

Sensitivity and Specificity of Chromogenic Media for Detection of Some Pathogens in Water

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Abstract

The main aim of the study was to evaluate the performance of some chromogenic media for detection of some pathogenic microorganisms in water such as; *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans*. Sensitivity, specificity and limit of detection of these media were studied. A total of 140 water samples were examined; groundwater (40), River Nile surface water (70), wastewater (20) and marine water (10). The first purpose of the study was to evaluate the specificity and sensitivity of chromogenic media; HiCrome Aureus Agar Base (HAA, Himedia, India), M-CP Agar Base (MCP, Himedia, India) and HiCrome Candida Differential Agar (HCD, Himedia, India) for detection of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans* in water samples, respectively. The second purpose was to determine the limit of detection for these chromogenic media. The isolated colonies were confirmed using PCR technique. Results showed that these chromogenic media were rapid, specific and sensitive for detection of the previous pathogenic microorganisms in different water samples, also, these chromogenic media showed a low recovery level of the examined microorganisms.

Keywords: *Chromogenic media, pathogenic microorganisms, membrane filtration, water.*

1. Introduction

One of the most important factors of water pollution is the microbial contamination; especially with pathogenic microorganisms. Enteric pathogens adversely affect the human health and the biodiversity in the aquatic ecosystem (Rangel et al., 2005). Most pathogenic microorganisms can be isolated and maintained on solid media. Approved methods and media for the isolation of the common bacterial pathogens and indicator bacteria from water and wastewater are well established and can be found in detail in standards produced by authorities in many countries, for example, the American Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Despite the fact that most microbial pathogens can be easily cultured, there are a number of problems associated with attempts to detect and quantify

them in water and wastewater samples. Problems associated with the direct culture of microbial species include difficulties in identification of pathogens, the time and expense involved in identifying and typing of pathogens isolates and the effect of selective media and/or selective isolation methods (Porter et al., 1995; Velusamy et al., 2010). In particular, waterborne infections like typhoid fever, cholera, dysentery and traveler's diarrhea, caused by different types of pathogens pose a major public health hazard (Hunter, 1997) especially in developing countries. The spectrum of waterborne infections is also expanding, and many infectious diseases once believed to be conquered are on the rise (Kong et al., 2002; Marshall et al., 1997). Regular monitoring of waterborne pathogens is required to protect public health.

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However, the lack of accurate and cost-effective diagnostic tests is a major obstacle in the prevention and control of infections and outbreaks transmitted by waterborne pathogens. To prevent pathogen infection, good monitoring and screening programs are required (Notermans et al., 1997). Recently in the United States and United Kingdom, and more recently in Australia, chromogenic substrate technologies have been accepted as standard methods (Anon, 2005; Brenner et al., 1996; Hallas et al., 2008; Oshiro, 2002). Over the last two decades, there has been a rapid expansion in the development and commercial availability of chromogenic agar media for the detection of pathogenic bacteria and yeasts (Aguilera-Arreola et al., 2012; Perry and Freydière, 2007). Such culture media typically contain multiple substrates that allow bacteria to form colored colonies based on their enzymatic activity. This facilitates the differentiation of species within polymicrobial cultures and the targeting of pathogens with high specificity. When specific pathogens are targeted, selective agents such as antibiotics are employed to limit the number of species able to grow. Most media rely upon the inclusion of indoxyl substrates in order to generate colonies with contrasting colors. For example, the release of green and red chromogens from two distinct substrates can result in the formation of green, red or purple colonies depending on whether one or both enzyme activities are present. Pathogens may therefore be differentiated from commensal bacteria by their possession of either one or both enzymes (Orenga et al., 2009). However, the main aim of the present study was to evaluate the specificity (the ability of media for detection of

actual negative colonies which are correctly identified) and sensitivity (the ability of media for detection of actual positive colonies which are correctly identified) of some commercial chromogenic media (Hicrome Aureus Agar Base, M-CP Agar Base and Hicrome Candida Differential Agar) for detection of some pathogenic microorganisms (*Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans*), respectively in different types of water. In addition, the limit of detection for these chromogenic media was investigated in the study.

