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Usefulness of Chromogenic CromoCen® AGN agar medium for the identification of the genus *Aeromonas*: Assessment of faecal samples

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ABSTRACT

Selective screening media for the detection and identification of *Aeromonas* strains are needed to guide primary isolation procedures in the clinical laboratory. This study compared the selective CromoCen® AGN chromogenic agar medium for the detection and identification of *Aeromonas* strains that were isolated from various samples against the conventional selective agar media that are commonly used for the isolation of this organism in food, environmental and clinical samples. The Miles and Misra and ecometric methods were used to evaluate the microbiological performance of CromoCen® AGN chromogenic agar medium, which was shown to be satisfactory. A total of 14 reference *Aeromonas* strains, 44 wild strains and 106 clinical stool specimens were examined using both non-chromogenic selective agars that are commonly used for *Aeromonas* isolation and CromoCen® AGN agar. The latter exhibited 94.73% sensitivity and 100% specificity for the various samples. On CromoCen® AGN agar medium, *Aeromonas* formed colonies with light green, greenish and salmon pigments with or without a surrounding wide transparent zone (halo) of 2–3 mm in diameter around the entire border. This medium is recommended for the isolation and potential identification of the *Aeromonas* genus.

1. Introduction

Over the past 20 years, *Aeromonas* species have received increased recognition as enteric pathogens in humans. In humans, *Aeromonas* species are associated with intestinal, skin and blood-borne infections, and they cause diseases in a variety of animals, including fish, amphibians, reptiles and mammals (Janda and Abbott, 2010). Consequently, there is growing interest in improving the selective or differential properties of culture media for the optimal isolation of *Aeromonas* from clinical and environmental sources. In the primary isolation of members of *Aeromonadaceae* from faeces of diarrhoeic patients, routine enteric agars such as MacConkey (McC), xylose lysine dextrose agar (XLD), Hecktoen enteric agar (HE) and Salmonella-Shigella agar (SS) can be used. However, it is clear that their use leads to a gross underestimation of the actual incidence. For example, in SS, lactose-negative isolates (e.g., *Aeromonas hydrophila*) must be differentiated from commonly isolated pathogens such as *Salmonella* and *Shigella*. In contrast, if the isolate is lactose- or sucrose-positive, it can be assumed to be normal flora (e.g., *Aeromonas caviae*) and therefore overlooked. For this reason, the current preference is to use a selective medium. Several selective media have been developed; however, few detailed comparative studies of the various media that have been proposed for the recovery of *Aeromonas* spp. from different origins have been conducted (Janda and Abbott, 2010; Huddleston et al., 2007; Gobat and Jemmi, 1995; Havelaar et al., 1987; Feige, 2001) (Table 1).

Chromogenic media have been developed for the identification of a large variety of pathogenic organisms (Tsoraeva et al., 2008). These media facilitate the identification of these organisms at the genus level and – in some cases – at the species level, thus requiring a shorter testing time and simplifying the detection of target bacteria and the interpretation of primary culture results. Bacterial growth in this type of media facilitates the easy recognition of suspicious colonies by the development of specific colours that can be detected using the naked eye. Currently, several pathogenic enteric microorganisms, including *Escherichia coli*, *Klebsiella* spp., *Proteus*, *Enterobacter* spp., *Salmonella* spp., *Vibrio cholerae* and *Vibrio parahaemolyticus*, can be differentiated in chromogenic culture media. Other pathogens that can also be identified include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Listeria monocytogenes*, and *Candida* spp. (Manafi, 2000). The routine use of a chromogenic medium for the isolation of *Aeromonas* spp. would be a great asset to accelerate identification for either research or diagnostic purposes. The research group of the Centro Nacional de Biopreparados (BioCen, Cuba) developed a chromogenic medium for the presumptive identification of *Aeromonas*. These media are based on the *Aeromonas* due to their capacity to hydrolyse proteins (proteolytic activity), for which the medium contains fat-free milk, whose casein is degraded

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Table 1
The various media that were tested for the recovery of *Aeromonas* spp. from several origins.

