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Evaluation of CHROMagar Acinetobacter and MacConkey media for the recovery of *Acinetobacter baumannii* from soil samples

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Abstract

The opportunistic pathogen *Acinetobacter baumannii* occupies niches in human and veterinary clinics and other environments. This comparative study was designed to assess the recovery rate of *Ac. baumannii* from 20 soil samples collected in Germany (from which *Ac. baumannii* had been previously isolated) on selective CHROMagar Acinetobacter (CMA) and MacConkey Agar (MCA). Suspected *Ac. baumannii* were confirmed by detection and sequencing of $bla_{\text{OXA-51-like}}$ genes. Overall, the recovery rate of *Ac. baumannii* from both media was similar. Out of the 20 soil samples enriched in mineral salt medium (MM) plus 0.2% acetate for 5 h, *Ac. baumannii* was recovered from 12 (60%) on CMA and 11 (55%) on MCA, and from 14 (70%) with both selective media after enrichment for 24 h. Typical and distinct colonies of *Ac. baumannii* were more often observed on CMA with soil enriched in MM for 5 h, while on MCA distinct colonies were more obvious after 24 h. In five soil samples (25%), strains harbouring different OXA-51-like variants were recovered on MCA. Late lactose fermentation (48 h) was observed on MCA. The study suggests that no single growth medium would efficiently recover *Ac. baumannii* from all soil samples.

Significance and impact of the study:

The study established that MacConkey and CHROMagar Acinetobacter culture media did not differ in the rate of recovering *Ac. baumannii* from soil samples. In addition to that, using the two media provides a greater discriminatory power to recover more variants of *Ac. baumannii* from soil samples. The study also indicated that enriching the soil samples in mineral salt medium supplemented with 0.2% acetate for up to 24 h could improve the chance of isolation from soil samples with low content of *Ac. baumannii*.

Keywords: *Acinetobacter baumannii*, soil, MacConkey agar, CHROMagar Acinetobacter, culture media

Introduction

The World Health Organization has ranked carbapenem-resistant *Acinetobacter baumannii* as a pathogen of critical priority to public health worldwide (WHO 2017). This is because they cause a wide range of opportunistic infections, which range from septicaemia to pneumonia in humans, especially in hospitalized and immune-compromised patients, and resist treatments with multiple antibiotics (Zilberberg et al. 2016).

Acinetobacter baumannii are strictly aerobic, Gram-negative, oxidase-negative, and nonflagellated bacteria that are microscopically short rods or cocco–bacilli in shape. They are highly ubiquitous and successful bacteria that have occupied an extremely diverse range of niches in different ecosystems, such as hospital environments (Ababneh et al.), aquatic environments (Benoit et al. 2020), municipal wastewater treatment plants (Hrenovic et al. 2016), fruits (Ababneh et al. 2022b), vegetables (Karumathil et al. 2016), and different type of soil (Jiang et al. 2018, Dekic et al. 2020).

In addition, *Ac. baumannii* has also been isolated from different animal sources, including birds, cattle, pigs, and chickens, and it is among the species implicated in animal diseases (Müller et al. 2014, Ewers et al. 2017). Their high adaptability to adverse conditions in soil (such as the ability to persist for months in dry and moist soil) and ability to quickly acquire multiple antibiotic-resistant genes from soil and from soil-dwelling bacteria, can facilitate their spread into other environments, including hospitals (Gallego 2016, De Silva and Kumar 2019, Sharma et al. 2021). Due to soil complexity, *Ac. baumannii* has been reported to coexist with closely related *Acinetobacter* species such as *Ac. bohemicus* (Krizova et al. 2014), *Ac. kookii* (Choi et al. 2013), *Ac. soli* (Kim et al. 2008), *Ac. pittii*, *Ac. nosocomialis*, *Ac. albensis* (Krizova et al. 2015), and distantly related *Pseudomonas aeruginosa*, *Klebsiella* spp. (Pramila et al. 2012), which makes their isolation more difficult based on morphology and antimicrobial resistance, as evidence of interspecies interactions between them has been reported to facilitate the exchange of resistance and virulence genes. For instance, a number of *Ac. baumannii* from soil samples in Brazil have been reported to harbour several ß-lactamase encoding genes with new sequence types (ST) similar to the multidrug phenotype that has been more commonly reported for clinical isolates of *Ac. baumannii* (Furlan et al. 2018).

