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Evaluation of Different Conditions and Culture Media for the Recovery of *Aeromonas* spp. from Water and Shellfish Samples

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Running title Culture methods for *Aeromonas*

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Abstract

Aims: To perform a comparative study for determining the optimum culture method (direct plating or enrichment) and medium (ADA, SAA, BIBG-m) for recovering *Aeromonas*

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species from water and shellfish samples. ***Methods and Results:*** By direct culture, *Aeromonas* was detected in 65% (13/20) of the water samples and in 54.5% (6/11) of the shellfish samples. However, when a pre-enrichment step was included, the number of positive water samples increased to 75% (15/20) and the ones of shellfish to 90.1% (10/11). The enriched culture significantly favored ($P < 0.05$) the isolation of *Aeromonas allosaccharophila* from water, *Aeromonas salmonicida* from shellfish, and *Aeromonas caviae* from both types of samples. The most specific ($P < 0.05$) culture medium for detecting *Aeromonas* from water was ADA. However, no differences were observed in the case of shellfish samples ($P > 0.05$). Isolation of *Aeromonas media* from water was favored ($P < 0.05$) in the ADA medium, while SAA enhanced ($P < 0.05$) the isolation of *A. salmonicida* from shellfish. ***Conclusions:*** The culture method and medium used influenced the recovery of some *Aeromonas* species from water and shellfish samples. ***Significance and Impact of study:*** This fact should be considered in future prevalence studies to avoid overestimating the above mentioned *Aeromonas* species.

Key words: *Aeromonas*, direct plating, enrichment, culture media, water, shellfish.

1. INTRODUCTION

The genus *Aeromonas* was described by Stainer in 1943 (Martin-Carnahan and Joseph 2005) and nowadays it contains 32 species (Beaz-Hidalgo *et al*. 2015; Marti and Balcázar 2015; Martínez-Murcia *et al*. 2016). These species are considered indigenous to aquatic environments because most of them have been isolated from fresh water, seawater, drinking water and sewage. However, some species can also be isolated from healthy and diseased fish, chironomid egg masses, and from intestinal and extraintestinal human samples (Borrell *et al*. 1998; Castro-Escarpulli *et al*. 2003; Figueras 2005; Janda and Abbott 2010; Figueras

et al. 2011: Beaz-Hidalgo *et al*. 2012; Figueras and Beaz-Hidalgo 2015). *Aeromonas* species are considered emerging pathogens in humans, and can affect both immunocompetent and immunocompromised individuals via the consumption of contaminated water and food, or through contamination of open wounds from environmental water sources (Figueras 2005; Janda and Abbott 2010; Figueras and Beaz-Hidalgo 2015).

Isolation and correct identification of *Aeromonas* spp. are complex and laborious (Jeppesen 1995; Perales 2003). A variety of selective and differential media has been proposed for isolating *Aeromonas*, but no single medium has received general acceptance (Jeppesen 1995; Perales 2003). Glutamate starch penicillin agar (GSP, Kielwein 1969), *Aeromonas* medium (Rippey and Cabelli 1979), ampicillin dextrin agar (ADA, Havelaar *et al*. 1987), ampicillin bile salts inositol xylose agar (Cunliffe and Adcock 1989), and starch glutamate ampicillin penicillin C-glucose agar (SGAP-10C, Huguet and Ribas 1991) were amongst the first media used for the isolation of *Aeromonas*. However, culture media such as starch ampicillin agar (SAA, Palumbo *et al*. 1985), MacConkey xylose agar (Okrend *et al*. 1987), MacConkey mannitol agar (Okrend *et al*. 1987), and bile salts irgasan brilliant green modified (BIBG-m, Neyts *et al*. 2000b) were specifically proposed for *Aeromonas* isolation from food samples. Other culture media used for isolating *Aeromonas* spp. from both food and environmental samples include Rimler Shotts agar (Shotts and Rimler 1973), peptone beef extract glycogen agar (Nishikawa and Kishi 1987), bile salt brilliant green starch agar (McCoy and Pilcher 1974), and Ryan medium (Anonymous 1990).

