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Efficiency of Hichrome *Enterococcus faecium* Agar in the Isolation of *Enterococcus spp*. and other Associated Bacterial Genera from Water

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Abstract

Members of the genus *Enterococcus* are intestinal microorganisms that have been used as indicators for recreational water fecal contamination. They are opportunistic pathogens that have been associated with nosocomial infections, therefore, their isolation and identification is important for proper estimation of their count. Accordingly, this study was conducted to identify the efficiency of Hichrome *Enterococcus faecium* agar in isolating enterococci to better monitor their presence in recreational water samples. Ten water samples were collected, filtered, and spread on the chromogenic Hichrome *Enterococcus faecium* agar in an attempt to isolate *Enterococcus spp*. depending on its ability to grow on this medium and produce color as illustrated by Himedia company. Results showed the ability of this medium to select for *Enterococcus spp.*, however, other non-enterococci genera were also capable of growing on this medium producing different colors that were not mentioned by Himedia company. Interestingly, some of these bacterial colonies were found to be Gram negative. Colonies were diagnosed by VITEK-2 system then further diagnosed by 16S rRNA gene sequencing. Our results, showed major differences in diagnosis between the two methods suggesting 16S rRNA gene sequencing for more precise identification. Antibiotic resistance of our environmentally isolated bacteria revealed the threat of antibiotic resistance spread among environmental bacteria with streptomycin being the highly resistant (64.2%) and chloramphenicol being the lowest (35.7%).

Keywords: *Enterococcus, Environmental bacteria, 16S rRNA gene sequencing*
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I. INTRODUCTION

Enterococci belong to a group of organisms known as lactic acid bacteria (LAB). This genus was not separated as an independent genus distinct from streptococcus until 1984 depending on DNA hybridization and 16S rRNA gene sequencing (Fisher and Phillips, 2009). *Enterococcus* are ubiquitous due to their ability to survive in a wide range of environmental conditions, as they exist in the intestines of humans, animals, and insects and are found in different environmental samples such as water, soil, and plants. *Enterococcus* are intestinal microorganisms and most of the isolated *Enterococcus* are fecal in origin (McAuley, 2016). The presence of enterococci has been used as an indicator of fecal contamination of recreational water. This water born pathogen is also correlated with the incidence of swimming-related illnesses especially those caused by *E. faecalis* and *E. faecium* (Lata *et al*., 2009). *Enterococcus* are opportunistic pathogens that have been associated with nosocomial infections and plays an important role in the distribution of antibiotic resistance, because they exhibit resistance to a wide variety of antimicrobial drugs and have the ability to exchange genetic information through conjugation (Lata *et al*., 2009). Accordingly, it is extremely important to monitor *Enterococcus* presence in recreational water and precisely distinguish these pathogens from other non-enterococci bacteria in order to provide accurate estimation for *Enterococcus*. Several types of selective media have been used to isolate *Enterococcus spp*. including mEI agar (Haugland *et al*., 2005), Bile esculin azide agar (Yilema *et al*., 2017), Chromocult enterococci agar (Miranda *et al*., 2005), and Hichrome *Enterococcus faecium* agar (Atanasova *et al*., 2014). In our study we attempted to study the efficiency of the chromogenic Hichrome *Enterococcus faecium* agar in isolating *Enterococcus spp*. based on colony

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morphology. Isolates were molecularly identified using 16S rRNA gene sequencing. Finally, antibiotic resistance spread among these environmentally isolated bacteria was identified.

II. Materials and Methods

A. Water Sample Collection

Ten water samples were collected from swimming pools and recreation areas from Tigris river in Mosul city during the period November 2020 to March 2021. Water samples were taken by immersing a 1L sterile plastic bottle upside down at a depth of 30 cm below water surface. Samples were labelled and transferred to the laboratory in cooled containers for further analysis (Lata *et al*., 2009).