Medium	Purpose	*Aeromonas* isolation	
		Main advantages	Main disadvantages
Salmonella and Shigella agar	Selective and differential medium for *Salmonella* and *Shigella* isolation	Grows well	• Lactose-negative isolates such as *A. hydrophila* must be differentiated. • Lactose-positive isolates such as *A. caviae* may be assumed to be normal flora and be overlooked.
Cefsulodin, irgasan, novobiocin agar (CIN)	Highly selective medium designed to isolate *Yersinia enterocolitica*	By reducing the concentration of cefsulodin in the media, *Yersinia* spp. grow well together	Oxidase test needed to differentiate *Aeromonas* from *Yersinia*, and identification of *Citrobacter* cannot be performed directly.
Ampicillin (20 μg/ml) sheep blood agar (ASBA)	Selective and differential medium useful only for the primary recovery of *Aeromonas* spp.	Haemolytic colonies can readily be tested for oxidase, which favours screening.	Non-haemolytic *Aeromonas* strains will be missed. *A. trota* is susceptible to ampicillin and would be inhibited.
Ampicillin (10 μg/ml) dextrin agar (ADA)	Selective medium that partially inhibits the growth of non-target bacterial species while allowing most *Aeromonas* spp. to grow	*Aeromonas* is identified by the fermentation of dextrin and the presence of yellow colonies.	*A. trota* is undetected by the selective agent, as in ASBA.
ADA with vancomycin (2 mg) (ADA-V)	Increases selectivity		
Ryan *Aeromonas* medium (AM)	Selective medium for the isolation of *Aeromonas*; a modified formulation of XLD agar	Supports the growth of *Aeromonas* spp. *Plesiomonas* spp. and the usual *Enterobacteriaceae*	

Xylose lysine deoxycholate agar (XLD agar) is a selective growth medium used in the isolation of Salmonella and Shigella species from clinical samples.

by the proteolytic enzymes of most *Aeromonas* species. Hydrolysis of these proteins gives rise to the characteristic transparent halo. Within the Gram negative bacteria isolated from faecal samples, *Pseudomonas* is the other genus that exhibits this capacity, but it differentiates from Aeromonas by the absence of glucuronidase and galactosidase activities.

Besides, differentiation of *Aeromonas* from other species is not based on their capacity to ferment carbohydrates, such as lactose or xylose, with formation of acids and changing pH indicators, a principle used in other media and which leads to incorrect identification, since other enterobacteria depict a similar capacity.

The only carbon source included in the medium is alcohol, and *Aeromonas* are not able to degrade it. CromoCen® AGN agar medium was previously used for the isolation of Gram negative bacteria including *Aeromonas* from food samples (Quesada-Muñiz et al., 2002).

The purpose of this study was to assess the usefulness of the chromogenic CromoCen® AGN agar medium for the isolation, differentiation, and potential identification of *Aeromonas* spp. isolated from several sources.

2. Materials and methods

2.1. Bacterial strains

A total of 62 strains were tested, including 13 type strains from the Spanish type culture collection (CECT) of the *Aeromonas* genus, 12 strains isolated from water, 9 isolated from frozen fish, 23 isolated from gastrointestinal infection, and 6 other strains from the American type culture collection (ATCC) (Table 2). The medium was tested using the ecometric streaking method described by Mossel et al. (1980) as a semi-quantitative alternative to other techniques.

2.2. Culture media

CromoCen® AGN agar (BioCen, Cuba) that was prepared from the medium base following the manufacturer's instructions was evaluated. The medium was designed for the preparation of the chromogenic agar medium for the rapid and simultaneous identification of *Aeromonas* and *Pseudomonas* and for the identification and/or enumeration of *Salmonella*, *E. coli*, and coliform bacteria. Two control media (MacConkey Bioxon®, Mexico, and Ryan media®, United Kingdom) were used for the comparative evaluation using the ecometric test that is described below.

