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Efficiency of HiCrome *Acinetobacter* Agar in identifying *A. baumannii* from different clinical specimens

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ABSTRACT

Acinetobacter baumannii is an opportunistic bacterial pathogen that is commonly linked to hospital-acquired infections. This pathogen is responsible for many life-threatening infections caused worldwide; therefore, it is crucial to isolate and identify *A. baumannii* rapidly and precisely. This study was carried out to determine the efficiency of HiCrome Acinetobacter Agar in identifying *A. baumannii* from different clinical specimens. In an attempt to isolate *Acinetobacter* species, 170 distinct specimens were collected from wounds, burns, sputum, and CSF. Samples were streaked on HiCrome *Acinetobacter* Agar and the isolation percentage of *A. baumannii* was determined according to colony color (yellow to light purple). Results showed that this medium was not only selective for *Acinetobacter* species, but also other genera that were not related to *Acinetobacter* may also grow and produce other colors that the Himedia company did not mention. Accordingly, the isolation rate for *A. baumannii* changed from 95.5% to 38.9% when adding the oxidase test and to 14.4% when adding lactose fermentation on MacConkey agar and finally to 13.3% when isolates was further identified using 16S rRNA sequencing. Results showed that 24/35 purple colonies belonged to members of the enterobacteriaceae and 51/90 total colonies were identified as *Pseudomonas aeruginosa.* The higher isolation rate observed for *P. aeruginosa* suggests that HiCrome *Acinetobacter* agar could be used as a selective and differential media for isolation of *P. aeruginosa* from clinical specimens.

Keywords: *Acinetobacter baumannii*, 16S rRNA, HiCrome Acinetobacter agar.

INTRODUCTION

The genus *Acinetobacter* belongs to the family *Moraxellaceae,* class γ-proteobacteria (Sheck *et al*., 2023). In 1954, Brisou and Prevot proposed the genus *Acinetobacter* to distinguish it from the motile organisms within the genus *Achromobacter* (Almasaudi, 2018). The genus *Acinetobacter* is diverse, with more than 50 species (Al-Atrouni *et al.* 2016) most of which are nonpathogenic environmental members with *A. baumannii, A. calcoaceticus, and A. lwoffii* being the most frequent species to cause infections (Wong *et al*., 2017). Additional species, have also been reported as pathogens such as *A. haemolyticus, A. johnsonii, A. junii, A. nosocomialis, A. pittii, A. schindleri, and A. ursingii* (Salzer *et al.* 2016).

The opportunistic bacteria *A. baumannii* has attracted the attention of researchers due to its high ability to develop resistance against a variety of antibiotics, its capacity to take in genetic material from different bacterial species, and its ability to form biofilms on several surfaces (Ibraheem and Baqer, 2021). *A. baumannii* infections are mostly acquired through a number of risk factors, including extended hospital stays, mechanical breathing, intravascular devices, advanced age, immunosuppression, past broad-spectrum antibiotic therapy, sepsis, and enteral feedings. *A. baumannii* is thought to be a low-virulence pathogen, until it was isolated from patients with comorbidities, such as elderly adults with chronic illnesses like cancer or newborns with low birth weights (Islahi *et al*., 2015). Four percent of all diseases connected to shunts and meningitis are caused by *A. baumannii* (Basri *et al*., 2015). Although it accounts for 2.1% of ICU-acquired wound infections, it can also cause infection in elderly patients who are too weak to care for themselves or in patients who have long-term indwelling catheter-related infections. *A. baumannii* has also been shown as a causative agent for ophthalmitis, keratitis, and endocarditis during contact lens wear and after eye surgery (Peleg *et al*., 2008).