Therefore, effective culture-based methods and techniques that will facilitate recovery and identification of *Ac. baumannii* from complex soil environment is critical, especially when carrying out researches that aim to survey and quantify levels

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Table 1. Characteristics of soil samples and *Ac. baumannii* isolated using CMA and MCA.

S/N	Sample ID	Collection date (month-day-year)	Collection site (GPS coordinates)	pH value*	OXA-51-like variant originally identified (CMA)	Novel OXA-51-like variants (MCA)
1	U20-Hope-S25	08-03-2020	51.863328, 10.831263	6.0	408	106
2	U20-ZW-S17.7	05-23-2020	51.827424, 10.788081	6.5	65	
3	U20-ZW-S26	09-18-2020	51.827424, 10.788081	6.5	180	
4	U20-Hope-S26	08-27-2020	51.863283, 10.831391	6.5	Truncated (GenBank: OL944015)	
5	U20-Hope-S30	08-27-2020	51.863328, 10.831263	7.0	314	69, 761
6	U21-Hope-S7	06-07-2021	51.863332, 10.831215	7.0	773	
7	U21-Hope-S5	06-07-2021	51.863328, 10.831263	6.5	65	
8	U20-Hope-S31	10-22-2020	51.863328, 10.831263	6.5	510	
9	U20-Hope-S33	10-22-2020	51.863283, 10.831391	6.5	91	
10	U21-Benzi-S4	05-17-2021	51.837082, 10.870252	6.5	51	
11	U21-Benz-S1	04-19-2021	51.837082, 10.870252	6.5	1093 (GenBank: OL944014)	
12	U21-Hope-S8	06-28-2021	51.863283, 10.831391	6.0	208	
13	U21-Hope-S13	07-15-2021	51.863283, 10.831391	6.5	208	
14	U21-Hope-S12	06-28-2021	51.863328, 10.831263	6.5	340	208
15	U21-Hebata-S17	07-15-2021	51.827404, 10.887331	6.5	314	
16	U21-Zibata-S4	07-14-2021	51.807115, 10.806543	6.5	104	
17	U21-ZW-S8.1	07-14-2021	51.827424, 10.788081	7.5	314	126
18	U21-ZWS-8.4	07-14-2021	51.827424, 10.788081	7.5	314	90,1092 (GenBank: OL944013)
19	U21-Hebata-S11	06-22-2021	51.826746, 10.886730	6.5	69	
20	U21-Schma-S4	06-07-2021	51.890750, 10.763045	6.5	51	

* pH value determined with Macherey–Nagel pH-Fix pH strips (pH 2–9) after resuspension of 1 g of soil sample into 5 ml double-distilled water.

Table 2. Growth index (0–4) of *Ac. baumannii* isolated on MCA and CMA.

S/N	Sample ID	Distilled water		MM incubation period (h)			
				5		24	
		CMA	MCA	CMA	MCA	CMA	MCA
1	U20-Hope-S25	0	1	1	2	2	3
2	U20-ZW-S17.7	0	1	0	1	0	1
3	U20-ZW-S26	1	0	1	1	4	4
4	U20-Hope-S26	1	0	1	1	3	3
5	U20-Hope-S30	0	1	1	1	3	4
6	U21-Hope-S7	1	0	1	0	3	0
7	U21-Hope-S5	0	0	0	0	1	2
8	U20-Hope-S31	0	0	0	0	0	0
9	U20-Hope-S33	0	1	1	1	4	3
10	U21-Benzi-S4	0	0	0	0	4	3
11	U21-Benzi-S1	0	0	0	2	2	3
12	U21-Hope-S8	0	0	2	0	4	3
13	U21-Hope-S13	0	0	1	1	2	3
14	U21-Hope-S12	0	0	2	1	3	3
15	U21-Hebata-S17	0	0	0	0	0	0
16	U21-Zebata-S4	0	0	1	0	0	0
17	U21-ZWS-8.1	0	0	1	1	2	2
18	U21-ZWS-8.4	0	1	1	1	4	3
19	U20-Hebata-S11	0	0	0	0	0	0
20	U21-Schma-S4	0	0	0	0	0	0

0 means no typical colony of *Ac. baumannii* observed, 1 means 1–10 colonies, 2 mean 11–50 colonies, 3 signifies semi confluent colonies (51–100 colonies), and 4 confluent colonies (too numerous to count).

of their contamination in the environment. Such methods may also be adaptable to the clinical context, thereby improving sensitivity of surveillance and diagnostics, and thus reducing the risk of *Ac. baumannii* infection outbreaks in hospitals and communities.

