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Research Article

Microdot method: used with chromogenic agar is a useful procedure for sanitary monitoring in aquaculture

Erwin Strahsburger[1], Patricio Retamales[2], Juan Estrada[3] & Michael Seeger[4]
[1]Laboratorio de Biotecnología Molecular, Facultad de Recursos Naturales Renovables
Universidad Arturo, Iquique, Chile
[2]Facultad de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile
[3]Centro de Investigaciones Marinas de Quintay (CIMARQ)
Universidad Andrés Bello, Quintay, Chile
[4]Laboratorio de Microbiología Molecular y Biotecnología Ambiental
Centro de Nanotecnología y Biología de Sistemas, Centro de Biotecnología
Universidad Técnica Federico Santa María, Valparaíso, Chile
Corresponding author: Erwin Strahsburger (estrahsb@unap.cl)

ABSTRACT. The microdot method is a downscaling methodology of traditional tenfold serial dilution procedure used in microbiology. The microdot method uses 100 µL for serial dilution and count colonies in a spot of 10 µL. In this study we counted colonies directly in a chromogenic agar plate to determine, at the same time, the presence and cell concentration of target bacteria required for sanitary monitoring of Chilean export fishery products. Due to among importers countries the most concerning bacteria included in sanitary monitoring are *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*, we used the chromogenic agar; CHROMagar ECC, CHROMagar Listeria and Baird Parker agar, respectively. The results shows no differences between quantitative results obtained with microdot and traditional method during the quantification of a culture of *Escherichia coli* (1.5 L). The sensitivity and specificity of the microdot method in association with each chromogenic agar was demonstrated *in vitro* with reference strains. In addition, the usefulness in sanitary monitoring of aquaculture procedures was evaluated in *Artemia salina* tanks. This method did not detected sanitary problems in surface water. Although other colonies grown in the chromogenic agar plate, their morphological and chromogenic properties not correspond to *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*, being identified as *Salmonella enterica* subsp. *enterica*, *Microbacterium* sp., *Bacillus* sp. and *Staphylococcus pasteuri* by 16S rRNA gene sequence analysis. Hence, we propose the microdot chromogenic method as a low cost, specific and reliable procedure for sanitary monitoring of aquaculture procedures.

Keywords: microdot method, sanitary monitoring, aquaculture, chromogenic agar.

Método de la microgota: usado con agar cromogénico es un procedimiento útil para el monitoreo sanitario en acuicultura

RESUMEN. El método de la microgota es una versión a menor escala del procedimiento tradicional de dilución en serie en base diez utilizados en microbiología. El método realiza diluciones en 100 µL y cuenta colonias crecidas en una gota de 10 µL. En este estudio se cuentan colonias directamente en placas cromogénicas para determinar densidad celular y presencia de bacterias requeridas en vigilancia sanitaria de productos pesqueros chilenos de exportación. Entre los requisitos de países importadores, la vigilancia sanitaria involucra frecuentemente a *Escherichia coli*, *Listeria monocytogenes* y *Staphylococcus aureus*, por lo que se utilizan los agares cromogénicos; CHROMagar ECC, CHROMagar Listeria y agar Baird Parker para su identificación. La comparación entre el método de microgota y el método tradicional no muestra diferencias al evaluar un cultivo de *Escherichia coli* (1,5 L). La sensibilidad y especificidad del método de microgota junto a cada agar cromogénico se demostró *in vitro* con cepas de referencia. Además, en estanques de *Artemia salina* se evaluó la utilidad de este método para el monitoreo sanitario. Este método no mostró problemas sanitarios en aguas super-

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ficiales, aunque otras colonias crecieron en la placa de agar cromogénico. Sus propiedades morfológicas y cromogénicas no corresponden a *Escherichia coli*, *Listeria monocytogenes* y *Staphylococcus aureus*, siendo identificadas según el análisis de la secuencia del gen 16S rRNA como *Salmonella enterica* subsp. *enterica*, *Microbacterium* sp., *Bacillus* sp. y *Staphylococcus pasteuri*. Por lo tanto, se propone el método de microgota cromogénico como un procedimiento de bajo costo, específico y fiable para el monitoreo sanitario en acuicultura.