Due to the wide range of infections caused by different species of *Acinetobacter* and that many of the infections are life-threatening, accurate identification of *Acinetobacter* species is critical for early treatment (Vijayakumar *et al*., 2019). *Acinetobacter spp*. can be isolated using several selective and differential media. One such medium contains bile salts and bromocresol purple. Another includes bile salts with a selection of antibiotics intrinsically resisted by *Acinetobacter* (Ciftci *et al*., 2015). HiCrome media has been used in many studies for rapid detection of pathogens (Jangla *et al*., 2020; Rameshkumar *et al*., 2015). However, the rate of isolation and identification was found to be significantly higher using Chromogenic based media compared to basic traditionally used laboratory media (Sachu and Samuel, 2022). Higher rates are sometimes doubtful that drive scientists to further investigate the identification process. In a previous study. Abdulrazzaq and Faisal (2022), studied the efficiency of Hichrome Enterococcus faecium agar. Results showed that this media not only selected *E. faecium and E. faecalis*, but also some Gram-negative bacteria as well. In a study conducted earlier by Al Najim (2023), they mentioned that the rate of *A. baumannii* isolation using HiCrome Acinetobacter agar was more than 80%. These results collectively made us propose that chromogenic based media require further evaluation. Therefore, we attempted to study the efficiency of the chromogenic HiCrome Acinetobacter agar from Himedia in identifying *A. baumannii* according to colony morphology. Finally, efficiency was determined by 16S rRNA gene sequencing for suspected isolates.

MATERIALS AND METHODS

Sample collection

One hundred and seventy specimens were collected from Al-Salam Teaching Hospital, Al-Jumhori Teaching Hospital, and Burns Ward Plastic Surgery in Mosul City during the period from September 2023 to December 2023. All specimens were collected from hospitalized patients from different clinical sources including wounds, burns, sputum, and CSF. Burns and wound swabs were taken from the sites of infection, then placed in sterile tubes containing transport media (amies with charcoal) to keep the swabs wet. CSF samples were collected by an experienced physician, while

urine and sputum samples were collected using tightly covered sterilized cups. All specimens were labeled and transferred to the lab.

Isolation of suspected *A. baumannii* isolates from clinical specimens

Samples collected from different specimens were cultured directly on HiCrome Acinetobacter Agar plates using the streaking method. Plates were incubated aerobically at 37°C for 24 hours. HiCrome Acinetobacter Agar (Himedia Labratories/ India) was prepared in accordance with the manufacturer's instructions. Briefly, 30.85g of HiCrome Acinetobacter Agar Base was dissolved in 1000 ml of distilled water. The media was sterilized by boiling to completely dissolve the components. Medium was cooled to 45–50°C and two vials of MDR Acinetobacter selective supplement (FD271) was added. The medium was mixed well, and poured into sterile petri dishes.

Diagnosis of *Acinetobacter spp.*

Suspected *A. baumannii* colonies that grew on HiCrome Acinetobacter Agar were subcultured on MacConkey agar plates by the streaking method and incubated aerobically at 37°C for 24h to test for their ability to ferment lactose. Isolates were then checked for obtaining cytochrome oxidase using the oxidase test. Colonies that turned purple when subjected to the oxidase test reagent after 60-90 seconds were recorded as positive for the oxidase test (Shields, 2010).

Molecular diagnosis via 16S rRNA gene sequencing

Genomic DNA of suspected isolates was extracted using an extraction kit supplied by Geneaid. The DNA was extracted following the instructions provided by the company. The 16S rRNA gene was amplified using the universal 16S rRNA primers 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1522R: 5′-AAGGAGGTGATCCARCCGCA-3’ (Khaleel *et al*., a2023). Polymerase Chain Reaction (PCR) was conducted in a 20 µL volume reaction using GoTaq® G2 Green Master Mix. A 20-µL PCR reaction contains the following ingredients: 10 µL GoTaq® G2 Green Master Mix 10X, 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer, a variable amount of DNA (up to 250 ng), and a variable amount of nuclease-free water. PCR conditions used to target the 16S rRNA gene was as follows: 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 (Khaleel *et al*., b2023). PCR products were run on a 1% agarose gel, 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).