B. Isolation of Enterococcus spp. from Water

Membrane filtration method was used for the isolation of *Enterococcus spp*. from water samples. One hundred milliliter of water was filtered through 0.45µm sized membrane filter. The flow of water was facilitated by using an air vacuum pump attached to a conical flask to collect the unwanted filtered water. The filter paper was placed on the surface of Hicrome *Enterococcus faecium* agar selective media plates were incubated at for 37ºC for 24h and colonies were selected according to their shape, size, and color as described by Himedia company (Mumbai, India). Growth and colony color as mentioned by Himedia is shown in table 1. Single colonies were streaked twice on Hicrome *Enterococcus faecium* agar to confirm purity of bacterial isolates to be used for following experiments.

Table 1. Selection of *Enterococcus* on Hicrome *Enterococcus fae*cium agar as mentioned by Himedia Company.

Expected organism	Growth and color of colony
Enterococcus faecalis	Blue
Enterococcus faecium	Green
Enterococcus hirae	Blue
Escherichia coli	Inhibited
Pseudomonas aeruginosa	Inhibited
Staphylococcus aureus	Inhibited

C. Diagnosis of Enterococcus spp.

1-Test for growth on Bile esculin agar

Bile esculin agar was inoculated with a pure culture grown on Hicrome *Enterococcus faecium* agar by the streaking method. Plates were incubated at 37oC for 24h. Plates were examined and the ability of isolates to hydrolyze esculin and tolerate bile salts was recorded (Garry and Hall, 2017).

2-Catalase test

This test demonstrates the presence of catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide into oxygen and water. The test was done by mixing a drop of 3% hydrogen peroxide on a glass slide with a small amount from a bacterial isolate transferred via a sterile wooden stick. Generation of air bubbles within 20 seconds of mixing was considered a positive result (Tille, 2015).

3-The ability to grow at 45ºC

This test was used to distinguish the genus *Enterococcus* from other genera. Nutrient agar plates were streaked with the tested bacteria and incubated for a period of 3 days at 45ºC.

4-Diagnosis by VITEK-2 system

Cultures that were believed to be *Enterococcus* according to the results of all tests mentioned above were sent for further identification using VITEK-2 System at Radhwan Al-Jamas diagnostic laboratory in Mosul city. This method is considered one of the most fastest and accurate methods for bacterial diagnosis that is being used locally to identify microorganisms.

5-Molecular diagnosis via 16S rRNA gene sequencing

Genomic DNA was extracted from isolates using the kit supplied by Geneaid. The 16S rRNA gene was amplified using the universal 16S rRNA primers, 27-F (5′-A GAGTTTGATCMTGGCTCAG-3′) and 1552-R (5′-AAGGAGGTGATCCARCCGCA- 3′). The following steps were used for 16S rRNA gene amplification: initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95°C for 30 sec, 54°C for 30 sec, and 72°C for 1:30 min, and a final extension step for 3 min at 72°C. Amplification was conducted in 20µl reaction using 1X GoTaq Green master mix (Promega, USA), 1 ng/µl genomic DNA, and 1mM final concentration for each primer. PCR products were analyzed on 1% agarose gel and were then purified and sent for DNA sequencing. Sequences obtained were compared to sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

D. Antimicrobial susceptibility

The antibiotic resistance pattern of isolates under study were tested using the agar dilution method by streaking isolates separately on Muller Hinton agar medium containing the antibiotics understudy with the final concentrations shown in table (2). Muller Hinton agar media was autoclaved and antibiotics (Sigma-Aldrich) were added after the media was cooled down to 45ºC. Isolates were then streaked and plates were incubated at 37ºC for 24-48h. Results were recorded after incubation period was ended (Wayne, 2011).

Table 2. Types of antibiotics and their final concentrations.

Antibiotics	Stock concentration mg/ml	Final concentration µg/ml	Solvent
Ampicillin	50	50	dH2O
Vancomycin	30	30	dH2O
Gentamycin	20	10	Solution
Streptomycin	20	20	dH2O
Ciprofloxacin	20	10	dH2O
Tetracycline	15	30	70% ethanol
Kanamycin	50	50	dH2O
Chloramphenicol	30	50	70%ethanol

III. RESULTS AND DISCUSSION

A. Isolation of Enterococcus spp. using chromogenic methods

Three out of ten water samples (30%) showed the presence of *Enterococcus* isolates by producing either blue or green colored colonies. Accordingly, these samples were selected for studying the efficiency of hicrome *Enterococcus faecium* agar. Four different colored colonies were seen on this media with two colors (pink and orange) that were not mentioned by Himedia and were isolated at a percentage of 30.76 (4/13) - 58.4% (31/53). From the three *Enterococcus* containing samples, 73 bacterial isolates with different colors were obtained. The percentage for enterococci isolation was 41.5 (22/53) - 69.22% (9/13). Table 3 below shows the percentages of isolation for bacterial colonies according to their colors.