Palabras clave: método microgota, monitoreo sanitario, acuicultura, agar cromogénico.

INTRODUCTION

In microbiology, the quantitative determination of microorganisms is performed by a traditional procedure involving 10-fold serial dilution, in a total volume of 10 mL and pours 0.1 mL of a dilution tube on a single agar plate to count around 200-1000 colonies after an incubation period. Considering also the triplicates, the method comprise many materials (plates and dilution tubes) and a laborious time to finally determine the CFU (colony forming unit) per mL of a bacteria present in the sample. With the intention of reducing samples volume, microbiological material and laborious time, several downscaling method has been reported in literature. One of them is the track dilution method which consists in performing tenfold serial dilution in a total volume of 1 mL, and then rolled along the surface of a square agar plate 10 µL obtained from the dilution tube. According to the dilution, the cells are spread in this track allowing counts between 20-200 colonies (Jett *et al*., 1997). Other methods involve serial dilution using a 96 microplate in a total volume of 200 µL and spotted onto agar plate using a 96-spronged stamp (Tornero & Dangl, 2001). A modified version of this last method was proposed by Sieuwerts *et al*. (2008), where 5 µL from each diluted well are spotted onto agar plate and after a period of incubation, between 1 and 200 minicolonies (200-500 µm) are counted with the help of a digital camera and a processing image software. The microdot method, proposed in this report, is the simplest downscaling method, because it is a low scale of the traditional method. The 10-fold serial dilution is performed in a total volume of 100 µL and from each dilution tube triplicate dots of 10 µL are deposited in a single agar plate. After a period of incubation and according to the cell dilution, among 10-90 colonies are counted within the dot. This microdot method has been applied to enumerate bacteria under different experimental conditions in studies of plant-bacteria relationship (Keppler & Baker, 1989), bacteriocin import mechanism (Strahsburger *et al*., 2005), bacterial metabolism (Chirino *et al*., 2013) and bacterial bioconversion (DiGioia *et al*., 2014). However, this method is unknown for many microbiologists and its procedure has not been explained in detail in literature. Nevertheless, due to its simplicity and reduced use of material, methodologies like this are desirable for routine laborious labor like those performed in sanitary monitoring of export fishery products.

In Chile, aquaculture is the main activity of the fishery sector and one of the most important export activities after mining. The foremost export products are trout and salmon (around 94%), followed in a minor range by bivalves, algae, osteons, abalone and oysters. These products are characterized by their high quality, good taste and safety, according to international standards. That is, due to Chilean fishery companies applying strictly national technical norms to ensure the approval of all sanitary and quality controls before its exportation to approximately 30 countries around the world. These technical norms correspond to a certification program of fishery export products (CER-NT3) that include all the sanitary demands of all importer countries. The sanitary request is supported by the technical norm 2 (CER-NT2), which establishes the microbiological, physical, chemicals, sensorial and toxicological parameters involved in this sanitary certification. The technical norm 7 (LAB-NT7) indicates the microbiological methods approved for certification according to the international standards. The criteria that determine which bacterial species are included in that sanitary control program change according to the concerns of each importer country. However, three bacterial species are frequently included in sanitary monitoring; *E. coli*, *L. monocytogenes* and *S. aureus*. They are expected absent or low presence in diverse raw or elaborate fishery products. The microbiological methods described in CER-NT2 involve selective and differential medium according to the target bacteria. The quantification of *E. coli* is performed by the most probable number (MPN; CFU mL^{-1}) (Feng *et al*., 1998). The quantification of *L. monocytogenes* and *S.* aureus is determined by counting colonies in agar plates and expressed as CFU g^{-1} (Hitchins & Jinneman, 2013; Bennett & Lancette, 2001). However, for routine sanitary monitoring, the procedures are laborious and require a number of materials and large incubator space. Alternative methods are disabling for periodic sanitary monitoring programs within the fishery company, with the purpose of suggesting corrective actions in the case of finding sanitary problems. This

alternative method has to be easy to perform, economic and not laborious, as well as, selective and sensitive, and does not exclude the practice of certified methods at the time of certification.