RESULTS AND DISCUSSION

Chromatogenic media are culture media that are commercially produced to make quick and easy identification of bacteria possible by identifying bacteria based on their colors. For the quick identification of colonization with microorganisms that cause hospital-acquired illnesses, a variety of commercially available chromogenic media are currently in use (Ajao *et al*., 2011). HiCrome Acinetobacter Agar is one of the selective media used, the selective mix it contains suppresses gram-positive organisms, while the chromogenic combination in the media distinguishes *Acinetobacter* species from other organisms (Higham *et al*., 2023).

One hundred and seventy specimens were collected from Mosul Hospital/ Iraq. The specimens included 34.1% burns, 29.4% urine, 22.3% wounds, 11.7% sputum, and 4.1% CSF samples. Out of 170 samples, 90 samples produced growth on HiCrome Acinetobacter Agar, all speciemns types except CSF produced growth probably due to the low number of CSF samples (7 samples) studied. There are two reasons to interpret the presence of *A. baumannii* infections in burns and wounds more than other clinical sources, burns and wounds are more susceptible to infection in hospital setting, which contributes to the resistant strain spread. In addition, most antibiotics are unable to deeply enter burn and wound sites making such samples more predominant (Sehree *et al*., 2021). *A. baumannii* on HiCrome Acinetobacter Agar should appear as yellow to light purple colonies while inhibiting all other types of bacteria. However, the current study showed that colonies appeared round and mucous and differed in their color. Most of the colonies appeared purple (35 light purple and 51 slightly darker purple) indicating that 95.5% of the isolates belonged

to *A. baumannii*. The remaining four isolates were 3 white and 1 yellow. The differentiation in colony color is more likely due to the production of different enzymes from different bacterial genera that break the chromogenic substrate present in this medium and thereby producing a variation in color (Panagea *et al*., 2011; Faisal and Rasol, 2022). The difference in colony color was not mentioned in the instructions provided by Himedia for HiCrome Acinetobacter agar. Therefore, further identification should be subjected to identify the isolates obtained.

Microscopic examination of isolates showed that they were all Gram-negative, however, microscopic examination identified the white colonies as *Candida spp.* Growth of the white colonies on HiCrome Acinetobacter agar is believed to be due to the ineffective bacterial antibiotics in the media towards *Candida spp*. in addition to lacking other antifungals.

The current results require more clarification as new phenotypes for colonies were noticed on this medium that were not mentioned previously by the company. Therefore, all colonies were subjected to oxidase test and checked for their ability to ferment lactose on MacConkey agar, knowing that *A. baumannii* is negative towards oxidase and does not ferment lactose (Constantiniu *et al*., 2004). Results showed that the 35 light purple Fig. (1B) were negative towards oxidase, while the 51 slightly darker purple colonies Fig. (2E and F), were positive towards oxidase. On the other hand, the light-yellow colony was also oxidase negative. Using oxidase reduced the isolation rate for *A. baumannii* from 95.5% to 38.9%. This a huge drawback for HiCrome Acinetobacter agar when used alone to identify *A. baumannii*.

To differentiate *A. baumannii* from lactose fermenters belonging to enterobacteriaceae, all isolated bacteria were grown on MacConkey agar. MacConkey agar is not selective for *Acinetobacter* and does not distinguish *Acinetobacter* from other gram-negative bacteria that do not ferment lactose, however, it can be a dependable method for differentiating *Acinetobacter* species from other lactose fermenters that can probably grow on HiCrome Acinetobacter agar (Ajao *et al*., 2011). Results showed that the single light-yellow isolate was non-lactose fermenter and all 51 slightly purple colonies were non-lactose fermenters. In addition, all 51 slightly purple colonies were pyocyanin producers suggesting that they belong to *Pseudomonas aeruginosa. P. aeruginosa and A. baumannii* commonly cause hospital acquired infections are linked together in several infections due to their resistance to antibiotics and detergents contributing to increased hospital morbidity and mortality (Djordjevic *et al*., 2013). As a result, it is reasonable to select *P. aeruginosa* on HiCrome Acinetobacter agar as it resists most of the antibiotics added in the medium (ampicillin and ceftazidine) for selection of *A. baumannii*. Interestingly, the 35 light purple oxidase negative isolates also differed in their ability to ferment lactose. Our results show that 23/35 (66%) were lactose fermenters. Excluding these isolates from our previous isolation percentage decreased the isolation rate to 14.4% when MacConkey agar is used as a second layer for identification.