In this study, we evaluated the selectivity and sensitivity of three culture media (ADA, SAA, and BIBG-m) to obtain *Aeromonas* spp. from water and shellfish samples. The ADA medium was originally described to analyze these microbes from different types of water (sewage, lake, river, sea, and drinking water), SAA was developed for testing foods such as fish and shellfish, and BIBG-m was designed to isolate *Aeromonas* spp. from feces and to analyze

food samples (shrimp, minced meat, and vegetables). Currently, ADA medium is the most widely used for detecting *Aeromonas* from water samples, and has been the reference medium used in our laboratory since 1998 (Borrell *et al*. 1998). SAA and BIBG-m were selected based on good results from sensitivity and specificity testing reported in previous studies (Okrend *et al*. 1987; Tsai and Chen 1996; Singh 1997; Mattick and Donovan 1998; Neyts *et al*. 2000a, b). An enrichment step is commonly used for analyzing *Aeromonas* from food products where it is suspected that the number of bacteria may be low or the bacterial cells are stressed. Alkaline Peptone Water (APW) and Trypticase Soy Broth (TSB), supplemented with antibiotics such as ampicillin and cephalotin, are the enrichment media most commonly used to detect *Aeromonas* (Bobat and Jemmi 1995; Sachan and Agarwal 2000). Therefore, we also aimed to determine the importance of the enrichment step in conjunction with the three culture media to obtain *Aeromonas* from food and water samples.

2. MATERIALS AND METHODS

Bacterial strains. This study included 27 *Aeromonas* type strains and four strains belonging to other genera that are commonly misidentified as *Aeromonas* (Table 1).

Culture media. The three culture media used in the comparison were ADA (HIMEDIA, Mumbai, India), SAA, and BIBG-m. The latter two were prepared as previously described (Table S1). Trypticase soy agar (TSA) (Difco, Le Pont de Claix, France) was used as a control medium. All strains were cultured under aerobic conditions at 30°C for 24 h, except for *Aeromonas salmonicida* (CECT 894^{T}), which was incubated at room temperature for 24–48 h. All tested strains were evaluated in triplicate on each culture medium.

Selectivity and sensitivity analysis of the culture media using artificially inoculated distilled water samples. All bacterial strains were directly inoculated from overnight culture onto the three culture media and TSA to verify their growth, colony characteristics, and the

selectivity of the media. To evaluate bacterial growth, each agar plate was streaked in three directions to cover the entire surface of the plate. When growth was observed in all three directions, the growth was considered abundant (+++), if bacteria only grew in two directions growth was considered intermediate (++), and growth in one direction was considered scarce (+).

To determine the sensitivity of the culture media, sterile distilled water samples were artificially contaminated with pure cultures of *Aeromonas hydrophila* CECT 389^T, *Aeromonas caviae* CECT 838^T, *Aeromonas salmonicida* CECT 894^T, and *Aeromonas trota* CECT 4255^T. These *Aeromonas* strains were chosen randomly, except for *A. trota* CECT 4255^T, which has been previously described as susceptible to ampicillin (Carnahan *et al*. 1991). Ampicillin is generally included both in ADA and SAA because all *Aeromonas* species other than *A. trota* are resistant to this antibiotic (Abbott *et al*. 2003). Strains were inoculated into 10 ml of TSB (Difco) and incubated at 30°C for 24 h. Following incubation, cultures were adjusted to an optical density of 0.8 (measured at 450 nm), which corresponded to a concentration of approximately 10^8 CFU/ml. Ten-fold serial dilutions were performed for each strain using sterile distilled water, and 100-µl aliquots of each dilution were inoculated onto TSA, ADA, SAA, and BIBG-m plates to determine the bacterial counts. Plate assays were performed in triplicate and the results were averaged.

Water and shellfish samples. Thirty-one samples were studied, including11 bivalve mollusk (mussels, oysters, and clams) and 20 water samples. Among the water samples, 12 were reclaimed water samples (secondary treated wastewater and stored tertiary treated wastewater used for vegetables' irrigation), four were seawater samples where the shellfish were harvested (Alfacs Bay, Ebro River Delta, Spain), and four were brackish water collected from a channel. The channel contained untreated wastewater from the village of Poble Nou, on the Ebro River Delta.