In general, the chromogenic medium is an alternative method to identify quickly the presence of pathogens or bacteria associated to sanitary problems. This cromogenic agar has the advantage of being selective and differential, because certain bacterial species are capable of growth and within them the target bacteria shows morphological and chromatic characteristic allowing their identification (Tavakoli *et al*., 2008; Rank, 2012). The high demands for this kind of medium have promoted broad offers of chromogenic agar by the market. Regarding its application, in a study about the sanitary state of water and sediments of an estuary in Matang Mangrove Forest Reserve in Malaysia, the chromogenic agar CHROMagar ECC allowed the determination of *E.* coli and coliforms in the surface water (Ghaderpour *et al*., 2014). In this medium, the *E. coli* colonies has blue colors due to the β-glucoronidase activity, meanwhile coliforms colonies has mauve colors due to β-galactosidase activity (Alonso *et al.*, 1999). In a study about the effect of high pressure procedure over the growth of resistant strain of *Listeria monocytogenes* in the greenshell mussel meat (Fletcher *et al*., 2008), the enumeration of resistant bacteria was done with CHROMagar™ Listeria, where *L. monocytogenes* has a typical turquoise blue color with a clear white halo around the colony. The blue color is due to the β-D-glucosidase activity over the chromogenic substrate 5-bromo-4-chloro-3-indoxl-β-D-glucopyranoside. Meanwhile, the halo zone is due to the enzymatic activity of both virulence factors phosphatidylinositol-phospholipase C (PI-PLC) and phosphatidylcholin-phospholipase C (PC-PLC) over the substrate L-α-phosphatidyl-inositol (Reissbrodt, 2004). In the same way, the environmental impact of shrimp farm effluents over costal area of Mexico was evaluated measuring pathogenic and fecal origin bacteria in white clamps (Martínez-Porchas *et al*., 2016). Among these bacteria, the presence of *S. aureus* was detected by Baird Parker agar, where this bacteria looks like grey-black shiny colonies with a clear halo. The grey-black appearance is due to the reduction of tellurite; meanwhile the clear zone is due to the protease activity over the egg yolk added previously to the medium. In general, in chromogenic agar the other bacteria did not show the same chromogenic properties as target bacteria or they just did not grow.

Therefore, considering the chromogenic agar ability to identify directly a target bacteria and the microdot method that reduce the material used to determine cell concentration in a sample. We propose an alternative method that combines the advantage of microdot method with the use of chromogenic agar to identify and quantify quickly target bacteria in a water sample. Thus, this microdot chromogenic method is a reliable, fast, cheap and less laborious procedure in comparison with traditional method. Here we propose its use for sanitary monitoring of *E. coli, L. monocytogenes* and *S. aureus* in aquaculture processes.

MATERIALS AND METHODS

Strains and culture media

E. coli strain ATCC 12651, *Listeria monocytogenes* strain IV (Instituto de Salud Pública, Chile) and *Staphylococcus aureus* strain ATCC 25923 are stored in the culture collection of the Laboratorio de Biotecnología, Facultad de Recursos Renovables, Universidad Arturo Prat, Chile. The strains were cultured in MacConkey agar (Becton & Dickinson Company, Sparks, MD), Luria-Bertani (LB) agar (Oxoid, UK), Baird Parker agar (Oxoid, UK), CHROMagar ECC™ (CECC) (Chromagar Microbiology, Paris, France) and CHROMagar LM™ (CLM) (Chromagar Microbiology, Paris, France). The medium agar was suspended in distillated water (DW) or filtered marine water (MW) following the manufacturer's instructions. The culture was incubated in aerobic conditions for 24-48 h at 37°C.