To further identify the isolates according to the color of colony, 16S rRNA from selected isolates were amplified and sequenced Fig. (3). Results showed that 11 light purple (oxidase negative, non-lactose fermenters) and the single light-yellow colony (oxidase negative, non-lactose fermenter) were identified as *A. baumannii*, while one colony (oxidase negative and non-lactose fermenter) was identified as *Morganella morganii* (PP727500) Fig. (2C). This reduces the isolation rate to 13.3% and shows that only 16S rRNA gene sequencing may provide accurate identification for *A. baumannii*. These strains were submitted to NCBI under the accession numbers shown in (Table 1). However, the 23 light purple colonies detected on HiCrome Acinetobacter agar were found to be non-*Acinetobacter* isolates. These colonies were also oxidase negative but fermented lactose. 16S rRNA sequencing for some representatives followed by BLAST revealed that these colonies belonged to enterobacteriaceae species including *Escherichia coli* (OR884234), *Enterobacter spp*. (PP727504), and *Klebsiella pneumoniae* (OR884233). Morphology of these colonies are shown in Fig. (2 A, B, C, and D respectively). Ciftci *et al.* (2015) showed that when using HiCrome Acinetobacter agar along with SBA supplement, three other species were detected particularly *P. aeruginosa, K. pneumoniae*, and *C. albicans*, however, their work did not show growth of *Enterobacter spp*. and *Morganella morganii*.

Furthermore, diagnosis of the slightly dark purple colonies by 16S rRNA sequencing showed that such isolates belonged to *P. aeruginosa* (accession numbers OR884236- OR884238 and PP727497) which had a higher isolation rate (56.7%) compared to *A. baumannii*. This suggests that HiCrome Acinetobacter agar could be used as a selective and differential media for isolation of *P. aeruginosa* from clinical specimens. Our results also suggests that *P. aeruginosa* is resistant to antibiotics used in this medium and is more common in Mosul hospitals than *Acinetobacter*. The growth of *P. aeruginosa* on this medium comes in agreement with a recent paper by Wiranto *et al*. (2024) whom detected this pathogen along with *A. baumannii* on HiCrome Acinetobacter agar.

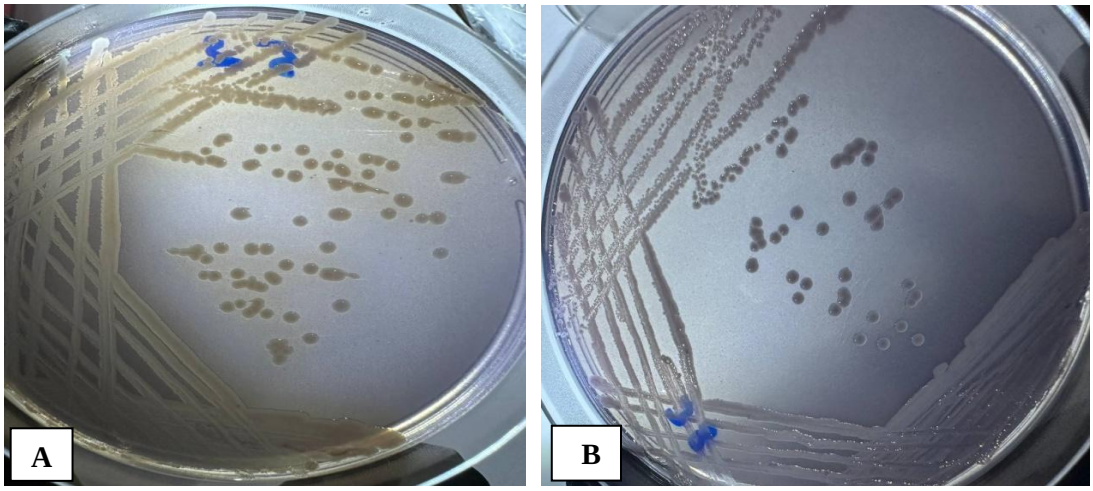

Fig. 1: Colony appearance of *Acinetobacter baumannii* on HiCrome Acinetobacter agar. A (light yellow colonies), B (Light purple colonies).

