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EVALUATION OF MODIFIED CONGO RED AGAR FOR DETECTION OF BIOFILM PRODUCING MDR KLEBSIELLA PNEUMONAIE CLINICAL ISOLATES

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Abstract: The notoriety of Multiple Drug Resistant (MDR) K. pneumoniae extends beyond nosocomial or community acquired infections, since it is a major cause of biofilm-related infections. The utilization of a very specific or distinct media, Congo Red Agar (CRA), for detection of biofilm, has significant drawbacks, including inconsistencies in the creation of black pigment. In this study, an assessment of Modified Congo Red Agar (MCRA), for detection of biofilm, was performed on 23 multidrug-resistant (MDR) K. pneumoniae strains obtained from various clinical samples. The aim of this study was to assess the effectiveness and dependability of Modified Congo Red Agar (MCRA) as an alternative medium for studying biofilm production to the non modified Congo Red Agar. Also to examine the prevalence of the fimH gene, which is necessary for biofilm formation among these isolates. Lastly to determine the relationship between antibiotic resistance, the presence of the fimH gene, and the ability to produce biofilm among K. pneumonaie strains. These strains were diagnosed according to their specific bacteriological characteristics. After assessing antibiotic susceptibility, all MDR K. pneumoniae isolates exhibited the presence of the fimH gene using the PCR method. On CRA 20 isolates were strong biofilm producer as they appeared as black colored colonies, two were moderate biofilm producer (pink colored) and only 1 was non biofilm producer (red colored) colony. On MCR the same results for biofilm production to CRA, except for one colony differed as it appeared red (non biofilm producer) on MCRA and pink (moderate biofilm producer) on CRA. However, the growth of 75% blackness pigment of MDR K. pneumoniae strains on the CRA decreased over time. The phenotypic pigmentation on CRA was enhanced by modifying the contents of the agar that led to the persistent development of a highly concentrated black pigment in isolates containing the fimH gene for 2 to 4 days, with no drop in pigmentation seen over time. The change of the agar ingredient enabled the stable synthesis of black pigment and also resulted in a reduction in the cost of agar preparation.

Keywords: Biofilm, Congo Red Agar, fimH gene, Modified Congo Red Agar, MDR K. pneumonaie.

Introduction

The prevalence of MDR K. pneumoniae nowadays in different parts of the world is an advancing issue and Iraq is not excepted. This needs an urgent methods for diagnosis, treatment, and prevention of such infections (1-4). The possibility of forming biofilms in K. pneumoniae is considered to be one of the components that determine strains' capability to overcome the host's immunologic response and antibiotics, especially in isolates with MDR. Among genes that have significant role in enhancing the biological process of biofilm formation, include fimH gene, essentially operating on type 1 fimbrial adhesin (5,6).

Type 1 fimbriae, found in many bacteria of the Enterobacteriaceae family, are one of the intensively researched fimbrial adhesins. The fimbriae are controlled by a gene cluster called fim, which encompasses all the essential genes needed for their structural makeup and formation. The assembly process follows the chaperone-usher pathway, as outlined by Gahlot (7).

The fimbrial structure mostly consists of recurring subunits named fimA, with an adhesin molecule called fimH situated at the apex of the fimbriae. The organization allows the fimbriae to attach to mannose-containing glycoconjugates present on the outer layer of host cells (8). It is important to note that the control of type 1 fimbriae in K. pneumoniae is influenced by phase variation, a regulatory mechanism that is similar to the observed regulation mechanism in Escherichia coli (9).

The MDR K. pneumoniae biofilm can be investigated utilizing different phenotypic techniques. The Congo red agar (CRA) test, devised by Freeman et al. (1989), involves the cultivation of bacterial strains on Brain Heart Infusion Agar (BHIA) that is enriched with sucrose and Congo red dye (10). Researches has shown that this approach has a low level of precision, nevertheless, it is cost-effective and simple to execute. The assessment criteria rely on a visual examination of the coloration of the colonies that develop on the agar (11,12). The accuracy of this method can be enhanced by adding or substituting certain compounds or modifying specific parameters, as shown in other studies (13,14). The objective of this work was to modify the composition of the Congo red agar medium to enhance the precision of detecting biofilm generated by different multidrug-resistant K. pneumoniae strains

Methods

Sample collections: A total of 230 different clinical samples were collected from infected patients attending two major hospitals in Mosul city namely Al-Jumhoree and Al-Salam Teaching Hospitals during the period from February 2023 to December 2023.

Patients: This study includes individuals of all age groups and both genders. Its objective is to document the medical background of each patient, which includes their personal details such as name, age, gender, and the specific type of specimen collected. Furthermore, it was verified that the patients participating in the study were not receiving any therapy, particularly no intake of antibiotics for a period of 3 days before collecting the specimen for culture.