Table 3. Percentage of isolation on Hichrome *Enterococcus faecium* agar

Isolate No.	Blue colonies n (%)	Green colonies n (%)	Enterococci n (%)	Pink colonies n (%)	Orange colonies n (%)	Non-Enterococci n(%)
1	7 (53.84)	2 (15.38)	9 (69.22)	3 (23.07)	1 (7.69)	4 (30.76)
2	19 (35.8)	3 (5.7)	22 (41.5)	27 (50.9)	4 (7.5)	31 (58.4)
3	3 (42.8)	1 (14.2)	4 (57)	2 (28.5)	1 (14.2)	3 (42.7)

Our goal was to diagnose the enterococci isolates by VITEK-2 system and 16S rRNA gene sequencing, in addition to identifying the non-enterococci in an effort to provide a guide for researchers using this media. Therefore, we selected 13 colonies of different colors for further identification using their ability to hydrolyze esculin on bile esculin agar, produce catalase, and grow at 45ºC. Results in table (4) shows the color of selected isolates and their results for the tests mentioned above.

Table 4: Diagnosis for selected colonies.

Isolate number	Colony color	Asculin Hydrolysis	Catalase production	Growth at 45ºC
1	Blue	+	-	+
2	Blue	+	-	+
3	Blue	-	-	+
4	Green	+	-	+
5	Green	+	-	+
6	Green	+	-	+
7	Green	+	-	+
8	Green	+	-	+
9	Pink	-	+	+
10	Pink	-	+	+
11	Pink	-	+	+
12	Orange	+	-	+
13	Orange	+	-	+

From the results obtained we may notice that all blue, green and orange colonies were catalase negative, able to grow at 45ºC, and hydrolyze esculin to esculetin and dextrose (except one) which gives a strong prediction that blue and green isolates belong to the genus *Enterococcus*. On the other hand, pink colonies were not able to hydrolyze esculin but were catalase positive and were able to grow at 45ºC. According to the differences in results of isolate 3 to other blue colonies we expected this colony to be different from isolate 1 and 2. However, orange colonies showed similar results to what was expected to be seen from *Enterococcus spp.* except that the color of the colony was different to what was mentioned by Himedia. In order to identify enterococci strains we diagnosed isolates 1-8 (blue and green colonies) using VITEK-2 system. Results shown in table 5 proved that 4 out of 8 isolates belonged to *Enterococcus* (2 *E. faecium*, *E. gallinarum*, *E. casselifelavus*), including those that failed in giving the actual identification. Interestingly, the blue colony (isolate 3) that was phenotypically similar to *Enterococcus* was identified by VITEK-2 as *Staphylococcus lentus*.

Table 5. Identification of isolates by VITEK-2 system.

Isolate number	**Isolate name**	**probability**
1	*Enterococcus casselifelavus*	89%
2	*Enterococcus gallinarum*	89%
3	*Staphylococcus lentus*	93%
4	Non-specific	
5	Non-specific	
6	Non-specific	
7	*Enterococcus faecium*	89%
8	*Enterococcus faecium*	98%

Results for 16S rRNA gene sequencing (table 6) shows the identification of our isolates by molecular method. This method of diagnosis showed that only 4 out of 8 suspected enterococci isolates truly belonged to *Enterococcus*. These results prove that some blue or green colonies belonged to different genera such as *Aerococcus*, *Aeromonas*, *Cellvibrio*, and *Paenibacillus* (Table 7). Because all pink and orange isolates were similar in biochemical and morphological characteristics, a single colony from each group was picked and its 16S rRNA gene was sequenced. Results showed that pink colonies belonged to *Brevundimonas spp.* While orange colonies belonged to *E. casseliflavus*.

