. fluorescens</i>	2	2	2	2	0	0
<i>V. anguillarum</i>	6	6	6	6	0	0
<i>Y. ruckeri</i>	14	14	14	3	2	3

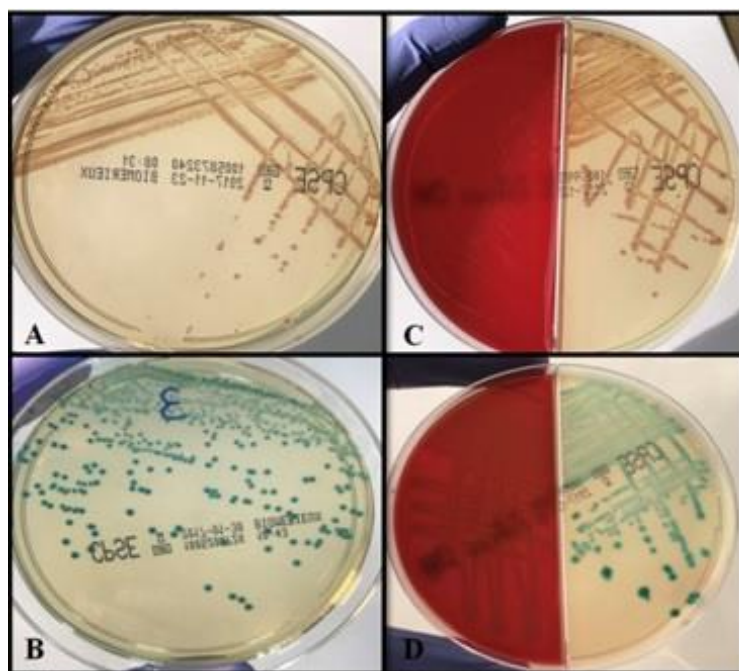


Figure 1. A: *A. hydrophila* on chromID® CPS® Elite; B: *L. garvieae* on chromID® CPS® Elite; C: *A. hydrophila* on chromID® CPS® Elite / Columbia CNA +5% sheep blood agar; D: *L. garvieae* on chromID® CPS® Elite / Columbia CNA +5% sheep blood agar

The colonial growth of *P. fluorescens* appeared on chromID® CPS® Elite / Columbia CNA +5% sheep blood and chromID® CPS® Elite, moreover on chromID® *Vibrio* as orange colonies (Figure 2, Table 1). No colonies were presented on chromID® MRSA Smart and chromID® *S. aureus* Elite. In addition, colonies were present on selective GSP and Cetrimide agar.

Vibrio anguillarum colonies were observed on chromID® CPS® Elite as pale brown colonies and on chromID® *Vibrio* as dark pink/purple colonies but there was no colonial growth on Columbia CNA +5% sheep blood agar, chromID® MRSA Smart and chromID® *S. aureus* Elite (Figure 2, Table 1). In

addition, yellow colored colonies appeared on TCBS agar which is the selective agar for *Vibrio* species.

Pale brown colonies of *Y. ruckeri* were present on chromID® CPS® Elite but there was only weak growth on chromID® MRSA Smart and chromID® *S. aureus* Elite with pink pigmentation and on chromID® *Vibrio* with green pigmentation. There were no colonies detected on Columbia CNA +5% sheep blood agar (Figure 3, Table 1). The precipitation of insoluble calcium salts around colonies were observed on Waltman-Shotts Medium and dark pink/red colonies were detected on Yersinia CIN Agar.