In an effort to achieve this, selective media have been developed to assist in quick isolation of multidrug-resistant (MDR) and non-MDR *Acinetobacter* species from different clinical and environmental samples such as blood, water, sewage, and soil. Currently, CHROMagar Acinetobacter (CMA) is considered a highly selective growth medium for the isolation of *Acinetobacter* species and frequently used in clinical laboratories (Gordon and Wareham 2009, Ajao et al. 2011). Some studies have compared the recovery rate of CMA and other

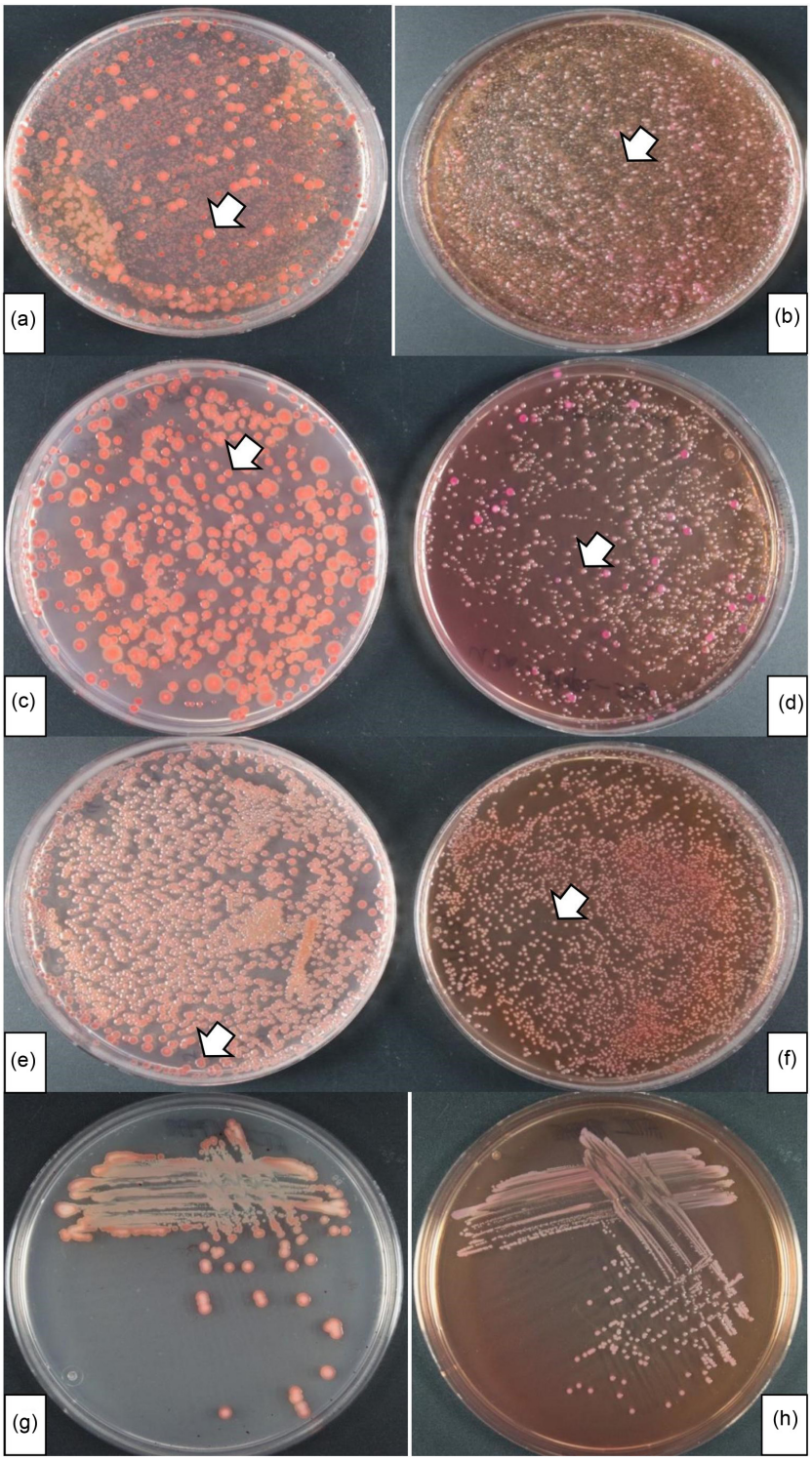

Figure 1. Colonial appearances of *Ac. baumannii* and other bacteria on CMA and MCA for soils enriched in MM + 0.2% acetate for 5 h. (a), (c), and (e) show growth on CMA while (b), (d), and (f) show growth on MCA. Depending on the soil type and the amount of *Ac. baumannii* in the soil, the number of typical colonies varies. In (a) and (b), the growth indexes are different. In (a), it is 1, with only a few typical colonies of *Ac. baumannii* while in (b), it is 2. In (c), more typical colonies of *Ac. baumannii* are prominent, but in (d), typical colonies are hardly distinguishable from other colonies. (e) and (f) show semiconfluent growth of colonies, with few colonies of *Ac. baumannii* embedded in colonies of other bacteria. Arrows exemplify typical *Ac. baumannii* appearance. (g) and (h) shows typical appearance of reference *Ac. baumannii* strain ATCC 17978 on CMA and MCA.

selective and nonselective media in isolation of *Ac. baumannii* from different samples. For instance, Ajao et al. (2011) compared CMA and MacConkey agar (MCA) efficiency in isolating *Ac. baumannii* from clinical samples and revealed that MCA was able to identify 89% of *Ac. baumannii* against 100% recorded for CMA. Benoit et al. (2020) compared selective CMA and nonselective Modified Karmali Agar (MKA) in the isolation of *Ac. baumannii* from different water sam-