Comparison between microdot and traditional method

E. coli strain ATCC 12651 was grown in 1.5 L of LB medium prepared with DW under aerobic conditions using an orbital shaker at 150 rpm h^{-1} for 24 h at 37°C. The cell concentration of this culture was determined by both traditional and microdot methods to compare both methods.

The traditional method comprised a sample of 1 mL taken directly from the *E. coli* culture (1.5 L) that was 10 fold serial diluted in a total volume of 10 mL using six tubes with 9 mL of physiological serum. An aliquot (0.1 mL) of each dilution tube was poured on a LB agar plate and incubated under aerobic condition for 18-24 h at 37°C. The plates containing between 200 and 1000 colonies were counted. The analysis was performed in triplicate.

The microdot method comprised an aliquot of 10 µL taken directly from the *E. coli* culture (1.5 L) or from a sample of 1 mL taken directly from the *E. coli* culture (1.5 L) or from a sample of 100 mL taken directly from the surface water of a rotifers tank. That aliquot was serially diluted 10 fold in a total volume of 100 µL until tube six. Triplicate aliquots of 10 µL from each dilution tube were spotted on the corresponding division,

previously marked at the external face of the base of the agar plate, prepared at least 24 h before in a sterile Petri dish of 180×11 mm. The plate distinguish 7 regions divided from the center of the plate and correlatively marked from 0 until 6, to indicate the dilution tube (0, correspond to the sample tube; 1, corresponding to dilution 10^{-1}; and so on, Figure 1). The plate was open under sterile condition until all the spots were adsorbed. Then, the plate was cover with its lid and incubated under aerobic condition for 18-24 h at 37°C. The colony-forming unit per mL were determined by counting between 20-80 colonies in each spot and multiplied by the dilution factor.

Sanitary monitoring in an *Artemia salina* culture

The sanitary monitoring of a rotifers tank of 200 L located in the live diet room of Centro de Investigaciones Marinas de Quintay (CIMARQ), Universidad Andres Bello, Quintay, Chile, was performed. A sample of 100 mL was collected from the surface water of the rotifers tank and analyzed by the microdot chromogenic method using CECC, CLM and Baird Parker agar to detect the presence of *E. coli*, *L. monocytogenes* and *S. aureus*, respectively. The colonies observed, after 18 h of incubation at 37°C, were identified according to their morphology and chromogenic characteristic and confirmed by 16S rRNA gene sequence analysis.

PCR and DNA sequencing

The colonies grown in Baird Parker, ECC and CLM agar plates were identified by colony PCR using 16S rRNA universal primers 27F and 1492R (Chirino *et al*., 2013). The PCR reaction mixture was composed of 5 µL 5× buffer, 1.25 µL of deoxynucleotide triphosphate 10 mM, 0.25 µL of each primer 1 µM, 0.25 µL of Gotaq DNA polymerase 5 u μL^{-1} (Promega) and 18 µL of nuclease-free water. PCR was performed in an Eppendorf Thermocycler (Eppendorf AG, Hamburg, Germany). The PCR amplification protocol comprised an initial incubation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final extension at 72°C for 7 min. The PCR products were separated in an agarose gel electrophoresis performed at 100 V for 45 min using an agarose gel (1%) in Tris acetate buffer containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA (pH 8.1). The agarose gel were stained with ethidium bromide (5 µg mL^{-1}) and registered in an ultraviolet (UV) trans-illumination with Gel Doc 2000 system (Bio-Rad). Each band was extracted from gel using the PureLink Quick Gel Extraction Kit (Life Technologies, UK). The purified PCR product was sequenced (Macrogen, Seoul, Korea) and the sequence analyzed by BLAST with NCBI database for bacteria identification (Altschul *et al*., 1990).