2. Materials and Methods

2.1. Materials

Water Samples and Sampling

140 different water samples were collected from Egypt and examined during the study; 40 ground-water samples from New Valley governorate; 70 River Nile water samples from Rosetta branch; 20 wastewater samples from Al-Rahawy drain and 10 marine water samples from Marsa Mattrouh governorate (Figures 1 and 2). Water samples were collected according to standard methods for the examination of water and wastewater (APHA, 2005). All samples were collected in sterile 1L glass bottle (Simax, Czech Republic) and stored on ice (4°C) for transportation to the laboratory.

Reference Strains

10 typical strains (5 ATCC and 5 local isolates) of each microorganism and 4 atypical strains (2 ATCC and 2 local isolates) were used as shown in Table (1).

Table 1

Used Reference Strains

Microorganism	Typical strains		Atypical strains	
	ATCC (n=15)	Local (n)	ATCC (n=6)	Local (n)
<i>Staphylococcus aureus</i>	25923, 29213, 33591, 43300, 12600	5	<i>S. epidermidis</i> 12228 & 14990	2
<i>Clostridium perfringens</i>	13124, 3624, 3626, 10873, 12918	5	<i>C. difficile</i> 43593 & 43579	2
<i>Candida albicans</i>	10231, 10259, 10261, 14053, 18527	5	<i>Can. tropicalis</i> 11006 & 750	2

Media Used

A) *Staphylococcus aureus*:

HiCrome Aureus Agar Base (HAA) (Himedia, India) is recommended for isolation and enumeration of coagulase positive *Staphylococcus aureus* (*S. aureus*) from environmental samples. *Staphylococcus aureus* gives brown black colonies with clear zone around the colony. Suspend 63.1 grams in 950 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50 ml concentrated Egg yolk Tellurite Emulsion (Himedia, India). Mix well and pour into sterile Petri plates. The inoculated plates are incubated at 35-37°C for 24-48 hours.

B) *Clostridium perfringens*:

M-CP Agar Base (CPA) (Himedia, India) with selective supplements is recommended for isolation and enumeration of *Clostridium perfringens* (*C. perfringens*) from water samples using membrane filtration. Suspend 35.60 grams of dehydrated powder in 485 ml distilled water. Bring to boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C. Aseptically add the rehydrated contents of 1 vial of M-CP Selective Supplement I (Himedia, India) and 1 vial of M-CP Selective Supplement II (Himedia, India). Mix well and pour into sterile Petri plates. The inoculated plates are incubated at 44°C for 24-48 hours under anaerobic conditions. Color differentiation on M-CP Agar Base is sometimes difficult, so typical colonies (yellow turning into pink-red after exposure to ammonia fumes for 30 seconds) as well as atypical colonies (green or those that remain yellow upon exposure to ammonia fumes) are picked for confirmation. Presumptive *C. perfringens* can be confirmed by sulphite reduction, gram-positive, sporulating rods, non-motile, reduction of nitrate, gelatine liquefaction and lactose fermentation or by using PCR.

C) *Candida albicans*:

HiCrome Candida Differential Agar (CDA) (Himedia, India) is recommended for rapid isolation and identification of *Candida* species from

mixed cultures. Suspend 42.72 grams in 1000 ml distilled water. Bring to boil to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 50°C and pour into sterile Petri plates. The inoculated plates are incubated at 30°C for 40-48 hours. HiCrome Candida Differential Agar is a selective and differential medium, which facilitates rapid isolation of yeasts from mixed cultures and allows differentiation of *Candida* species namely, *C. albicans*, *C. krusei*, *C. tropicalis* and *C. glabrata* on the basis of coloration and colony morphology. *C. albicans* appear as light green colored smooth colonies, *C. tropicalis* appear as blue to metallic blue colored raised colonies. *C. glabrata* colonies appear as cream to white smooth colonies, while *C. krusei* appear as purple fuzzy colonies.