Isolation and detection of *Aeromonas* spp. Bacteria were cultured directly from all samples by inoculating 10 ml of the original water sample into 90 ml of buffered peptone water (BPW). Ten-fold serial dilutions were performed, and 100-µl aliquots were inoculated onto the three different culture media and incubated at 30°C for 24 h. In parallel, an enrichment step was performed for all samples using APW supplemented with ampicillin (10 mg/l, APW-A). For this step, 1 ml of water sample was diluted in 9 ml of APW-A, or in the case of shellfish samples, 10 g of sample were homogenized with 90 ml of APW-A. The diluted samples were incubated at 30°C for 24 h, and thereafter ten-fold serial dilutions were performed using BPW. Aliquots (100 µl) of each dilution were inoculated onto the three tested media and were incubated under the same conditions. Following incubation, eight suspected *Aeromonas* colonies were picked from each of the direct and enriched culture plates for each sample, and were transferred to TSA to obtain a pure culture for genotyping and molecular identification.

Typical *Aeromonas* colonies (CFU/100 ml) were counted from the direct culture plates for each of the three media, and verified using the most probable number method (MPN/100 ml) from five tubes for each dilution on ADA medium. In both cases, the incubation conditions were the same as mentioned above.

Genotyping and molecular identification. Typical *Aeromonas* colonies were transferred to TSA, and one colony was used for DNA extraction using InstaGene Matrix (BioRad, Hercules, CA, USA) according to manufacturer's instructions. All isolates were identified to genus level using PCR-based analysis of the glycerophospholipid cholesterol acyltransferase (GCAT) gene region as previously described (Chacón *et al*. 2002; Latif-Eugenín *et al*. 2015). To avoid working with clonally related isolates, enterobacterial repetitive intergenic consensus (ERIC) PCR typing was performed for each isolate using the primers and conditions described by Versalovic *et al*. (1991). Molecular identification to species level was

performed by PCR and sequence-based analysis of *rpoD* using primers and conditions described by Soler *et al*. (2004).

Statistical analyses. The data were analyzed using a two-way ANOVA model with respect to the type of culture (direct or enrichment culture) and culture medium (ADA, SAA, or BIBG-m) tested, using the Bonferroni correction for multiples comparison. The significance level was fixed at $P < 0.05$. All analyses were performed using SigmaPlot 11.0 (SSI, USA).

3. RESULTS

Selectivity and sensitivity of the culture media using artificially inoculated distilled water samples. Of the 27 *Aeromonas* type strains directly inoculated onto the three culture media, all except for *A. trota* CECT 4255^T (26/27, 96.3%) showed abundant growth on ADA Table 1). All strains except for *A. media* CECT 4232^T and *A. trota* CECT 4255^T (25/27, 95.3%) grew on SAA medium, with 22 strains showing abundant growth. For BIBG-m, all *Aeromonas* type strains were recovered, with all strains apart from *A. schubertii* CECT 4240^T and *A. salmonicida* CECT 894^T growing abundantly. For the four non-*Aeromonas* comparison strains (*Pseudomonas aeruginosa* CECT 110^T, *Plesiomonas shigelloides* CECT 597, *Vibrio parahaemolyticus* CECT 588, and *Escherichia coli* CECT 744), BIBG-m was the most selective (100%) of the three media tested, while ADA allowed the growth of all four organisms and was therefore considered non-selective (Table 1). The four bacteria produced colorless or whitish colonies of 1–2 mm in diameter on ADA medium.

Analyses using artificially contaminated distilled water samples produced the same results as direct plating for *A. hydrophila* CECT 839^T, *A. caviae* CECT 838^T, and *A. salmonicida* CECT 894^T on both ADA and SAA (Table 2). Both ADA and SAA were significantly more sensitive for detecting these strains compared with BIBG-m ($P < 0.05$). *A. trota* CECT 4255^T only grew on BIBG-m.