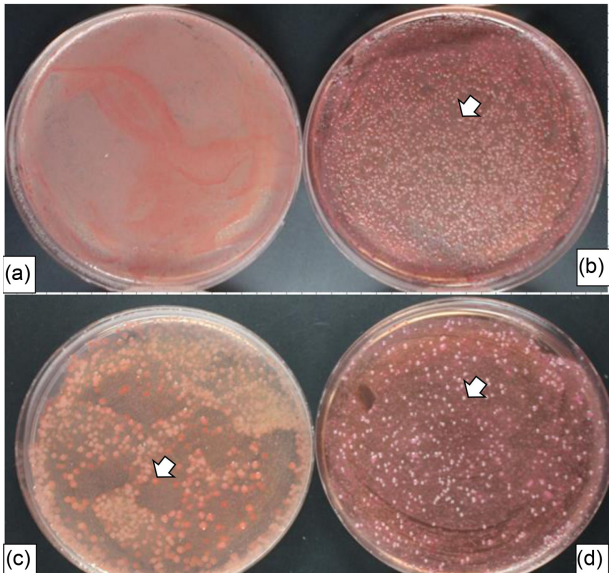

Figure 2. Different colonial appearances of *Ac. baumannii* and other bacteria on CMA and MCA after enrichment of different soil samples in MM + 0.2% acetate for 24 h. Plates (a) and (c) are CMA, while (b) and (d) are MCA. Growth index in (a) and (b) is 4. In (a), a more confluent growth with no typical identifiable colonies of *Ac. baumannii* while in (b), the confluent growth with less identifiable *Ac. baumannii* colonies. In (c) and (d), more typical colonies of *Ac. baumannii* are more prominent and distinguishable from other bacteria (growth index 3).

ples and concluded that *Ac. baumannii* is highly adaptable to grow on selective and nonselective growth media at different temperature and incubation conditions and that no single growth media would efficiently recover *Ac. baumannii* from all environmental water types. So far, no study has compared the recovery rate of CMA with other media in the isolation of *Ac. baumannii* from soil samples, and in determining if single media could isolate all variants of *Ac. baumannii*. This study aimed to compare the recovery rate of *Ac. baumannii* from soil samples using selective CMA and MCA growth media.

Materials and methods

Soil sample collection and culture media

In this study, two types of culture media were used, standard MCA and CMA without MDR supplements. All plates were prepared according to the manufacturer's instructions and were stored at 4°C for up to 1 week until used. Quality control was ensured by growing on the media 10 characterized strains of *Ac. baumannii* previously isolated from soil samples.