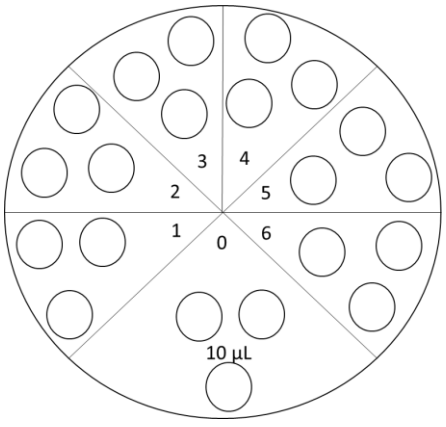

Figure 1. Distribution of 10 µL dots in the agar plate. These dots were deposited in triplicate and taken directly from the sample or from the corresponding dilution tube 1 to 6. The cell concentration was calculated counting between 10 and 90 colonies within the spot and multiple by dilution factor to express as cell per mL.

RESULTS

Comparison between microdot and traditional method

In this study, the cell concentration of *E. coli* in a liquid culture (1.5 L) was measured by conventional and microdot methods. The conventional method registered a cell concentration of 7.93 ± 0.05 log CFU mL^{-1}, whereas the microdot method registered a cell concentration of 7.78 ± 0.04 log CFU mL^{-1} and 7.91 ± 0.02) log CFU mL^{-1}, using a sample volume of 10 µL and 1 mL, respectively. Since each method showed the same cell concentration, we used only the microdot method in the next cell concentration determination.

The chromogenic agar has the advantage of identifying directly a target bacterium according to the morphology and chromogenic characteristic of its colony. In this study we evaluated these properties during cell concentration determination by the microdot method. In this sense, we compare the cell concentration results obtained by the microdot method, when diverse agar medium including chromogenic and not chromogenic agar were used. At the same time, we also evaluated if solvent used in medium suspension (distilled water or marine water) did not interfered with its chromogenic properties or its use during cell concentration determination. As showed in Table 1, for

Table 1. Cell concentration of Gram-positive bacteria grown in different media. LB-MW: Luria Broth in marine water, LB-DW: Luria Broth in distilled water, TSA-MW: Trypticase soy agar in marine water, TSA-DW: Trypticase soy agar in distilled water, Baird Parker-MW: Baird Parker agar in marine water, Baird Parker-DW: Baird Parker agar in distilled water, CLM-MW: Chromagar Listeria Monocytogenes in marine water, CLM-DW: Chromagar Listeria Monocytogenes in distilled water.

	Growth culture log (CFU mL^{-1}) (±SD)							
Strain	LB-MW	LB-DW	TSA-MW	TSA-DW	Baird Parker-MW	Baird Parker-DW	CLM-MW	CLM-DW
S. aureus ATCC-25923	9.40 (± 0.03)	9.36 (± 0.06)	9.34 (± 0.06)	9.29 (± 0.05)	9.36 (± 0.07)	9.45 (± 0.06)	-	-
L. monocytogenes IV	9.72 (± 0.05)	9.64 (± 0.04)	9.75 (± 0.03)	9.54 (± 0.08)	9.73 (± 0,01)	9.57 (± 0.03)	9.84 (± 0.02)	9.63 (± 0.05)

Table 2. Cell concentration of Gram-negative bacterium *E. coli* grown in different media. LB-MW: Luria Broth in marine water, LB-DW: Luria Broth in distilled water, TSA-MW: Trypticase soy agar in marine water, TSA-DW: Trypticase soy agar in distilled water, McConkey-MW: McConkey agar in marine water, McConkey-DW: McConkey agar in distilled water, CECC-MW: Chromagar *E. coli* chromogenic media in marine water, CECC-DW: Chromagar *E. coli* chromogenic media in distilled water.

	Growth culture log (CFU mL^{-1}) (±SD)							
Strain	LB-MW	LB-DW	TSA-MW	TSA-DW	McConkey-MW	McConkey-DW	CECC-MW	CECC-DW
E. coli ATCC 12651	9.65 (± 0.01)	9.47 (± 0.04)	9.46 (± 0.04)	9.20 (± 0.09)	9.49 (± 0.03)	9.48 (± 0.02)	9.48 (± 0.07)	9.48 (± 0.06)

Gram-positive bacteria, and Table 2 for Gram-negative bacteria, the cell concentration of each reference strain was the same, independently of the medium used or the solvent used (distilled water or marine water) during medium agar preparation. As expected, the morphology and chromatic characteristic of each reference strain in their respective chromogenic medium was as describe by the manufacture company (Table 3). Thus, the chromogenic agar used in conjunction with the microdot method is a reliable method to use in monitoring sanitary state of an aquaculture process, identifying and quantifying target bacteria as *E. coli*, *L. monocytogenes* and *S. aureus*.