Recovery of *Aeromonas* from water and shellfish samples. Of the 20 water samples analyzed, 15 (75%) were positive for the presence of *Aeromonas* following enrichment and MPN enumeration, while 13 samples (65%) were positive by direct culture (Table 3). The four seawater samples and one tertiary treated reclaimed water sample used for irrigation, were negative for *Aeromonas* by all methods (data not shown). For the shellfish samples, *Aeromonas* were isolated from 10 of the 11 samples (90.9%) using enrichment and MPN enumeration, and were detected in six samples (54.5%) by direct culture (Table 3). No significant differences ($P > 0.05$) were observed between the three media in regards to the *Aeromonas* plate counts from water and seafood samples.

The highest levels of specificity for *Aeromonas* recovery from water and shellfish samples were observed using ADA (98.4%) and BIBG-m (90.5%) media (Table 4). Statistical analysis revealed that in the case of water samples, the specificity of ADA medium was significantly greater ($P < 0.05$) than that of SAA and BIBG-m. No significant difference in the specificity of the three culture media was observed for seafood samples.

Of the 705 *Aeromonas* isolates recovered from water samples, 658 (93.3%) were positively identified by GCAT-PCR analysis, and belonged to 436 different ERIC genotypes or strains (66.3% genetic diversity), as shown in Table 5. Recovery rates of *Aeromonas* by direct culture (46.3%) and by culture following enrichment (53.7%) were similar ($P > 0.05$). Independently of the culture approach, the number of isolates obtained from ADA medium was significantly ($P < 0.05$) higher than the numbers recovered from either SAA or BIBG-m. In relation to the shellfish samples, 335 isolates were obtained, and 289 (86.3%) were identified as *Aeromonas* spp. following GCAT-PCR analysis. These isolates corresponded to 156 (54.3%) ERIC patterns or strains (Table 5). The majority of isolates (63.5%) were recovered from the enrichment cultures ($P < 0.05$), and the three culture media produced a similar percentage of isolates ($P > 0.05$) (Table 5).

As shown in Table 6, the 436 *Aeromonas* strains recovered from water samples corresponded to 12 *Aeromonas* species. Five of these species (*A. media*, *A. caviae*, *A. salmonicida*, *A. allosaccharophila*, and *A. veronii*) accounted for 94.2% of the total number of strains recovered by using the two culture approaches (Table 6). The remaining seven species corresponded to 25 isolates (5.7%) (Table 6) and were treated as a whole for the purpose of statistical analyses. The recovery rates for *A. caviae* and *A. allosaccharophila* were significantly higher using enrichment culture ($P < 0.005$) compared with direct culture. Moreover, *A. media* was mainly isolated from ADA plates ($P < 0.005$) and *A. salmonicida* from SAA plates ($P < 0.05$), apart from the culture technique.

Ten species of *Aeromonas* were recovered from shellfish samples (Table 7). The most prevalent were *A. salmonicida*, *A. media*, and *A. caviae*, which together represented 82.7% of the total number of isolates. The remaining seven species corresponded to 27 (17.3%) isolates, which were again analyzed together for statistical purposes. *A. salmonicida* and *A. caviae* were more frequently isolated from shellfish following enrichment culture ($P < 0.05$), and significantly ($P < 0.05$) more *A. salmonicida* isolates were recovered using SAA medium compared with ADA.

Aeromonas bivalvium, *Aeromonas sanarellii*, and *Aeromonas eucrenophila* were only found in water samples following enrichment, as did the four isolates identified as *Aeromonas* sp. from shellfish. Several species (*Aeromonas popoffii*, *Aeromonas bestiarum*, and *Aeromonas fluvialis*) were only isolated from water samples by direct culture (Tables 6 and 7).