For comparing the sensitivity and specificity of CMA and MCA in recovering *Ac. baumannii* from soil samples, 5 g of previously collected soil samples from different locations in Germany were used for the study. From each of the 20 soil samples, *Ac. baumannii* had been previously isolated using CMA (Wilharm et al. 2017). The original isolation protocol consisted of an enrichment step for 5 h at 37°C in MM +0.2% acetate as detailed below, followed by spreading on CMA and incubation for 18 h at 37°C. The pH of all soil samples was in the range between 6.0 and 7.5. The sample site and date of collection as well as $bla_{\text{OXA-51-like}}$ variant of each *Ac. baumannii* isolated from the soils are given in Table 1.

Preparation of samples and isolation of *Ac. baumannii*

To achieve recovery of *Ac. baumannii*, 5 g of each soil sample was transferred into two separate sterile 50 ml bottles, and 5 ml of (i) mineral salt medium (MM) supplemented with 0.2% acetate and (ii) distilled water was added to each bottle. The composition of the MM per liter of water is 10 g KH_2PO_4, 5 g Na_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.001 g $CaCl_2$ $(2H_2O)$, 0.001 g $FeSO_4.7H_2O$, and 0.2% sodium acetate (pH 7.0) (modified after Baumann 1968 and Nemec et al. 2009). The soil suspension in MM +0.2% acetate was vigorously vortexed and incubated in a rotary shaker for 5 h and up to 24 h at 37°C. Similarly, 5 g of soil resuspended in distilled water was also vortexed and incubated for 5 h at 37°C. After incubation, 100 μl of the soil suspensions were simultaneously inoculated onto CMA and MCA by means of a sterile glass spreader and were incubated at 37°C for 18–24 h.

After incubation, growth of typical *Ac. baumannii* on both plates was examined based on colonial appearance and was assessed semi quantitatively based on 4 scale growth indexes (i.e. 0–4) previously adopted by Moran-Gilad et al. (2014), where 0 means no typical colony of *Ac. baumannii* observed, 1 means 1–10 colonies, 2 represents 11–50 colonies, 3 signifies semi confluent colonies (51–100 colonies), and 4 confluent colonies (too numerous to count). For easy identification of typical *Ac. baumannii* morphology and counting, the plates were viewed under a magnifying lens, and an average number of colonies for the duplicate plates was recorded. A typical colony of *Ac. baumannii* on CMA was based on the manufacturer's instruction while on MCA, it was based on observation of nonlactose fermenting colonies with raised, smooth edge appearance of varying size. For each morphotype suspected to pertain to *Ac. baumannii*, a representative colony was picked and subcultured onto Mueller Hinton blood agar (MHBA), incubated at 37°C for 18–24 h. After incubation, colonies were examined and used for further confirmation by applying molecular techniques.

Genotypic confirmation of *Ac. baumannii*

The DNA of the suspected *Ac. baumannii* isolated from both culture media was extracted by a boiling method (heating at 95°C for 10 min), after emulsifying a slight portion of the pure bacterial colony in 40 μl of sterile distilled water. Amplification of $bla_{\text{OXA-51-like}}$ genes was carried out as previously reported (Turton et al. 2006). For each $bla_{\text{OXA-51-like}}$ positive strain, the full length sequences of $bla_{\text{OXA-51-like}}$ genes were determined after amplification with primers OXA-69A (5′-CTAATAATTGATCTACTCAAG-3′) and OXA-69B (5′-CCAGTGGATGGATGGATAGATTATC-3′) (Héritier et al. 2005). Newly identified variants were registered and deposited in the NCBI database.

Results and discussion

Research laboratories continue to face challenges of an appropriate and affordable method of recovering *Ac. baumannii* from complex soil environments, due to their varying population density in different soil and their ability to adapt to different environmental conditions. Considering the need to explore many soil ecologies for *Ac. baumannii* colonization and other surveillance sites, selecting an appropriate medium that has selective and differential properties that will enable easy

isolation of soil-dwelling *Ac. baumannii* is very crucial. This study was, therefore, designed to evaluate two common growth media and time of soil enrichment in MM + 0.2% acetate for their ability to support the easy recovery of *Ac. baumannii* from various soil sources for the purpose of surveying and monitoring *Ac. baumannii*.