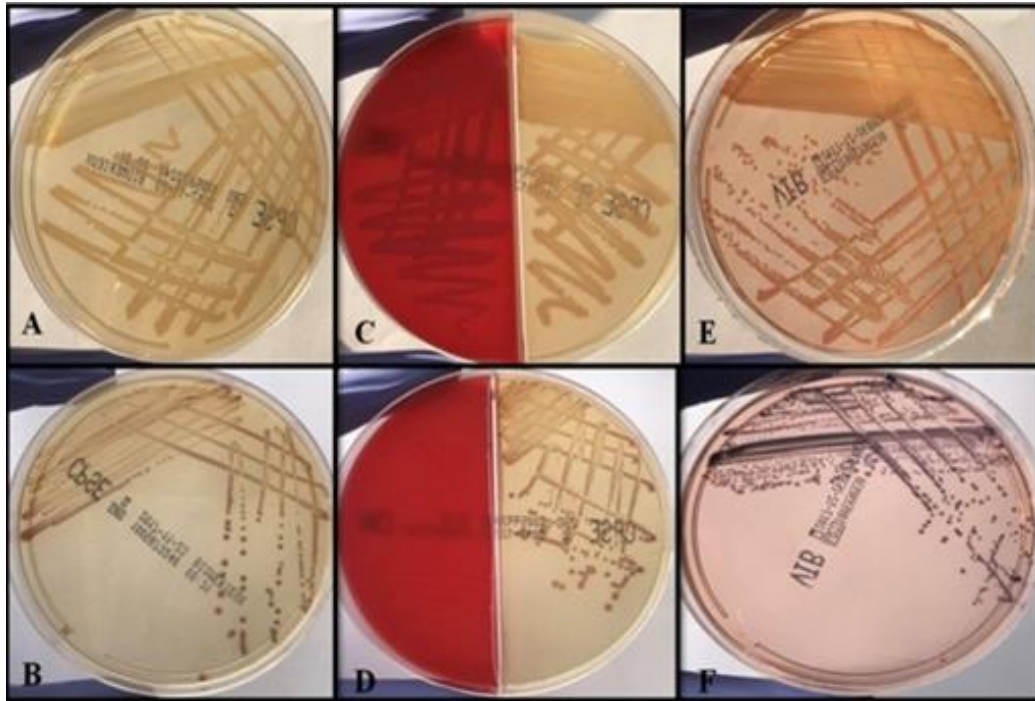


Figure 2. A: *P. fluorescens* on chromID® CPS® Elite; B: *V. anguillarum* on chromID® CPS® Elite; C: *P. fluorescens* on chromID® CPS® Elite / Columbia CNA +5% sheep blood agar; D: *V. anguillarum* on chromID® CPS® Elite / Columbia CNA +5% sheep blood agar; E: *P. fluorescens* on chromID® Vibrio; F: *V. anguillarum* on chromID® Vibrio