Application of microdot chromogenic method in sanitary monitoring of *Artemia salina* culture

Artemia salina was cultured as live diet for fish production in the Marine Research Center of Quintay, Andres Bello University, Chile. In Table 3 is shown that no sanitary problems related with the presence of *E. coli*, *L. monocytogenes* and *S. aureus* were detected by microdot method in conjunction with chromogenic agars. The colonies observed in the chromogenic agar plate were identified as *Salmonella* sp., *Microbacterium* sp., *Bacillus* sp. and *Staphylococcus pasteuri* strains according to the16S rRNA gene sequencing analysis by BLAST algorithm in NCBI database (Table 3).

DISCUSSION

In this study, we showed the usefulness of the microdot method with chromogenic agar to simultaneously detect and quantify the presence of *E. coli*, *L. monocytogenes* and *S. aureus* in water samples. As expected, the chromogenic agar was specific for these bacteria and did not interfere with the bacterial quantification. Thus, the microdot chromogenic method was fast, low cost and easily to perform. In addition, the method shows same quantitative results as the traditional method during quantification of an *E. coli* culture (1.5 L), even though sample volume between both methods was 100 fold different. This last observation means that *E. coli* population was in a homogenous dispersion within 1.5 L of culture, being irrelevant the sample volume used. Therefore, we propose that in tanks or ponds (≥200-1000 L) of aquaculture production, the target bacteria will also be in a homogenous dispersion, being able to be detected and quantified by the microdot chromogenic method. In principle, the limit of sensitivity of this method is >100 UFC mL^{-1} *i.e.*, one colony in the dot of 10 µL taken directly from the sample.

In addition, the method was used to monitor the sanitary state of a tank of culture of *Artemia salina*, revealing the absence of *E. coli*, *L. monocytogenes* and

Table 3. Colony description and PCR identification by 16S rRNA gene sequence analysis.

Strain	Culture media	Colony description	16S rRNA identification
E. coli ATCC 12651	CHROMagar™ ECC	blue colony	*E. coli*
ECC-1	CHROMagar™ ECC	white opaque	*Salmonella enterica* subsp. *enterica*
ECC-2	CHROMagar™ ECC	white rose	*Salmonella enterica* subsp. *enterica*
L. monocytogenes IV	CHROMagar™ Listeria	blue colony with white halo	*L. monocytogenes*
CL-1	CHROMagar™ Listeria	green	*Microbacterium* sp.
CL-2	CHROMagar™ Listeria	white opaque	*Microbacterium* sp.
S. aureus ATCC- 25923	Baird Parker Agar	round black shiny colony with opaque halo	*S. aureus*
ABP-1	Baird Parker Agar	round opaque	*Bacillus* sp.
ABP-2	Baird Parker Agar	round white	*Staphylococcus pasteuri*

S. aureus in the surface water. The artificial contamination of the tank with *Artemia salina* by reference strains of *E. coli*, *L. monocytogenes* and *S. aureus* was discarded due to the hazard risk of environmental contamination or possible contamination of other cultures situated closer to this tank in the marine research center. Thus, we just monitor the bacterial presence in the surface water of the tank with *Artemia salina* using the chromogenic agar and the microdot method. Although other colonies appears, the chromogenic agar did not distinguish other bacteria that not correspond to the target bacteria because, for example, many other bacteria like *Salmonella* spp. and *Hafnia alvei* shows white colonies in Chromagar ECC. Therefore it was necessary to identify them by sequence analysis of 16S rRNA gene. The inclusion of other chromogenic agar like Chromagar Salmonella could be useful to specifically detect *Salmonella* in water sample. For example, *Salmonella* spp. is usually not desired in fishery products like surimi or freeze cooked mollusk (CER-NT2). In this study, against the presence of *Salmonella* spp. in the surface water of *Artemia salina* tank, corrective actions were performed. The UV lamp of the marine water filtration system was changed. Then, *Salmonella* spp. disappeared from the tank of *Artemia salina* (data not shown). Hence, our recommendation is to implement a periodic monitoring of sanitary state of water by microdot method in conjunction with chromogenic agar, to prevent the colonization on aquatic organisms by undesirable bacteria. Otherwise, it will be less effective and more expensive eradicating the undesired bacterial from the aquatic organism. Besides, this method could be done with others chromogenic agar, like those used to monitor other bacteria associated to foodborne disease (Tavakoli *et al*., 2008) or to monitor pathogens that damages aquatic organisms (Kriem *et al.*, 2015).