Table 6: Identification of isolates by 16S rRNA gene sequencing.

Isolate number	Isolate name	Identity
1	*Aerococcus viridans* strain F42	99.79%
2	*Enterococcus gallinarum* strain 644	99.89%
3	*Staphylococcus lentus*	99.80%
4	*Aeromonas sanarellii* strain A2	99.57%
5	*Cellvibrio fontiphilus* strain MVW-40	99.05%
6	*Paenibacillus sp.* AHK180-5	99.09%
7	*Enterococcus faecium* strain VBO96	99.49%
8	*Enterococcus faecium* strain SrsV161	99.02%
9	*Brevundimonas sp*	83.98%
12	*Enterococcus casseliflavus*	85.53%

Table 7. Types of genera that may grow on Hichrome *Enterococcus faecium* agar media and produce blue or green colonies expected to belong to *Enterococcus*.

Blue colonies	Green colonies
Enterococcus facealis	*Enterococcus faecium*
Enterococcus gallinarum	*Aeromonas sanarellii*
Aerococcus viridans	*Cellvibrio fontiphilus*
Staphylococcus lentus	*Paenibacillus sp*

B. Antibiotic resistance

The isolates from water samples exhibited different patterns of antibiotic resistance as shown in table 8. Out of 14 environmental isolates, 3 were sensitive to all antibiotics used. However, the rest were resistant to at least 2-8 antibiotics (25-100%). The highest resistance towards antibiotics in our study was seen towards streptomycin (64.2%) while the lowest resistance was seen towards chloramphenicol.

Table 8. Antibiotic resistance for *Enterococcus* and other environmentally isolated bacteria mentioned as frequency of resistance.

Isolate No.	Antibiotics (µg/ml)*								
	AMP (50)	STR (20)	Gm (10)	VA (30)	CIP (10)	TE (30)	C (30)	KAN (50)	Total resistance (%)
1	S	S	R	S	R	S	S	R	37.5
2	S	S	R	S	S	R	S	S	25.0
3	S	S	S	S	S	S	S	S	0
4	R	R	R	R	R	R	R	R	100
5	S	S	S	S	S	S	S	S	0
6	S	S	S	S	S	S	S	S	0
7	R	R	R	S	R	S	S	R	62.5
8	S	R	R	S	R	S	S	R	50.0
9	R	R	S	R	R	R	R	R	87.5
10	R	R	S	R	R	S	S	S	50.0
11	R	R	R	R	R	S	R	R	87.5
12	R	R	R	R	S	R	S	R	75.0
13	S	R	S	R	S	R	R	S	50.0
14	S	R	S	S	S	R	R	S	37.5
% Resistance	42.8%	64.2%	50%	42.8%	50%	42.8%	35.7%	50%	

* R: resistant, S: sensitive, AMP: ampicillin, STR: streptomycin, Gm: gentamycin, VA: vancomycin, CIP: ciprofloxacin, TE: tetracycline, C: chloramphenicol, Kan: kanamycin

IV. DISCUSSION

Hicrome *Enterococcus faecium* agar is a chromogenic selective media supplied from Himedia (Mumbai, India) used for the isolation of *Enterococcus spp*. During an attempt to isolates *Enterococcus* from water samples, our results show the possibility of different microorganisms not mentioned by the company to grow on this medium and that their percentage for isolation was relatively high compared to *Enterococcus* isolation. Four different colored colonies were observed on this media (blue, green, pink, and orange). The highest observed was in sample 2 which contained 50.9% (27/53) of pink colonies (table 3). Two out of the three blue colonies that were phenotypically similar to *Enterococcus* isolates turned out to belong to *Staphylococcus lentus* and *Aerococcus viridans*. This indicated that not necessary all blue colonies growing on this media belongs to *Enterococcus faecalis* as stated by the company and that additional diagnostic tests should be performed. Similar results were noticed from the analysis of green isolates.

Identification of pink colonies showed that they belong to *Brevundimonas spp*. Members of this genus are Gram negative bacteria oxidase and catalase positive that are common causes of nosocomial infections and are found in soil and water sources. They are mostly resistant to multiple antibiotics (Ryan and Pembroke, 2018). These characteristics mentioned above were all observed in our results for pink isolates. Looking at table 8 we observe that all pink colonies (isolate 9,10, and 11) were highly resistant to antibiotics understudy, as the percentage of antibiotic resistance for *Brevundimonas spp.* was 50-87.5%.