2.2. Experimental setup

Microbiological Examination

Membrane filtration (MF) technique (APHA, 2005) was used for detection of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans* in the collected water samples. The sterile cellulose nitrate membrane filter (Whatman®, 47 mm diameter, 0.45µm pore size) was placed over a sterilized porous plate receptacle using sterile forceps, and then sterilized funnel unit was placed over receptacle and locked. The samples were filtered through the membrane filter. After filtration the funnel was removed and the membrane filter was immediately placed on HiCrome Aureus Agar Base (Himedia, India) for detection of *Staphylococcus aureus*, M-CP Agar Base (Himedia, India) for detection of *Clostridium perfringens* and HiCrome Candida Differential Agar (Himedia, India) for detection *Candida albicans*.

Confirmation of Isolates

Polymerase chain reaction (PCR) technique was used for the confirmation of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans* isolates picked up from the Petri dishes of the examined water samples. The following primers were used in the study (Table 2).

Table 2
Nucleotide sequences of PCR primers.

Organism	Target gene	Primer	Length (bp)	Reference
<i>S. aureus</i>	<i>clfA</i>	5'GCAAAATCCAGCACAAACAGGAAACGA3' 5'CTTGATCTCCAGCCATAATTGGTGG3'	638	Mason <i>et al.</i> , 2001
<i>C. perfringens</i>	<i>CPE</i>	5'ACAGGTACCTTTAGCCAATC3' 5'AATCTTTCTGTAGCAGCAGC3'	425	Miwa <i>et al.</i> , 1996
<i>Cand. albicans</i>	<i>CHS1</i>	5'CGCCTCTTGATGGTGTATGAT3' 5'TCCGGTATCACCTGGCTC3'	122	Jordan, 1994

Table 3

Typical ($n=30$) and atypical ($n=12$) isolates on chromogenic media showing characteristic colonies appearance.

Media	Positive strains	n (%)	False negative colonies	n (%)	Negative strains	n (%)	False positive n (%)
HAA	Brown-black	10 (100)	ND*	0 (0)	Yellow-brownish	4 (100)	0 (0)
CPA	Yellow turning into pink	10 (100)	ND*	0 (0)	Green	4 (100)	0 (0)
CDA	Light green	9 (90)	Blue	1 (10)	Blue to purple	4 (100)	0 (0)

*ND: Not Detected

2.3. Statistical analysis

For evaluation of the used media, percentages of sensitivity and specificity were calculated, as follows:

Sensitivity (%) = True Positives X 100 / (True Positives + False Negatives).

Specificity (%) = True Negative X 100 / (True Negative + False Positive).

Determination of Limit of Detection for Chromogenic Media

The previous ATCC reference strains were grown overnight at 35°C in brain heart infusion broth (Himedia, India) and enumerated by heterotrophic colony counts using R2A agar and incubated at 35°C for 48 h. Overnight cultures were consis-

tently found to have levels of 10^9 – 10^{10} CFU mL⁻¹. All brain heart infusion broths were serially diluted in sterilized saline solution and then added to filtered sterilized potable water to obtain a final spiked concentration of approximately 1-20 CFU 100 mL⁻¹, 20-80 CFU 100 mL⁻¹ and 80-500 CFU 100 mL⁻¹ according to the protocol of Hallas *et al.* (2008). All samples were analyzed by using chromogenic media.

3. Results

Sensitivity and Specificity of Chromogenic Media for Reference Strains

The results of the identification of typical (ATCC and local isolates) *S. aureus*, *C. perfringens* and *Candida albicans*, and atypical (ATCC and local

isolates) reference strains on different examined chromogenic media were summarized in Table (3). Positive results were 100 % for *S. aureus* and *C. perfringens* and 90% for *Candida albicans*. False negative colonies were not detected on HAA and CPA media while, one (10%) typical *Candida albicans* colony showed different coloration (blue) to the typical colony (light green). False positive colonies were (0%) for all tested media. No atypical strain showed the typical coloration of typical strains on these chromogenic media. The usefulness of tested chromogenic media for identification of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans* strains was shown in Table (4). In the present study, by using reference strains (typical and atypical), we found that, HAA and CPA media had sensitivity and specificity of 100 % for detection of *S. aureus* and *C. perfringens*, respectively, whereas, CDA media had sensitivity (90 %) with specificity (100 %) for *Candida albicans* detection.