Regarding to the colonies grown in chromogenic agar, two observations are necessary to remark. First, In CHROMagar Listeria, *Microbacterium* spp. showed green or white opaque colonies, in contrast to the blue colonies of *L. monocytogenes*. According to the literature, *Microbacterium* spp. and other species like *Cellulomonas* spp., *Bacillus circulans*, *B. pumilus*, *Enterococcus* spp., and *Staphylococcus xylosus* may form similar colonies like *L. monocytogenes* (Stessl *et al*., 2009), however, this was not the case although the identification of *Microbacterium* spp was confirmed by sequence analysis of 16SrRNA. *Microbacterium* is a genus that includes at least 92 species with high similarity in its 16S rRNA sequence (Laffineur *et al*., 2003; Hadjadj *et al*., 2016). Therefore, our observation could be explained because within this diversity some species could give false positive and others not for *Listeria monocytogenes* identification. On the other hand, in Baird Parker agar, *Bacillus* sp. showed dark brown colonies as expected (Baird-Parker, 1962), meanwhile *S. pasteuri* appeared as a round white colony. This last bacterial species has been isolated in sausage food using Baird Parker agar, but the colony description was not mentioned (Rebecchi *et al*., 2015). *S. pasteuri* is a new *Staphylococcus* species described 20 years ago, isolated from animals, humans, and foods (Chesnau *et al*., 1993), and apparently this is the first report that described its presence in aquaculture water.

Other applications of microdot chromogenic method

The potential application of this microdot chromogenic method in aquaculture could be like those studies in which *E. coli*, *L monocytogenes* or *S. aureus* were detected in water; in finfish aquaculture, monitoring the sanitary status of recirculating and non-recirculating water system (Pullela *et al*., 1998), in water of pounds of shrimp culture (Koonse *et al*., 2005), or in surface water and sediments of oyster ponds culture (*Crassostrea virginica*) (Sonier *et al*., 2008), among others. In the same way, it could be applied as well in combination with other chromogenic agar as CHROMagar *Vibrio* to distinguish several *Vibrio* species or CHROMagar *Salmonella* to perform the bacteria surveillance. Although all these studies are concerned about the sanitary state of waters, in general, those subjects do not represent a priority performed

periodically in aquaculture system. However, that nonattendance could produce negative results compromising not only the final products which has a commercial value, but rather the employments of many worker associated directly or indirectly to the company, as occurred in Chilean salmon industry (Iizuka & Zanlungo, 2016). Therefore, we propose a simple method to support this issue periodically in aquaculture.

CONCLUSIONS

We have demonstrated that the microdot method produce same results as the traditional method and its use with chromogenic agar allow, at the same time, the identification of target bacteria specie. This application is proposed to monitor is *E. coli*, *L. monocytogenes* and *S. aureus* in surface water of aquaculture system using the respective chromogenic agar; CHROMagar ECC, CHROMagar Listeria and Baird Parker agar. Although other colonies could appear during sanitary monitoring, their chromogenic properties are different from target bacteria, without interfering in their identification. In addition, the microdot method allows reducing material and time consuming labor during execution of the procedure, being a reproducible method to perform periodical monitoring in aquaculture systems.

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