4. DISCUSSION

Overall, between 95.3–100% of the 27 *Aeromonas* type strains grew on SAA, ADA, and BIBG-m, showing the typical colony size and color described for each culture medium. However, five type species, *A. schubertii* CECT 4240^T, *A. salmonicida* CECT 894^T, *A. sobria* CECT 4245^T, *A. media* CECT 4232^T, and *A. trota* CECT 4255^T showed poor or no growth on

the three culture media. To corroborate these results, we evaluated four additional strains of *A. sobria* and *A. media* on SAA medium, and four *A. salmonicida* and *A. schubertii* strains on SAA and BIBG-m. In all cases, the additional strains showed abundant growth on SAA and/or BIBG-m media with the expected phenotypic characteristics of *Aeromonas*. Therefore, these results indicate that the recovery of *Aeromonas* species may be strain dependently. Previous studies performed by Gavriel and Lamb (1995), who compared culture media ADA, GSP, and SGAP-10C, showed that *A. sobria* grew in ADA only, while *A. schubertii* did not grow in any of the tested culture media. The results obtained for *A. trota* CECT 4255^T in our study (growth on BIBG-m only) agree with previous descriptions of this species, which is ampicillin-susceptible (Carnahan *et al*. 1991; Abbott *et al*. 2003).

We determined that BIBG-m was the most selective medium, only allowing the growth of *Aeromonas* strains (Table 1). However, it showed the lowest plate counts and recovery rates for the *Aeromonas* strains. Neyts *et al*. (2000b) used 27 *Aeromonas* strains, 10 species of Enterobacteriaceae, four *Pseudomonas* strains, and one strain of *Shewanella putrefaciens* to compare the selectivity of BIBG and BIBG-m media by direct inoculation. The study found that all bacterial strains tested grew on BIBG medium, while only one Enterobacteriaceae (*Citrobacter freundii*) and four *Pseudomonas* strains grew on BIBG-m. Moreover, these results were confirmed by the authors when artificially contaminated food samples (shrimp, minced meat, pre-cut leek, and shredded carrot) were tested with the same media. Our current results agree with those presented by Villari *et al*. (1999), who compared the selectivity of six culture media using 12 water samples artificially contaminated with fecal material, but free of *Aeromonas* sp., and confirmed that ADA and SAA are less selective than BIBG-m.

The present study demonstrated that ADA and SAA show similar sensitivity, which is superior to that of BIBG-m when analyzing artificially inoculated distilled water samples (Table 2). These results are concordant with those reported in 1999 by Villari *et al*. (1999),

who tested the sensitivity of six culture media using 18 sterile tap water samples artificially contaminated with nine *Aeromonas* strains at concentrations ranging from 1–10 CFU/ml.

As expected, the number of positive samples for the presence of *Aeromonas* was higher when using an enrichment step. The percentage of positive samples increased by 10% (65% to 75%) for the water samples and by 36.4% (54.5% to 90.9%) for the shellfish samples. Gobat and Jemmi (1995) analyzed 829 food samples and identified *Aeromonas* in 7.4% and 20.5% of the samples when using direct and enriched culture methods, respectively. In the current study, analysis of water samples showed that ADA medium had superior specificity (98.4%) compared with SAA and BIBG-m ($P < 0.005$). Holmes and Sartory (1993) obtained specificities of 90% and 95% using ADA for analysis of 146 drinking water samples and 153 water samples from different sources, respectively, and concluded that ADA was the most specific of the media tested.

The present study revealed that the culture approach (with or without enrichment) influenced the recovery of some *Aeromonas* spp. The detection rates of *A. allosaccharophila* from water, *A. salmonicida* from shellfish, and *A. caviae* from both types of samples were significantly higher ($P < 0.05$) when using an enrichment step. In addition, following enrichment, three rare species (*A. bivalvium*, *A. sanarellii*, and *A. eucrenophila*) were identified in water samples, and a potentially novel *Aeromonas* species (study in preparation) from seafood was discovered. On the other hand, *A. fluvialis* was obtained by using direct culture, and this represents the first report of this species since its initial isolation from river water (Alperi *et al*. 2010).

We also demonstrated that the culture medium influences the recovery of some *Aeromonas* spp. from water and shellfish samples. For instance, ADA and SAA favored the recovery of *A. media* and *A. salmonicida*, respectively. These results disagree with those obtained by

Holmes and Sartory (1993), who reported that the diversity of *Aeromonas* species obtained from water samples was the same for all culture media tested.