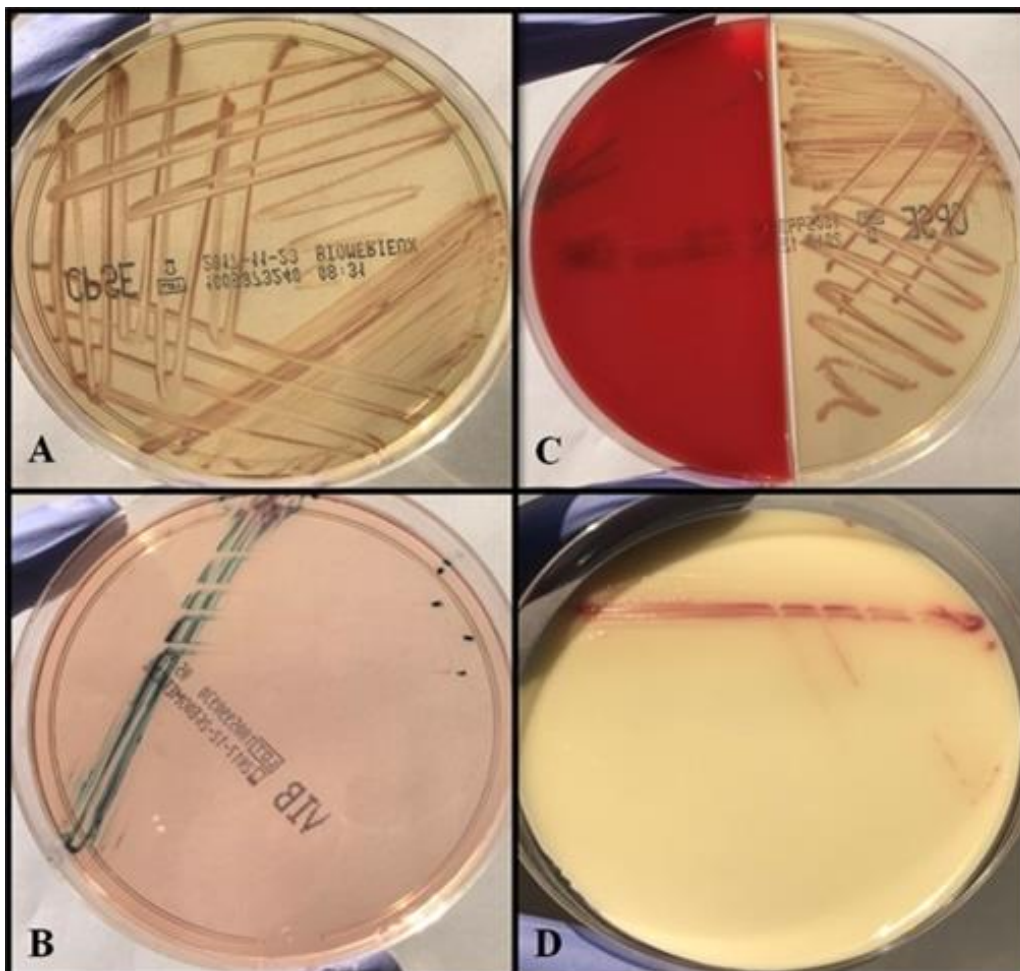


Figure 3. A: *Y. ruckeri* on chromID® CPS® Elite; B: *Y. ruckeri* on chromID® Vibrio; C: *Y. ruckeri* on chromID® CPS® Elite / Columbia CNA +5% sheep blood agar; D: *Y. ruckeri* on chromID® *S. aureus* Elite

Discussion

The bacterial fish pathogens were plated on 5 different commercialized media and compared to selective media in order to detect colony morphologies, growth performance and efficiency for detecting and identifying infectious fish pathogens.

Based on the colony characteristics, chromID® CPS® Elite agar has been recently commercialized and used for isolation and identification of urinary tract infections, especially *E. coli* in clinical microbiology. In addition, it is possible to detect *Enterococcus* spp., some members of *Proteeae* group and some members of *Enterobacteriaceae*, culture them and amplify antibiotic susceptibility tests. This media allows a preliminary identification for various microorganisms with genus or group level on the strength of colony appearance and Gram stain. Also, it is able to directly identify *E. coli* by the colony appearance without any requirement of Gram stain or biochemical testing (Yarbrough et al. 2016). The bacterial load of fish is less than urinary samples, it is likely that the selectivity of fish pathogens would be higher. In this study, all 5 bacterial fish pathogens grew on chromID® CPS® Elite, especially green colony was observed with *L. garvieae* which is one of Gram-positive bacteria but *Y. ruckeri* which belongs to the family *Enterobacteriaceae* showed no distinctive colony coloration. Moreover, we have tested chromID® CPS® Elite / Columbia CNA +5% sheep blood which contains two different media on the same agar plate in half. Columbia CNA +5% sheep blood agar is specific for the isolation and differentiation of Gram-positive microorganisms. Our results clearly showed that the chromID® CPS® Elite / Columbia CNA +5% sheep blood agar was highly selective to *L. garvieae*. Also, observing growth on both media on the same plate can lead to reliably compare the colony morphologies and coloration easily.

The members of the genus *Vibrio* have great importance because of the association with human disease as *V. cholerae* (the etiological agent of epidemic cholera) and *V. parahaemolyticus* (the cause of gastroenteritis associated with the consumption of seafood) (Farmer et al. 1985; Grimes 1991; Blanco-Abad et al. 2009; Eddabra et al. 2011). chromID® *Vibrio* was developed for determining *V. cholerae* and *V. parahaemolyticus* from other *Vibrio* species by the blue/green colonies of *V. cholerae* and pink colonies of *V. parahaemolyticus* on the medium. The TCBS *Vibrio* selective media was developed for isolation of pathogenic *Vibrio*