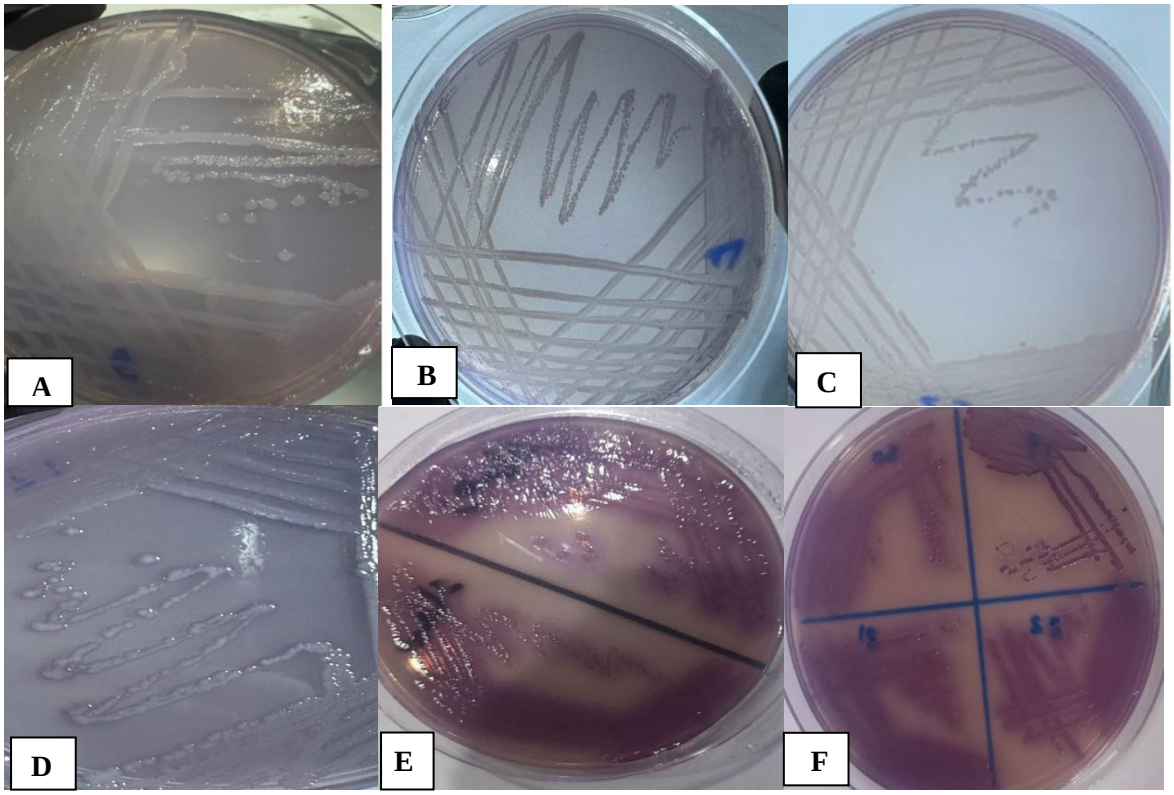

Fig. 2: Colony appearance on HiCrome Acinetobacter Agar for A. (E. *coli*), B. (*Enterobacter spp*.), C (*Morganella morganii*), D (*Klebsiella pneumoniae*), E and F (*Pseudomonas aeruginosa*).