In conclusion, ADA and SAA were the most sensitive of the tested media for analysis of the artificially contaminated water samples, while BIGB-m was the most selective. On the other hand, ADA was ratified as the most specific medium for the recovery of *Aeromonas* from water samples. However, as expected, the use of a pre-enrichment step enhanced the number of positive water and shellfish samples, and allowed the recovery of rarely isolated *Aeromonas* species. Finally, we demonstrated that both the culture approach and the culture medium influenced the prevalence of some *Aeromonas* species, and this should be considered in future studies.

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Table 1 Growth and colony characteristics of the 27 *Aeromonas* type strains and four competitive microorganisms on the three tested culture media (compared with TSA)

Organisms	Growth on			
	TSA	**ADA**	**SAA**	**BIBG-m**
A. hydrophila CECT 839^T	+++	+++	+++	+++
A. salmonicida CECT 894^T	+++	+++	+	+
A. sobria CECT 4245^T	+++	+++	+	+++
A. media CECT 4232^T	+++	+++	-	+++
A. veronii CECT 4257^T	+++	+++	+++	+++
A. caviae CECT 838^T	+++	+++	+++	+++
A. eucrenophila CECT 4224^T	+++	+++	+++	+++
A. schubertii CECT 4240^T	+++	+++	++	++
A. jandaei CECT 4228^T	+++	+++	+++	+++
A. trota CECT 4255^T	+++	-	-	+++
A. allosaccharophila CECT 4199^T	+++	+++	+++	+++
A. encheleia CECT 4342^T	+++	+++	+++	+++
A. bestiarum CECT 4227^T	+++	+++	+++	+++
A. popoffii CECT 5176^T	+++	+++	+++	+++
A. simiae IBS S6874^T	+++	+++	+++	+++
A. molluscorum CECT 5864^T	+++	+++	+++	+++
A. bivalvium CECT 7113^T	+++	+++	+++	+++
A. tecta CECT 7082^T	+++	+++	+++	+++
A. piscicola CECT 7443^T	+++	+++	+++	+++
A. fluvialis CECT 7401^T	+++	+++	+++	+++
A. taiwanensis CECT 7403^T	+++	+++	+++	+++
A. sanarellii CECT 7402^T	+++	+++	+++	+++
A. diversa CECT 4254^T	+++	+++	+++	+++
A. rivuli CECT 7518^T	+++	+++	+++	+++
A. cavernicola CECT 7862^T	+++	+++	+++	+++
A. australiensis CECT 8023^T	+++	+++	+++	+++
A. dhakensis CECT 5744^T	+++	+++	+++	+++
Pseudomonas aeruginosa CECT 110^T	+++	+++	+++	+++
Plesiomonas shigelloides CECT 597	+++	+++	-	-
Vibrio parahaemolyticus CECT 588	+++	+++	+	-

Escherichia coli CECT 744	+++	+++	-	-

TSA: trypticase soy agar; ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified agar. Type of growth: +++, abundant; ++, intermediate; +, scarce; -, no growth.

Table 2 Percent recovery of *Aeromonas* (sensitivity) from artificially contaminated distilled water samples using the three culture media

***Aeromonas* sp. (initial inoculum in TSA, cfu/ml)**	**Culture media**	***Aeromonas* count in cfu/ml by dilution (% recovery)**		
		10^{-6}	**10^{-7}**	**10^{-8}**
A. hydrophila CECT 839^{T} ($2.5x10^{8}$)	ADA*	223 (89.2)	24 (96)	3 (120)
	SAA*	225 (90.0)	26 (104)	2 (80)
	BIBG-m	190 (76)	11 (44)	0 (0)
A. caviae CECT 838^{T} ($2.9x10^{8}$)	ADA*	267 (92.1)	29 (100)	2 (69)
	SAA*	263 (90.7)	26 (89.7)	2 (69)
	BIBG-m	220 (75.9)	19 (65.5)	1 (34.5)
A. salmonicida CECT 894^{T} ($2.4x10^{8}$)	ADA*	203 (85.6)	24 (100)	2 (83.3)
	SAA*	205 (85.4)	32 (133.3)	1 (41.7)
	BIBG-m	161 (67.1)	9 (37.5)	0 (0)
A. trota CECT 4255^{T} ($3.3x10^{8}$)	ADA	ND	ND	ND
	SAA	ND	ND	ND
	BIBG-m	281 (84.8)	19 (57.6)	1 (30.3)

ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified agar; ND: not detected.