2.3. Mossel's ecometric technique

This technique was designed specifically to test the quality of solid media. Overnight cultures that were adjusted to McFarland Nephelometer Standards No. 0.5 (1.5×10^8 CFU/ml) of the test strains were inoculated onto the agar media in a defined, standardised way. One loop of inoculum was sequentially diluted from streak to streak. Growth on the plates was not recorded as a colony count but as a score. Five streaks of growth in each quadrant were scored as one (0.2 each). A maximum score of five was obtained when all of the streaks in the four quadrants showed growth, and the final streak in the centre of the plate was also colonised (Corry et al., 1999). Thirteen

Table 2
The bacterial strains that were used in the simple growth control and evaluation of culture media.

Bacterial species	Referenced	Bacterial species[a]	Origin		
			Clinical	Fish	Water
A. hydrophila	CECT839[b]	*A. caviae*	13		
A. bestiarum	CECT4227	*A. hydrophila*	10		
A. caviae	CECT838	*A. salmonicida*		6	
A. media	CECT4232	*A. bestiarum*		2	12
A. eucrenophila	CECT4224	*A. veronii* bv. sobria		1	
A. sobria	CECT4246	Total $n=44$	23	9	12
A. veronii	CECT4257				
A. jandaei	CECT4228				
A. trota	CECT4255				
A. allosaccharophila	CECT4199				
A. encheleia	CECT4343				
A. popoffii	LMG17541[c]				
A. culicicola	CECT5761				
S. aureus	ATCC25923[d]				
E. coli	ATCC25922				
K. pneumoniae	ATCC13883				
S. enterica Typhimurium	ATCC14028				
Vibrio fluvialis	ATCC338809				
P. aeruginosa	ATCC27853				

[a] Genetically identified in Castro-Escarpulli et al. (2003) and Aguilera-Arreola et al. (2005).
[b] CECT, The Spanish type culture collection, Universidad de Valencia, Valencia, Spain.
[c] LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium.
[d] ATCC, American Type Culture Collection, Rockville, USA.

type strains of *Aeromonas* spp. and *S. aureus* (ATCC 25923) were inoculated onto the McC agar (control agar), Ryan (*Aeromonas*) media and CromoCen® AGN agar medium. The media plates were inoculated and incubated in triplicate for 24–48 h at 28 °C or 37 °C. The scores for the selective and control agars were compared to estimate the degree of inhibition that was due to the selective agents. The results are expressed as six numbers divided in two groups. The first group represents the observed growth of *Aeromonas* strains on the specific media, and the second represents the absence of growth of the reference strains that were tested on the specific media for *Aeromonas* (Table 3).

2.4. Miles and Misra assay

Retrospectively, we also subcultured 44 wild strains of *Aeromonas* that were isolated from diarrhoeic faeces, frozen fish or water as well as 6 non-*Aeromonas* strains. These isolates were used as test strains to evaluate the CromoCen® AGN with non-reference or type strains such as non-*Aeromonas* strains (Table 2). Overnight cultures that were adjusted to McFarland Nephelometer Standards No. 0.5 (1.5×10^8 CFU/ml) of the test strains were inoculated onto the agar surface with 3 replicates by dropping 20 µl each using an automatic pipette (Corry, 1979).

2.5. Evaluation of CromoCen® AGN for the primary isolation of Aeromonas from faecal clinical specimens and for the identification of Aeromonas strains