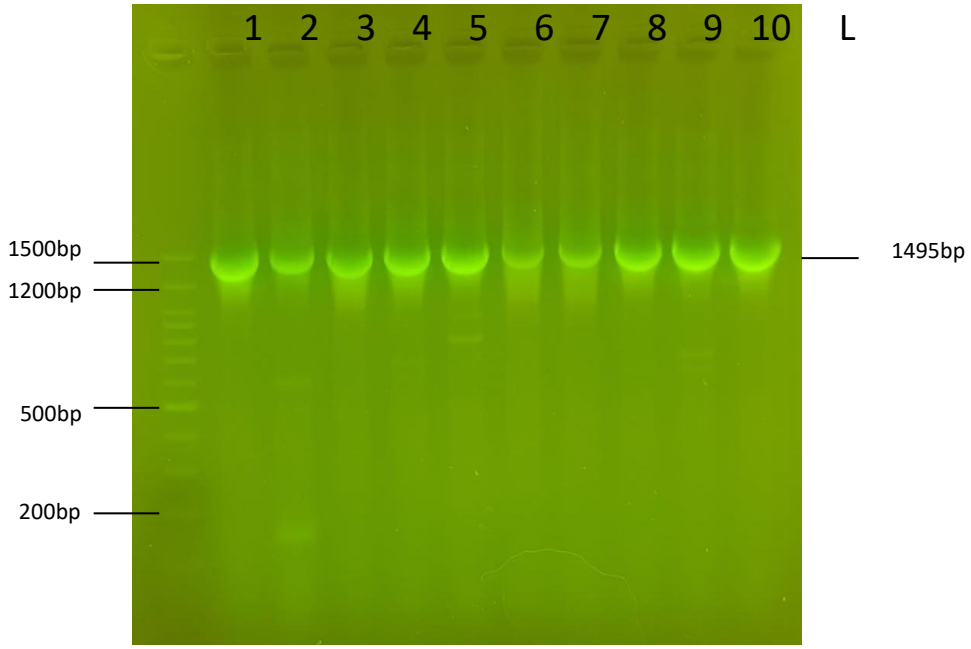

Fig. 3: Amplicons of 16S rRNA gene for a selection of isolates in this study. L: 1 Kb DNA ladder, 1-10: a selection of bacterial strains understudy.

The use of HiCrome Acinetobacter agar is an advantage for the selection of *A. baumanii* from different clinical specimens. Higher yield of isolation is noticed when using this medium compared to using traditional media. Sehree *et al*. (2021) showed that using traditional culture media and VITEK-2system was effective in detecting *A. baumanii* at lower percentages (14%) compared to another study conducted by Al-Najim *et al.* (2023) whom used HiCrome Acinetobacter agar and obtained more than 80% isolation rates, which is more likely related to the misuse of the medium in identification. To obtain more accurate results, this work suggests the possible use of HiCrome Acinetobacter agar for the isolation of *A. baumanii* only when used along with the oxidase test and growth on MacConkey agar.

Table 1: Identity and accession numbers of strains submitted to NCBI in this study.

Isolate number	Isolate name	Accession Number	Identity (%)
1	*Acinetobacter baumanni* NR1	OR884229	99.85
2	*Acinetobacter baumanni* NR2	OR884230	99.33
3	*Acinetobacter baumanni* NR3	OR884231	99.13
4	*Acinetobacter baumanni* NR4	OR884232	98.55
5	*Klebsiella pneumonia* NR5	OR884233	97.08
6	*Esherichia coli* NR6	OR884234	94.00
7	*Enterobacter.spp* NR7	OR884235	97.15
8	*Pseudomonas aeruginosa* NR8	OR884236	99.35
9	*Pseudomonas aeruginosa* NR9	OR884237	98.46
10	*Pseudomonas aeruginosa* NR10	OR884238	95.38
11	*Acinetobacter baumanni* NR11	PP727496	99.88
12	*Pseudomonas aeruginosa* NR12	PP727497	99.41
13	*Acinetobacter baumanni* NR13	PP727498	99.78
14	*Acinetobacter baumanni* NR14	PP727499	99.78
15	*Morganella morganii* NR15	PP727500	100
16	*Acinetobacter baumanni* NR16	PP727501	100
17	*Acinetobacter baumanni* NR17	PP727502	100
18	*Acinetobacter baumanni* NR18	PP727503	100
19	*Enterobacter hormaechei* NR19	PP727504	100
20	*Acinetobacter baumanni* NR20	PP727505	98.14
21	*Acinetobacter baumanni* NR21	PP727506	99.77