species but it has poor sensitivity in natural specimens, labor-intensive and time-consuming. In addition, TCBS may not differentiate *V. cholerae* from *V. alginolyticus* or *V. parahaemolyticus* and *V. fluvialis* from *V. mimicus* or *V. vulnificus* (Eddabra et al. 2011). There is a slight selective advantage of chromID® *Vibrio* because of the color colonies. It has indicated that its sensitivity is equivalent to TCBS medium and two times more specific as well as no confirmation is needed (Eddabra et al. 2011). *Vibrio cholerae* appears as blue-green colonies because of beta galactosidase-produce and *V. parahaemolyticus* grows pink colonies based on arabinose assimilation on chromID® *Vibrio* media. Pinto et al. (2011) reported that *Vibrio* CHROMagar plates (CAV, PBI International, Milan, Italy) displayed higher specificity and accuracy than TCBS agar for isolating *V. parahaemolyticus* and *V. fluvialis*. Furthermore, unlike TCBS medium, the CAV medium was more efficient to isolate *V. parahaemolyticus* for DNA-based typing analysis. The fish pathogen *V. anguillarum* was tested on chromID® *Vibrio* media for the first time in this study and observed dark pink/purple colonies. The color characteristic of *V. anguillarum* was not clear; therefore, further studies are needed to certify the reason of dark pink/purple coloration.

The direct screening of methicillin-resistant *S. aureus* (MRSA) is practicable with using chromID® MRSA Smart in clinical microbiology. ChromID® *S. aureus* Elite is another media for the direct identification of *S. aureus* by spontaneous green coloration of glucosidase-producing colonies. *Staphylococcus* genus has been implicated as pathogen for fish and causing septicemia-like signs, exophthalmia and lesions around fins (Shotts and Teska 1989; Gil et al. 2000). Another important fish pathogen *L. garvieae* affects mainly rainbow trout farms that cause losses approximately 50% of the total production (Ghittino and Prearo 1992; Alrabadi 2012; Tanrikul 2012; Balta and Dengiz Balta 2019). Also, infected or carrier fish may spread the disease to healthy rainbow trout and lead to serious economic damage (Algöet et al. 2009). Several antibiotic treatments were applying such as enrofloxacin, florfenicol and oxytetracycline in order to control the outbreaks in Turkey (Kayış et al. 2009; Türe and Boran 2015). The antimicrobial resistant bacteria and its potential transfer between animals and human are the main concern and the antibiotic treatment may be not efficient in fish farms (Cabello et al. 2013; Muziasari et al. 2014; Türe and Boran 2015). In this study, we aimed to use both media for isolation of *Staphylococcus* while inhibiting the growth of *L. garvieae* but none of the fish pathogens were able to

grow on chromID® MRSA Smart and chromID® *S. aureus* Elite. These results clearly show that both of these media are not suitable for isolation and identification of fish pathogens.

The use of chromogenic media brings some advantages such as easy detection of target pathogens and differentiation of mixed cultures. On the contrary, these products are more expensive than conventional media but the disadvantage may be compensated by the reduction of complementary reagents and different culture plates for suspected pathogens (Perry and Freydière 2007). Yarbrough et al. (2016) reported that using chromID® agar decrease the laboratory costs with reducing standard media consumption such as inoculation on both blood agar and MacConkey agar plates and additional biochemical tests.

In conclusion, the bacterial fish pathogens grew well in chromID® CPS® Elite agar and chromID® CPS® Elite / Columbia CNA +5% sheep blood agar, *V. anguillarum*, *P. fluorescens* and *Y. ruckeri* grew well in chromID® *Vibrio* but pigment production was only observed for *V. anguillarum*. ChromID® CPS® Elite agar, chromID® CPS® Elite / Columbia CNA +5% sheep blood agar and chromID® *Vibrio* may be a practicable alternative to routinely employed chromogenic medium may be a feasible alternative to routinely used media. They are cost saving with reducing the number of agar plates and provide presumptive data by coloration specificity. Also in some cases, it is unnecessary to do confirmation tests such as Gram stain, oxidase test and determination of biochemical properties for presumptive identification.

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