One hundred and six stool samples from asymptomatic children between 6 and 8 years of age were collected from the Ezequiel A. Chávez elementary school in Mexico City, inoculated in alkaline water, incubated overnight at 25 °C ± 3 °C and then subcultured overnight at 37 °C on McC, Ryan medium, ampicillin-dextrin agar (ADA), and ampicillin-dextrin agar with vancomycin ADA-V or CromoCen® AGN agar medium. Lactose-negative isolates on McC, dextrin-positive (yellow) isolates on ADA-V, and those isolates showing an *Aeromonas* characteristic morphotype on Ryan and CromoCen® AGN agar media were sub-cultured on Mueller Hinton agar (Bioxon®, Mexico). Five presumptive colonies of *Aeromonas* (showing a typical colonial morphology and Gram stain, but not necessarily hemolytic) were tested for oxidase (Bactident® oxidase, Merck-Mexico), and oxidase positive colonies were then grown in the presence of the vibriostatic agent 2,4 diamino-6,7 diisopropylpteridine (150 µg/ml) (Castro-Escarpulli et al., 2003). Only one or two colonies able to grow on plates with vibriostatic agent were selected for genetic identification to the species level by the 16S rDNA restriction fragment length polymorphism (RFLP) technique of Borrell et al. (1997), as reported by Figueras et al. (2000). Only one confirmed *Aeromonas* isolate (colony) per sample. Briefly, the PCR-amplified *16SrRNA* gene (1502 bp) was amplified by PCR and purified (Concern Rapid PCR, Gibco-BRl). The purified amplicon was digested with two endonucleases (*Alu*I and *Mbo*I (Invitrogen, Mexico)) simultaneously; the restriction products were electrophoresed for 3 h on 17% polyacrylamidegel at 300 V (Aguilera-Arreola et al., 2005).

Table 3
Interpretation examples of the results using the ecometric technique.

Acceptance code:		Interpretation	Rejection code:		Interpretation
Aeromonas	*S. aureus*		*Aeromonas*	*S. aureus*	
555	000	Selective media (ideal)	444	444	Not acceptable
554	001	Acceptable	441	332	Rejected
544	110	Acceptable	320	332	Rejected

2.6. Statistics

Sensitivity and specificity were calculated for the following pure type cultures: clinical or reference strains on CromoCen AGN ® agar medium ($n=57$) versus non-*Aeromonas* reference strains ($n=6$). The ability of the selective media to correctly promote the growth of the typically expected *Aeromonas* colonies was considered to be true positives (light green, greenish or salmon with or without a wide transparent halo). However, if the *Aeromonas* strains grew as atypical colonies (from rose to orange with a wide transparent halo and greenish fluorescence), they were considered to be false negatives. Non-*Aeromonas* strains that grew as light green, greenish or salmon colonies with or without a wide transparent halo were recorded as false positives. A true negative was a non-*Aeromonas* strain with a different colony colour than the expected colour for *Aeromonas*. A test with 100% sensitivity correctly identifies all of the tested strains as *Aeromonas* spp. A test with 100% specificity correctly identifies all of the tested non-*Aeromonas* strains (Ghaaliq and McCluskey, 2008).

3. Results

3.1. Growth promotion and typical expected colonies on CromoCen® AGN agar medium

All 13 *Aeromonas* reference or type strains grew on each medium after direct culture at 28 °C. *Aeromonas encheleia* CECT 4343 did not grow at 37 °C on CromoCen® AGN agar medium. Eight out of thirteen strains exhibited the characteristic and expected light green colour at 28 °C as reported by the CromoCen® AGN agar medium manufacturer, whereas the remaining five strains exhibited a light pink-greenish colour. A total of 12 of the strains (with the exception being *Aeromonas allosacharophila*) grew with a wide transparent halo. In contrast, at 37 °C, most of the strains exhibited a rose colour that was similar to salmon, and *Aeromonas bestiarum* and *Aeromonas eucrenophila* grew without a transparent halo due to a lack of proteolytic activity.

3.2. Mossel's ecometric test

All 13 type strains of the *Aeromonas* genus that were tested yielded a growth index of 555 and 000 on both selective (CromoCen® AGN agar medium) and control (McC and Ryan (*Aeromonas*)) media. In contrast, the *S. aureus* ATCC 25923 strain yielded a growth index of 000 and 000, demonstrating that Gram-positive bacteria can be inhibited; therefore, the medium is considered to be selective.