CONCLUSIONS

HiCrome Acinetobacter agar is a selective medium used to select *Acinetobacter spp*. from a variety of clinical sources depending on chromogenic methods. Our results demonstrated that this medium successfully isolated *A. baumanii* but growth was not restricted to this species alone. *Candida spp*. and *P. aeruginosa* grew on this medium and produced colonies with slightly different colors, while *E. coli, Enterobacter sp., Morganella morgani* and *Klebsiella pneumoniae* were able to grow but produce the same color for colonies undifferentiated from *A. baumanii*. Use of HiCrome Acinetobacter agar was simple and rapid, but was not precise. For better results, oxidase and lactose fermentation on MacConkey should be used along with the ability of an isolate to grow on this medium. This work also suggests the use of this medium for isolating *P. aeruginosa* from clinical specimens as it gave higher isolation rate for this bacterium.

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كفاءة وسط HiCrome Acinetobacter agar في تشخيص جرثومة *Acinetobacter baumannii* من عينات سريرية مختلفة

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قسم علوم الحياة/ كلية العلوم/ جامعة الموصل

الملخص

Acinetobacter baumannii هي احدى مسببات الأمراض البكتيرية الانتهازية التي ترتبط عادة بالعدوى المكتسبة في المستشفيات. هذه الجرثومة مسؤولة عن العديد من حالات العدوى المهددة للحياة والتي تحدث في جميع أنحاء العالم ولهذا السبب من الضروري عزل وتحديد *A. baumannii* بسرعة وبدقة. أجريت هذه الدراسة لتحديد كفاءة وسط HiCrome Acinetobacter Agar في التعرف على *A. baumannii* من عينات سريرية مختلفة. في محاولة لعزل أنواع جرثومة Acinetobacter، تم جمع 170 عينة من الجروح والحروق والبلغم وسائل النخاع الشوكي ثم زرعت بطريقة التخطيط على وسط HiCrome Acinetobacter Agar وتم تحديد نسبة عزل *A. baumannii* وفقًا للون المستعمرة (الأصفر إلى الأرجواني الفاتح). أظهرت النتائج أن هذا الوسط لم يكن انتقائياً لأنواع *Acinetobacter* فحسب، بل إن أجناس أخرى غير مرتبطة بجرثومة *Acinetobacter* قد تنمو أيضاً وتنتج ألواناً مغايرة لم تذكرها الشركة المصنعة. وبناء على ذلك، تغيرت نسبة عزل *A. baumannii* من 95.5% إلى 38.9% عند إضافة اختبار الاوكسديز وأخيرا إلى 14.4% عند إضافة اختبار تخمر اللاكتوز على وسط اكار الماكونكي والى 13.3% عند اعتماد التشخيص باستخدام تقنية تحديد تسلسل الجين 16 .S rRNA أظهرت النتائج أن 35/24 مستعمرة أرجوانية تنتمي إلى افراد العائلة المعوية و90/51 مستعمرة تم تحديدها على أنها *Pseudomonas aeruginosa*. يشير معدل العزل العالي الذي تم الحصول عليه لـ *P. aeruginosa* إلى أنه يمكن استخدام وسط HiCrome Acinetobacter كوسط انتقائي وتفريقي لعزل *P. aeruginosa* من العينات السريرية.

الكلمات الدالة: *Acinetobacter baumannii*، 16S rRNA، وسط HiCrome Acinetobacter agar.