Ethical approval: Ethical approval of this study was obtained from the Iraqi Ministry of Health/Mosul Health Department, with an assigned approval letter Number 9295 at 19th February 2023.

Study design: The present study is a cross-sectional study.

Bacterial strain identification: All specimens were cultured on MacConkey, Eosin Methylene Blue and on Blood agar and Brain Heart Infusion broth (BHI) then incubated over-night at 37 ºC, examined under microscope, and tested for oxidase, catalase and urease production and biochemical reactions for exact strains identification, which had been confirmed by VITEK®2 GN-ID card [(106)].

Test for determining the sensitivity of bacteria to antibiotics: The Kirby-Bauer disc diffusion method, following the guidelines set by the Clinical and Laboratory Standard Institute (CLSI) in 2022 (187), was employed to determine the susceptibility of isolated bacteria to different antibiotics. Antibiotic discs produced by Oxoid in England were utilized. These

antibiotic discs came in the following types and concentrations: 10µg/disc of imipenem, 30µg/disc of augmentin, 10 mg/disc of amikacin, 10µg/disc of tetracycline, 10 µg/disc of cefotrixone, and 10 µg/disc of gentamycin, chloramphenicol 10 µg/disc, cefotaxime 10 µg/disc, ceftazidime 10 µg/disc, and colistin 10 µg/disc.

Phenotypic method detection of biofilm by Congo Red Agar (CRA) and Modified Congo Red Agar (MCR): All isolated colonies which were confirmed to be *K. pneumoniae* were cultured on CRA and MCRA at 37 C^0 for 24 and 48 hour respectively. The main component of these agars are as shown in the following **Table** 1.

Table 1: The composition of both Congo red agar and Modified Congo Red Agar used in this study

Composition/Litter	Congo Red Agar	Modified Congo Red Agar
Congo Red Dye	0.8 g	0.4 g
Sucrose	36 g	----------
Glucose	----------	10 g
BHIA*	52	----------
BAB**	----------	40 g
Water	1000 ml	1000 ml

***BHIA (Brain Heart Infusion Agar), **BAB (Blood Agar Base)**

PCR method for amplification of *fimH*

Genomic DNA from all isolates was extracted using a specific kit (Geneaid kit , Taiwan). Genomic DNA was extracted from bacterial colonies cultured for 18-24 hours on nutrient agar plates. The process of DNA extraction was carried out in accordance with the instructions provided by kit.

The *fimH* gene was detected using conventional PCR. The PCR reaction was conducted in 0.2ml eppendrof tubes. Each 20µl reaction of PCR contained 10µl master mix, 2µl of each (forward, reverse primer, and nuclease free water). Four µl of DNA template to complete the final volume were added. The amplification process was conducted using a standard thermal cycler, as shown in following **Table 2**. The *fimH* primer had been designed by primer1 program: primer design web service for ASA-PCR is accessible through the internet at: http://primer1.soton.ac.uk/primer1.html. Primer's information as shown in **Table 3**.

Table 2: PCR amplification parameters

Process	
Initial denaturation	94 C^0 for 5 minutes
Denaturation	94 C^0 for 2 minutes
Primer Annealing	45 **C^0** for 2 minutes
Primer Extension	72 C^0 for 2 minutes
Final Extension	72 C^0 for 10 minutes
Cycle	30

Table 3: Primers information' including its sequence, amplicon size and its reference

Primer Name	Sequence	Amplicon Size	Reference
fimH	F AAATAATCCCCCTGTTCACC R GGTAAGAGGTGCCGTTATATT	306	This article

Statistical analysis

The statistical analyses were conducted using IBM SPSS statistics software version 25.0 (IBM Corp., Armonk, NY, USA). Data were described as tables, charts and diagrams. Statistical significance was defined as *p*-values less than 0.05. The Pearson correlation coefficient factor (r) was computed for the purpose of conducting correlation analysis. Kappa agreement coefficient was used to assess the deference in accuracy between two methods (CRA and MCRA)

Result and Discussion

Incidence of Klebsiella spp. isolates in various clinical specimens

A total of 230 distinct clinical isolates were obtained from patients in two prominent hospitals in Mosul city. The isolates were collected from individuals ranging in age from 3 months to 75 years, encompassing both genders, during a period of 10 months. In this study, out of the 230 clinical isolates, 33 samples were found to contain Klebsiella spp (10 of them *K. oxytoca* and 23 as *K. pneumonaie*), their overall percentages were as follow: 11 isolates (33.3%) from urine, 10 isolates (30.3%) from sputum, 7 isolates (21.2%) from pus, 2 isolates (6.06%) from tracheostomy, and 1 isolate (3.03%) each from pleural fluid, CSF, and Foley's catheter, as depicted in **Figure 1**.