3.3. Assessment of the CromoCen® AGN medium with isolated or type Aeromonas strains and non-Aeromonas type strains

To evaluate the behaviour (i.e., abundant growth, colour development and proteolysis halo) of the wild strains on CromoCen® AGN medium, the wild strains that were isolated from clinical and water samples and from frozen fish were tested. Overall, 44 isolates were streaked on media plates. As with the reference strains, differences in the growth at 28 °C and 37 °C were observed. One strain (18Hgo) did not grow at 37 °C. At 28 °C, all 44 tested strains grew (100%), 36 of which showed proteolytic activity (82%). In contrast, only 43 strains grew (98%) at 37 °C, and all 43 strains showed proteolytic activity (100%) (Table 4). In the medium, among all of the growing strains, some of the *Aeromonas* strains grew as colonies with various colours and tonalities ranging from a light (pale) green or salmon-like colour – with differing tonalities – to violet. The colour of the colonies was independent of the tested species; therefore, identification at the species level was not possible.

The results for the other Gram-negative bacteria that were tested are shown in Table 5. The strains grew on each medium after direct

Table 4
Evaluation of the colony appearance and proteolytic activity of wild *Aeromonas* strains on CromoCen® AGN agar medium.

Colony appearance	Growth temperature, characteristics and origin					
	28 °C			37 °C		
	Colony appearance/ proteolysis activity			Colony appearance/ proteolysis activity		
	Clinical $n=23$	Fish $n=9$	Water $n=12$	Clinical $n=23$	Fish $n=9$	Water $n=12$
Greenish to light green	10/10	8/9	10/11	8/11	8/8	4/4
Light pink-greenish tonality	3/3			0/0	1/1	
Rose/salmon-like colour	5/6		0/1	6/8		7/8
Bluish violet	0/3			0/2		
Violet	0/1			0/1		
Total strains tested, $n=44$	18/23	8/9	10/12	14/22[a]	9/9	11/12

[a] One strain did not grow at 37 °C.

inoculation, with the exception of *Salmonella enterica* Typhimurium, which was unable to grow on ADA medium. CromoCen® AGN agar medium allowed the expected differentiation of all of the Gram-negative bacteria as shown by colour and proteolytic activity. In addition, CromoCen® AGN agar medium had the advantage of also differentiating other clinically relevant Gram-negative genera of bacteria by the specific colour of the colonies or the presence of a halo (Table 5).

3.4. Evaluation of CromoCen®AGN agar medium for the primary isolation of Aeromonas from faecal clinical specimens and the identification of Aeromonas strains

CromoCen® AGN agar medium was assessed for the potential identification of *Aeromonas* spp. in faecal samples that were collected from asymptomatic school children. Out of 106 processed stool cultures, 5 were putatively positive (4.7%) and identified as *Aeromonas* colonies. These five strains were pale green in colour (light green), and only four had evident proteolytic activity. From each of these positive samples, a colony was picked and genetically identified. The five strains were genetically identified as *A. hydrophila* using *16S rDNA* RFLP. Although all clinical isolates have well grow on the McC, Ryan medium without ampicillin, ampicillin-dextrin agar (ADA), and ampicillin-dextrin agar with vancomycin ADA-V and CromoCen® AGN agar medium most of the *Aeromonas* described as rose-salmon like colour and light pink-greenish tonality showed characteristic transparent halo at 28 °C and 37 °C which make it easier to differentiate *Aeromonas* colonies from enteric bacteria colonies; thus the halo can be used for the presumptive identification of Aeromonas in faecal samples.

3.5. Statistics

The sensitivity and specificity values for CromoCen® AGN agar medium were 94.73% and 100%, respectively. The ideal (but unrealistic) diagnostic performance should be a 100% accurate test. Three false negatives were detected, corresponding to two *A. hydrophila* strains and one *A. caviae* strain. No false positives were found.

4. Discussion

The selection of a reliable and inexpensive medium for the isolation of *Aeromonas* that is also practical for used in routine laboratory testing is essential to avoid the underestimation of the incidence in clinical and environmental samples. Therefore, we tested the CromoCen® AGN agar medium to evaluate whether it could serve as a viable option.