*ADA and SAA were significantly more sensitive ($P < 0.05$) than BIBG-m.

Table 3 Average counts of *Aeromonas* recovered from water and shellfish samples by direct plate count and the MPN method

Total samples	Direct plate count (cfu/100ml)*			MPN/100ml†
	ADA	SAA	BIBG-m	
Water	8.03×10^4	6.28×10^4	5.01×10^4	2.89×10^5
Shellfish	1.11×10^3	1.14×10^3	1.17×10^3	1.15×10^4

ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified agar.

*Average from 13 positive water and six positive shellfish samples.

†Average from 15 positive water and 10 positive shellfish samples.

Table 4 Specificity of the three culture media used for the detection of *Aeromona*s from water and food samples

Samples (isolates)	Culture media	Total number of isolates	GCAT positive isolates	Specificity (%)
Water (705)	ADA	254	250	98.4*
	SAA	255	229	89.8
	BIBG-m	196	179	91.3
Shellfish (335)	ADA	119	97	81.5
	SAA	121	106	87.6
	BIBG-m	95	86	90.5

ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified agar.

*ADA medium was more specific ($P < 0.05$) than SAA and BIBG-m for the detection of *Aeromonas* from water samples.

Table 5 Number and percentage of *Aeromonas* recovered from water and shellfish samples based on culture method and medium used

Samples	Total number of isolates	Number (%) of isolates GCAT-PCR (+)	Number of genotypes (% of diversity)	Number (%) of isolates by direct culture				Number (%) of isolates after enrichment *			
				Culture media				Culture media			
				Total	ADA†	SAA	BIBG-m	Total	ADA†	SAA	BIBG-m
Water	705	658 (93.3)	436 (66.3)	202 (46.3)	83 (41.1)†	67 (33.2)	52 (25.7)	234 (53.7)	94 (40.2)†	82 (35.0)	58 (24.8)
Shellfish	335	289 (86.3)	156 (54.0)	57 (36.5)	21 (36.8)	21 (36.8)	15 (26.3)	99 (63.5)*	29 (29.3)	38 (38.4)	32 (32.3)

ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified agar.

*The use of enrichment favors ($P < 0.05$) the recovery of *Aeromonas* from shellfish samples.

†The use of ADA positively influenced ($P < 0.05$) the recovery of *Aeromonas* from water samples, with and without a pre-enrichment step.

Table 6 Prevalence of the different *Aeromonas* spp. recovered from water samples on the basis of culture method and medium used