Two agar-based culture media (CMA and MCA) were selected for the study because they are widely used in the isolation of *Acinetobacter* species from different samples. CMA, in particular, has been recommended for the isolation of *Acinetobacter* spp. from clinical samples and has been tested by various comparative studies along with other media to isolate specifically carbapenem-resistant *Acinetobacter* species from clinical and to some extent environmental samples (Ajao et al. 2011, Moran-Gilad et al. 2014). However, results from such studies have not indicated a total superiority of the media over other selective or differential media, specifically, in terms of differentiating *Ac. baumannii* from other *Acinetobacter* species based on colonial appearance. Similarly, MacConkey and blood agar have also been used to isolate *Acinetobacter* species from clinical and other samples (Tjoa et al. 2013). In both cases, other types of Gram-negative bacteria also grew on the media, making the media not exclusive for isolation of *Ac. baumannii*. However, for studies designed to isolate *Ac. baumannii* with natural resistance level from different soil environments in temperate climates, such comparative analysis has not been done.

In this study, isolation and characterization of different variants of *Ac. baumannii* from different soil samples collected from different locations in Germany between 2020 and 2021 was compared on two differential media (CMA and MCA) after preincubation in distilled water, and MM + 0.2% acetate for 5 and 24 h. The characteristic growth index of *Ac. baumannii* from soil samples on both culture media was shown in Table 2.

In both media, a mixture of *Ac. baumannii* and other Gram-negative bacteria (including other *Acinetobacter* species) grew on the media after 18–24 h at 37°C. Typical colonies (bright red, smooth, and convex colonies) of *Ac. baumannii* from soils enriched in MM + 0.2% acetate for 5 h fall within 0–2 growth index, while those enriched in MM + 0.2% acetate for 24 h produced confluent colonies of *Ac. baumannii* on CMA and typical colonies between 2–3 growth index and confluent growth on MCA (Fig. 1).

The results indicated a comparable rate of recovery of *Ac. baumannii* by the two culture media when soil samples were enriched in MM + 0.2% acetate for 5 and 24 h. Enrichments in MM + 0.2% acetate for 5 h resulted in fewer but easily identifiable (typical) colonies of *Ac. baumannii* on CMA compared to MCA, while samples enriched for 24 h in MM + 0.2% acetate resulted in the yield of confluent colonies on CMA and more typical colonies on MCA. A comparative study of MCA, CMA, and other mineral salt-based agars for *Ac. baumannii* isolation from clinical and soil samples has utilized a similar approach and a comparable result was obtained (Baumann 1968, Moran-Gilad et al. 2014).

MCA being nonselective media for *Ac. baumannii* allowed mixed growth of various aerobic and lactose-fermenting bacteria in the soil, with few colonies of *Ac. baumannii* that are hardly distinguished from the mixed culture. However, with further enrichment up to 24 h, *Ac. baumannii* became more prominent with a lower amount of lactose-fermenting bacteria, which indicates that increased duration of enrichment favours the subsequent suppression of other Gram-negative bacteria on MCA.

Overall, out of the 20 samples, *Ac. baumannii* were isolated from 12 (60%) and 11 (55%) plates of CMA and MCA, respectively. Typical *Ac. baumannii* colonies are easily identifiable from other *Acinetobacter* species and Gram-negative bacteria, with soil samples enriched in MM + 0.2% acetate for 5 h on CMA. However, on MCA, typical colonies of *Ac. baumannii* were more prominent and easily distinguishable from other bacteria when the soil samples are enriched in MM + 0.2% acetate for 24 h (Fig. 2). Samples U20-ZW-S26, U20-Hope-S25, U21-Hope-S12, and U21-ZWS-8.4 enriched in MM + 0.2% acetate for 5 h produced a high growth index on both media while the lower indexes (0–1) were only observed in other samples. The isolates recovered from both media produced comparable morphology on MHBA, with characteristic creamy, opaque, and nonhemolytic colonies. Except in a few cases, *Ac. baumannii* on MCA appears as a nonlactose fermenter, with raised creamy and smooth-edged colonies, which are distinguishable from other colonies of bacteria (mostly tiny and flat) and lactose fermenters.

In samples U20-ZW-S17.7 and U21-Hope-S7, *Ac. baumannii* was only recovered on either MCA or CMA but not the other medium. The recovery of *Ac. baumannii* was poor (7 out of 20) from soil samples suspended in distilled water alone. Interestingly, *Ac. baumannii* was not recovered from four samples (U21-Hebata-S17, U20-Hebata-S11, U21-Schma-S4, and U20-Hope-S31) by any of the two culture media, which indicates comparable sensitivity.