The ecometric technique has been used successfully by several authors to validate the qualities and properties of solid media or to trace errors in medium preparation. The primary advantages of this technique are that it is not labour-intensive and the material costs are low, as the overnight culture is diluted on one plate. However, the ecometric technique requires training to obtain reproducible results. As a dilution in colony numbers should be obtained from streak to streak, slight changes in the angle of the loop or the pressure that is applied to the loop during inoculation can lead to a perturbation of the desired dilution pattern and can render the score unreliable. The performance also depends on the type of microorganism. During the evaluation, the CromoCen® AGN agar medium provided a good yield due to the inhibition of Gram-positive bacteria and the growth promotion of *Aeromonas* species that was at least equivalent to that of the control media, which means that the media that were tested with the ecometric technique showed satisfactory performance. Upon completion of the training period, no problems were observed when the strains were streaked on media or during the interpretation of the test results.

CromoCen® AGN agar medium is a valuable medium for the primary isolation of *Aeromonas* spp. based on the enzymatic substrates that were included in its final formulation and which provide the characteristic colour reactions in the colonies and the surrounding area. CromoCen® AGN agar medium was therefore useful in the differentiation and presumptive identification of some Gram-negative bacteria genera based on the morphology and colour of the colour and colony. This feature enabled the medium to identify the tested strains

Table 5
Growth of Gram-negative bacteria on various media and the colony appearance on CromoCen® AGN agar medium.

Strain	MH[a]	McC[b]	ADA[c] 10 μg/ml	ADA-V[d] 2 μg/ml	CromoCen® AGN agar medium			
					Growth	Colour	Appearance	Proteolysis
E. coli ATCC[e] 25922	+	+	+	+	+	Intense bluish violet with violet halo		-
K. pneumoniae ATCC 13883	+	+	+	+	+	Violet		-
S. enterica Typhimurium ATCC 14028	+	+	-	+	+	Intense rose		-
Vibrio fluvialis ATCC338809	+	+	+	+	+	Dark pink		+
P. aeruginosa ATCC 27853	+	+	+	+	+	Light pink[f]		++
A. hydrophila ATCC 7966[Tg]	+	+	+	+	+	Light green		+++

[a] MH, Müller-Hinton agar; MH was the control medium for growth promotion.
[b] McC, MacConkey agar.
[c] ADA, ampicillin-dextrin agar.
[d] ADA-V, ampicillin-dextrin agar with vancomycin.
[e] ATCC, American Type Culture Collection.
[f] Fluorescent colonies were observed under UV light.
[g] Included for comparison purposes.

at the genus level with 94.73% sensitivity and 100% specificity. Identification of the *Aeromonas* genus was achieved in less time and with less financial and technical resources compared with traditional tests, considering that the latter required at least six classic tests for identification at the genus level. The plating efficiency of CromoCen® AGN agar medium for *Aeromonas* spp. that was isolated directly from stool specimens was performed in small samples in which the absence of swarming of *Proteus* spp. normal flora was observed, whereas some wild *A. hydrophila* strains could be isolated without problems, as shown by genetic identification. However, a wider sampling plan needs to be performed. These findings reinforce the utility of CromoCen® AGN agar medium. In contrast, some difficult issues included i) the spectrum of the colour display among the 44 strains that were tested on the agar and ii) the temperature dependence of the colour development and the appearance of a proteolysis halo. Nevertheless, most of the strains were in the greenish to light green colour spectrum at 28 °C. Because individual strains showed a significant dependence on temperature, it is recommended that at least two different incubation temperatures (i.e., 28 and 37 °C) be used to attempt to improve the isolation performance of the media. CromoCen® AGN agar medium is useful for the inhibition of Gram-positive bacteria, is easy to use, is simple to interpret and suitable for the identification of the *Aeromonas* genus. However, due to the incidence of false negative results, it should not be used for species identification.

4.1. Conclusions

If the incidence of *Aeromonas* diarrhoea in the patient population of a given laboratory is high, the routine use of CromoCen® AGN agar medium – a selective agar that is specifically designed for *Aeromonas* spp. and has a relatively high plating efficiency for most strains – can simplify the initial genus identification prior to genetic identification.

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