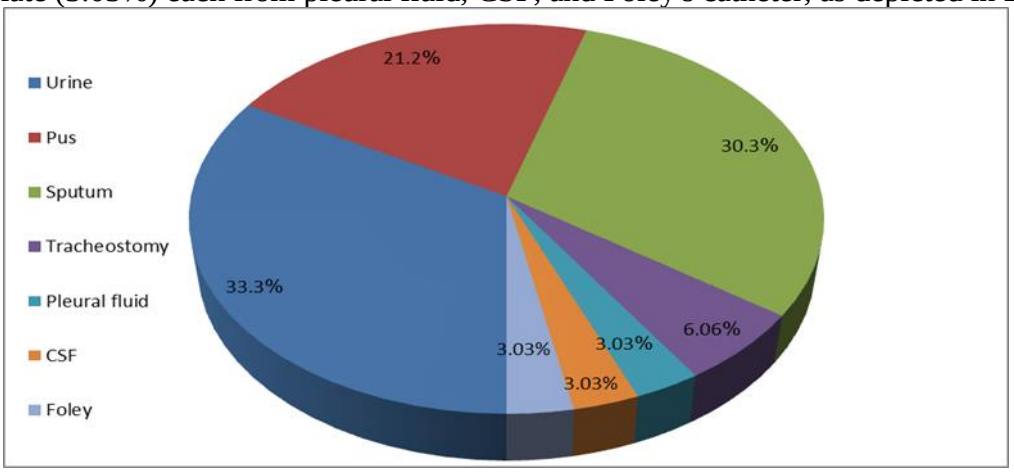

Figure 1: Percentage of Klebsiella Spp isolates from different clinical samples

Distribution of isolates according to gender and age of patients

The current research findings revealed that the frequency of *K. pneumoniae* isolated from various clinical samples was greater in female patients (39.39%) compared to males (18.18%) in relation to gender, regarding urine, pus, and Foleys catheter samples. However, in the case of sputum, tracheostomy tube, pleural fluid and CSF samples, the frequency of the isolates was higher in males (42.42%) than females (6.06%) as shown in following **Table 4**.

Table 4: Male and female distribution among different clinical samples

Sex	Urine	Pus	Sputum	Tracheostomy Tube	Foleys catheter	Pleural Fluid	CSF	Total
Male	4	2	8	2	0	1	1	18
Female	7	5	2	0	1	0	0	15
Total	11	7	10	2	1	1	1	33

Regarding age correlation with *K. pneumoniae* infection revealed that most *K. pneumoniae* isolates were from old aged group (60-80) years, median age group 68 year.

Antibiotic sensitivity test (AST)

All *K. pneumoniae* in this investigation were multidrug resistant isolates, exhibiting the maximum level of resistance to tetracycline, colistin, and ceftriaxone, with a 100% resistance rate. However, all isolates exhibited sensitivity to imipenem. The antibiotic resistance pattern of the isolates was determined by performing disc diffusion using the Kirby-Bauer method on Muller Hinton agar. The diameter of the inhibition zone was measured and compared to the CLSI guidelines for the year 2022. The results are as follows: the bacterium exhibits complete resistance (100%) to tetracycline, colistin, and ceftriaxone. It shows a resistance rate of 78.26% to augmentin, 65.21% to amikacin, 39.13% to cefotaxime, 34.78% to ceftazidime, 13.04% to chloramphenicol and ciprofloxacin, and no resistance (0%) to imipenem.

PCR detection of *fimH*

All *K. pneumoniae* isolates had *fimH* in their genome as appeared in **Figure 2**