Sensitivity and Specificity of Chromogenic Media for Water Samples

140 different water samples were collected and analyzed by membrane filtration technique for detection of *S. aureus*, *C. perfringens* and *Candida albicans* using HAA, CPA and CDA media, respectively. After the incubation periods of Petri dishes, some typical and atypical colonies were picked up for confirmation using PCR technique. Positive (true and false) and negative (true and false) results were summarized in Table (5). HAA media showed sensitivity for detection of *S. aureus*

ranged from 98.5% to 100% with specificity ranged from 75% to 100%. CPA media showed sensitivity for detection of *C. perfringens* ranged from 95% to 100% with specificity ranging from 69.2% to 100%. CDA media showed sensitivity for detection of *Candida albicans* ranged from 95% to 100% with specificity ranging from 66.7% to 100%. False positive colonies were identified using GEN III OmniLog® ID System (Biolog, USA) as showed in Table (6).

The Limit of Detection for Chromogenic Media

The filter sterilized potable water samples which were spiked with known concentrations (1-20, 20-80 and 80-500 CFU 100 mL⁻¹) of the tested reference strains (*S. aureus*, *C. perfringens* and *Candida albicans*) were examined using membrane filtration technique for the determination of detection limit of the media for the examined microorganisms. All examined chromogenic media showed detection levels of target microorganisms less than 20 CFU 100 mL⁻¹.

4. Discussion

Conventional methods for detection and enumeration of pathogenic microorganisms in water generally are time consuming and require a lot of confirmatory tests. In addition, there is a great need for rapid and sensitive tests for detection of pathogenic microorganisms in water, especially in drinking water (Tavakoli et al., 2008).

Table 4

Sensitivity and specificity of tested media for isolation of the reference strains (typical and atypical)

	Media		
	HAA	CPA	CDA
True positive results	10	10	9
True negative results	4	4	4
False positive results	0	0	0
False negative results	0	0	1
Sensitivity (%)	100	100	90
Specificity (%)	100	100	100

Table 5

Positive and negative PCR confirmed isolates from 140 different water samples on examined chromogenic media.

Media	Water type*	Positive isolates			Negative isolates			Sensitivity (%)	Specificity (%)
		(n)	T.P. † n (%)	F.P. † n (%)	(n)	T.N. † n (%)	F.N. † n (%)		
HAA	Groundwater	12	11 (91.7)	1 (8.3)	6	6 (100)	0 (0)	100	85.7
	River Nile	70	67 (95.8)	3 (4.2)	11	10 (90.9)	1 (8.9)	98.5	76.9
	Wastewater	20	20 (100)	0 (0)	13	13 (100)	0 (0)	100	100
	Marine water	10	9 (90)	1 (10)	3	3 (100)	0 (0)	100	75
CPA	Groundwater	9	8 (88.9)	1 (11.1)	5	5 (100)	0 (0)	100	83.3
	River Nile	45	41 (91.2)	4 (8.8)	10	9 (90)	1 (10)	97.6	69.2
	Wastewater	20	19 (95.0)	1 (5)	5	4 (80)	1 (20)	95	80
	Marine water	5	5 (100)	0 (0)	2	2 (100)	0 (0)	100	100
CDA	Groundwater	2	2 (100)	0 (0)	2	2 (100)	0 (0)	100	100
	River Nile	62	60 (96.8)	2 (3.2)	8	7 (87.5)	1 (12.5)	98.4	77.8
	Wastewater	20	19 (95.0)	1 (5.0)	5	4 (80)	1 (20)	95	80
	Marine water	13	11 (84.6)	2 (15.4)	4	4 (100)	0 (0)	100	66.7

† T.P.: true positive, F.P.: false positive, T.N.: true negative, F.N.: false negative.

Table 6

The identification of false positive isolates from examined chromogenic media.