Pure colonies of *Ac. baumannii* recovered from both media and grown on fresh MCA show a nonlactose fermenting colony after 18–24 h at 37°C, which later turned to lactose fermenters after extended incubation at temperatures between 4 and 37°C (Fig. 3). Additional OXA variants of *Ac. baumannii* were recovered from five (25%) soil samples grown on MCA in addition to the already identified variants on CMA.

It was also observed that the recovery rate of *Ac. baumannii* varies from one soil to another. In some soil samples, high densities of *Ac. baumannii* with typical colonies of *Ac. baumannii* that are easily distinguishable from other bacteria were observed on both media after 5 h of incubation in MM at 37°C. However, less typical colonies that are often mixed with other colonies, especially the nonlactose fermenters are observed on MCA inoculated with soil samples incubated for 5 h in MM. But after 24 h preculture in MM + 0.2% acetate, lesser growth of lactose fermenting bacteria and more typical colonies of *Ac. baumannii* were observed.

The soil samples used for the study are previously positive soil samples of our collection and have been originally confirmed to contain *Ac. baumannii* using CMA. This is to serve as quality control that viable and culturable *Ac. baumannii* are present in the samples. The absence of *Ac. baumannii* in four soil samples with all the media and enrichment time indicates their equal sensitivity in recovering *Ac. baumannii* from soil samples when subjected to the same treatment. This may be due to a very low concentration of *Ac. baumannii* in the samples, due to differing survival rates of strains in the soil, or due to the possibility that the available ones are nonculturable or inactivated upon storage at −20°C (Dekic et al. 2018). It was interesting to observe that different OXA-51-like variants of *Ac. baumannii* were detected in MCA in five (25%) samples, in addition to previous variants detected by CMA only, which suggests that using different media will enhance

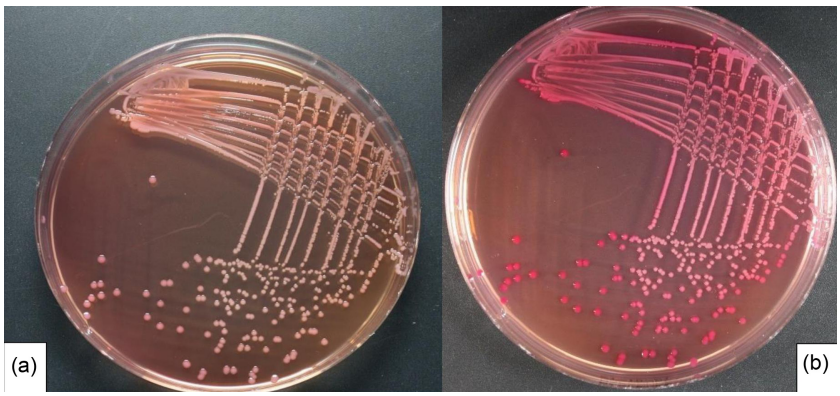

Figure 3. Pure culture of *Ac. baumannii* isolated from U21-Hope-S12. In (a), *Ac. baumannii* is nonlactose fermenting on MCA after 24 h incubation while in (b), the same *Ac. baumannii* turned to red after another 24 h, indicating late lactose fermentation.

recovery of morphologically similar but genetically varied strains.

Notably, we found that even though *Ac. baumannii* are labelled as nonlactose fermenters, some strains tend to become lactose fermenters on MCA after 24 h of storage in the fridge at 5°C. The observed results demonstrate that some *Ac. baumannii* could be late lactose fermenters and that late fermentation will enable them to be differentiated from other lactose fermenters after 48 h.

In conclusion, our data suggest that MacConkey and CMA culture media did not differ in terms of overall recovery of *Ac. baumannii* from soil samples, thereby none can be recommended as the sole medium for isolation and screening of *Ac. baumannii* from soil samples.

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Conflict of interest

No conflict of interest declared.

Author contributions

W.G. and I.Y. conceived and designed the study; I.Y. and E.S. carried out the laboratory investigation; I.Y., W.G., and E.S. interpreted and analysed the data; I.Y. drafted the manuscript; and W.G. critically revised the manuscript.

Data availability

The genomic datasets generated during the study are available in the Gene bank repository, (https//www.ncbi.nlm.nih.gov/genebank/).

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