Interestingly, diagnosis of the orange colony by 16S rRNA sequencing showed that this colony belonged to *E. casseliflavus.* This species is phylogenetically the closest to *E. gallinarum*, as they are both intrinsically resistant to vancomycin and cause less frequent enterococcal infections compared to *E. feacalis* and *E. faecium* (Monticelli *et al*., 2018). The detection of orange colonies that were diagnosed as *Enterococcus* is considered a false negative result when isolating *Enterococcus* using Hichrome *Enterococcus faecium* agar media, as such colony turned out to belong to the genus *Enterococcus*.

By comparing the diagnosis of isolates using VITEK-2 system and 16S rRNA gene sequencing, we noted that the VITEK-2 system cannot be totally relied upon to obtain accurate diagnosis and such results should be confirmed by 16S rRNA gene sequencing. In addition, VITEK-2 system was not efficient in detecting environmental bacteria compared to 16S rRNA gene sequencing. A comparative study between 16S rRNA gene sequencing, API20E, and VITEK-2 showed that 16S rRNA gene sequencing was efficient in identifying all tested bacteria to at least species and genus level. While using API20E and VITEK-2 failed in identifying 39% and 46% of tested bacteria, respectively (Bosshard *et al*., 2006). Our results showed that using hicrome *Enterococcus faecium* agar alone is inaccurate in the identification of *Enterococcus spp.* and for proper identification additional biochemical tests are required. According to the results obtained from VITEK-2 we found that this method was not effective in identifying environmentally isolated bacteria, therefore, we suggest further identification of isolates by sequencing their 16S rRNA gene and comparing their sequence to NCBI data base.

The highest resistance towards antibiotics in our study was seen towards streptomycin (64.2%) while the lowest resistance was seen towards chloramphenicol. Developing a resistance to streptomycin in *Enterococcus* is relatively easier compared to other antibiotics which may be the reason behind high resistance to this antibiotic by environmental bacteria. Resistance may occur basically by a single point mutation in the ribosomal gene or by acquiring a streptomycin modification enzyme. However, acquiring a modification enzyme usually creates resistance to higher levels of streptomycin (Hollenbeck and Rice, 2012). The low resistance to chloramphenicol is related to its limited use in most medical treatments, as this antibiotic was available for use in 1949 but after 48 years it was banned from use. Many reports showed that chloramphenicol may cause aplastic anaemia, bone marrow suppression, and reduce myeloperoxidase activity in bovine granulocytes and for that reason its use is limited (Bystrzycka *et al*., 2017). Due to the fact that antibiotic resistance is developed when bacteria is exposed to low concentrations of antibiotic in nature, we believe that less prescriptions of chloramphenicol lead to lower levels of chloramphenicol in our environment thus low chloramphenicol resistant strains.

The resistance of bacteria to antibiotics was relatively high in some of the isolates which is probably due to the lack of proper wastewater treatment process in Mosul which leads to the development of antibiotic resistance in environmental isolates. The presence of remaining antibiotic concentrations in waste water is probably the major driver for antibiotic resistance in Tigris river. On a study conducted on *Acinetobacter spp*., the antibiotic resistance pattern for these isolates increased in *Acinetobacter* isolated near raw influent compared to those isolated from the final effluent. Such results illustrates the influence of wastewater on selecting antibiotic resistance in bacteria (Zhang *et al*., 2009).

V. Conclusion

Hicrome *Enterococcus faecium* agar is a selective media used for the isolation of *Enterococcus spp*. from different sources depending on chromogenic methods. Even though the procedure for isolation is relatively easy, the accuracy could be low. Our results showed that other organisms besides *Enterococcus* grew on this media and produced colonies that were morphologically and biochemically similar to *Enterococcus spp.* In addition, we found that different genera grew on this medium and produce colonies with different colors that was not mentioned by Himedia company. Collectively, these results may facilitate isolation and proper identification of *Enterococcus spp.* for researchers using this medium.

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