Media	Isolates (n)	Bacterial strains (n)
HAA	5	<i>Staphylococcus epidermidis</i> (4) and <i>Listeria monocytogenes</i> (1).
MCP	6	<i>Clostridium bifermentans</i> (2), <i>Clostridium sordelli</i> (2), <i>Enterococcus faecalis</i> (1) and <i>Salmonella typhimurium</i> (1).
CDA	5	<i>Candida krusei</i> (3) and <i>Candida tropicalis</i> (2).

In recent years, a number of selective chromogenic plating media for detection and enumeration of the most important bacteria in food and water have been developed and marketed (Manafi, 2000; Orenge et al., 2009). In the present study, some commercial chromogenic media; Hicrome Aureus Agar Base (HAA, Himedia, India), M-CP Agar Base (CPA, Himedia, India) and Hicrome Candida Differential Agar (CDA, Himedia, India) were examined for detection of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans*, respectively in different types of water through studying its sensitivity, specificity and the detection limit of these microorganisms. By using reference (ATCC and local) strains (typical and atypical), it was found that, HAA gave 100% sensitivity and specificity for detection *S. aureus* reference strains, while it gave 98.5% to 100% sensitivity with 75% to 100% specificity for detection of *S. aureus* when water samples were examined. Many studies examined another chromogenic media for detection and enumeration of *S. aureus* in water and food like CHROMagar Staph aureus and *S. aureus* ID (Gaillot et al., 2000; Perry et al., 2003) with sensitivity and specificity 76% and 90%, respectively (Cherkaoui et al., 2007). The high sensitivity and specificity of HAA media may be due to presence of sodium pyruvate in the medium which protects the injured cells, helps recovery and enhances growth of *S. aureus*. Also, presence of lithium chloride and addition of egg yolk tellurite emulsion inhibit most of contaminating microflora except *S. aureus*. CPA gave 100% sensitivity and specificity for detection *C. perfringens* reference strains, while it gave 95% to 100% sensitivity with 69.2% to 100% specificity for detection of *C. perfringens* when water samples were examined. Adcock and Saint, (2001) evaluated the chromogenic media for rapid

detection of *C. perfringens* and showed 99.3% sensitivity with 93.3% specificity. CPA media showed high sensitivity and specificity due to CPA media includes indoxyl- β -D-glucoside as a chromogenic substrate for β -D-glucosidase and addition of D-cycloserine and polymyxin B (M-CP selective supplement I) makes the medium inhibitory to accompanying non-clostridial microflora and thus allows analysis of both clostridial vegetative cells and spores. Also, further selectivity is provided by incubation under anaerobic conditions. Many reviews studied the evaluation of chromogenic media for detection of *Candida albicans* such as; Albicans ID2 and CHROMagar Candida (Eraso et al., 2006; Patricio et al., 2006), these chromogenic media showed 91.7% sensitivity with specificity 97.2% and 97.4% sensitivity with specificity 98.7%, respectively. In the present study, CDA media showed 90% sensitivity with 100% specificity by using reference strains and 95% to 100% sensitivity with 66.7% to 100% specificity in all analyzed water samples. CDA was used as selective and differentiation media for *Candida* species due to presence of chloramphenicol which suppresses the accompanying bacterial flora. CDA media contain β -N-acetyl-galactosaminide as a chromogenic substrate for the enzyme β -N-acetyl-galactosaminidase produced by *Candida albicans*. HAA, CPA and CDA media showed very low detection limit (1-20 CFU 100 mL⁻¹) of *S. aureus*, *C. perfringens* and *Candida albicans*, respectively in the spiked filter sterilized potable water samples. Hallas et al. (2008) studied the limit of detection of chromogenic media for detection of coliforms and *E. coli*, which showed a detection limit less than 20 CFU 100 mL⁻¹.

5. Conclusion

The obtained results showed the suitability of using the chromogenic media for detection and enumeration of *S. aureus*, *C. perfringens* and *Candida albicans* in different types of water since these chromogenic media were sensitive, specific

and able to detect low levels of examined microorganisms making these media very suitable for routine work of laboratories especially it did not require any confirmatory tests.

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