Total number (%) of *Aeromonas* spp.	Direct culture				Post-enrichment culture*			
	total n (%) of strains	n (%) strains by culture media			total n (%) of strains	n (%) strains by culture media		
		ADA†	SAA‡	BIBG-m		ADA†	SAA‡	BIBG-m
176 (40.4) *A. media*	93 (52.8)	42 (45.2)†	25 (26.9)	26 (28.0)	83 (47.2)	42 (50.6)†	28 (33.7)	13 (15.7)
101 (23.2) *A. caviae*	40 (39.6)	16 (40)	13 (32.5)	11 (27.5)	61 (60.4)*	24 (39.3)	14 (23.0)	23 (37.7)
75 (17.2) *A. salmonicida*	32 (42.7)	9 (28.1)	16 (50.0)‡	7 (21.9)	43 (57.3)	10 (23.3)	22 (51.2)‡	11(25.6)
30 (6.9) *A. allosaccharophila*	9 (30.0)	5 (55.6)	3 (33.3)	1(11.1)	21 (70.0)*	13 (61.9)	6 (28.6)	2 (9.5)
29 (6.7) *A. veronii*	13 (44.8)	3(23.1)	5 (38.5)	5 (38.5)	16 (55.2)	3 (18.8)	7 (43.8)	6 (37.5)
9 (2.1) *A. popoffii*	9 (100)	7 (77.8)	1(11.1)	1 (11.1)	0 (0)	0 (0)	0 (0)	0 (0)
7 (1.6) *A. hydrophila*	1 (14.3)	0 (0)	1 (100)	0 (0)	6 (85.7)	0 (0)	4 (66.7)	2 (33.3)
3 (0.7) *A.bestiarum*	3 (100)	1 (33.3)	1 (33.3)	1(33.3)	0 (0)	0 (0)	0 (0)	0 (0)
2 (0.5) *A.sobria*	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2 (0.5) *A. bivalvium*	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	1(50)	1(50)	0 (0)
1 (0.2) *A. sanarellii*	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)
1 (0.2) *A. eucrenophila*	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
TOTAL (n= 436)	**202 (46.3)**	**83 (41.1)**	**67 (33.2)**	**52 (25.7)**	**234 (53.7)**	**94 (40.2)**	**82 (35.0)**	**58 (24.8)**

DA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified.

*The use of enrichment favors ($P < 0.05$) the detection of *A. caviae* and *A. allosaccharophila*.

†, ‡The use of ADA and SAA positively influenced ($P < 0.05$) the recovery of *A. media* and *A. salmonicida*, respectively.

Table 7 Prevalence of the different *Aeromonas* spp. recovered from shellfish samples based on culture method and medium used

Total number (%) of *Aeromonas* spp.	Direct culture				Enrichment culture*			
	total n (%) of strains	n (%) strains by culture media			total n (%) of strains	n (%) strains by culture media		
		ADA	SAA†	BIBG-m		ADA	SAA†	BIBG-m
59 (37.8) *A.salmonicida*	18 (30.5)	4 (22.2)	9 (50.0)†	5 (27.8)	41 (69.5)*	8 (19.5)	18 (43.9)†	15 (36.6)
41 (26.3) *A.media*	22 (53.7)	9 (40.9)	6 (27.3)	7 (31.8)	19 (46.3)	7 (36.8)	5 (26.3)	7 (36.8)
29 (18.6) *A.caviae*	7 (24.1)	3 (42.9)	4 (57.1)	0 (0)	22 (75.9)*	7 (31.8)	8 (36.4)	7 (31.8)
10(6.4) *A.allosaccharophila*	3 (30.0)	1 (33.3)	1 (33.3)	1 (33.3)	7 (70.0)	3 (42.9)	3 (42.9)	1 (14.3)
5 (3.2) *A.veronii*	2 (40.0)	1 (50.0)	0 (0)	1 (50.0)	3 (60.0)	1 (33.3)	1 (33.3)	1 (33.3)
4 (2.6) *Aeromonas* sp.	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)	3 (75.0)	1 (25.0)	0 (0)
3 (1.9) *A.hydrophila*	1 (33.3)	0 (0)	0 (0)	1(100)	2 (66.7)	0 (0)	1 (50.0)	1 (50.0)
3 (1.9) *A.popoffii*	2 (66.7)	2 (100)	0 (0)	0 (0)	1 (33.3)	0 (0)	1 (100)	0 (0)
1 (0.6) *A.fluvialis*	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1 (0.6) *A.bestiarum*	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TOTAL (n= 156)	**57 (36.5)**	**21 (36.8)**	**21 (36.8)**	**15 (26.3)**	**99 (63.5)**	**29 (29.3)**	**38 (38.4)**	**32 (32.3)**

ADA: ampicillin dextrin agar; SAA: starch ampicillin agar; BIBG-m: bile irgasan brilliant green-modified.

*Enrichment significantly favors ($P < 0.05$) the detection of *A. salmonicida* and *A. caviae*.

†The use of SAA positively influenced ($P < 0.05$) the recovery of *A. salmonicida*.

Supporting Information:

Table S1 Characteristics of the culture media compared in this study