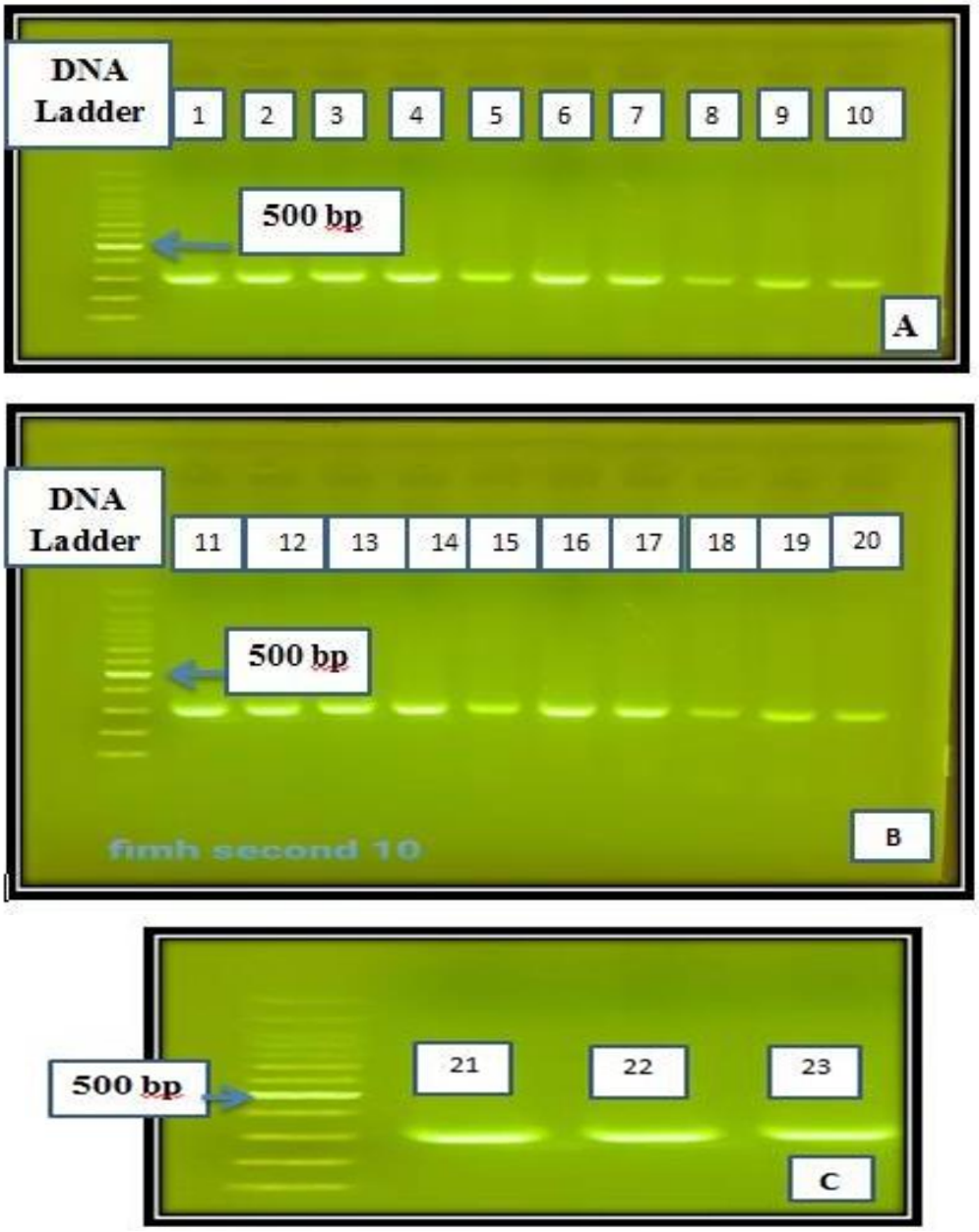

Figure 2 (A, B and C): 2% Agarose gel electrophoresis at 75 volte for 50 minutes had run for PCR products of *fimH* gene in the 23 isolated *K. pneumoniae* its size 306bp

Biofilm production assessment by CRA and MCRA

The results had shown that all isolates are biofilm producer with slime production on Congo Red Agar as they appeared gray to black (75% blackness) in 20 isolates, colored colonies which were strong biofilm producers and 2 were pinkish in color, moderate biofilm producers, and 1 was non biofilm producer appeared as red colored colonies as shown in **Figure 3**.

The degree of strength was decreased second day till 4 days later at room temperature, become white and red colored colonies, and the phenotypic characterization of biofilm generation was conducted using non modified Congo Red Agar, and the presence of the *fimH* gene was examined in the MDR *K. pneumoniae* as seen in **Table 5**.

Table 5: Phenotypic characterization of biofilm generation was conducted using non modified Congo Red Agar

Isolate No.	Biofilm formation on CRA after 24h incubation at 37 C^0	Biofilm formation on CRA after 2 to 4 days at room temperature	*fimH*
1	Strong black	Decreased blackness become white in color	+Ve
2	Strong black	Decreased blackness become white in color	+Ve
3	Strong black	Decreased blackness become white in color	+Ve
4	Moderate pink	Red	+Ve
5	Moderate pink	Red	+Ve
6	Strong black	Decreased blackness become white in color	+Ve
7	Strong black	Decreased blackness become white in color	+Ve
8	Strong black	Decreased blackness become white in color	+Ve
9	Strong black	Decreased blackness become white in color	+Ve
10	Strong black	Decreased blackness become white in color	+Ve
11	Strong black	Decreased blackness become white in color	+Ve
12	Strong black	Decreased blackness become white in color	+Ve
13	Strong black	Decreased blackness become white in color	+Ve
14	Strong black	Decreased blackness become white in color	+Ve
15	Strong black	Decreased blackness become white in color	+Ve
16	Strong black	Decreased blackness become white in color	+Ve
17	Strong black	Decreased blackness become white in color	+Ve
18	Strong black	Decreased blackness become white in color	+Ve
19	Strong black	Decreased blackness become white in color	+Ve
20	Red	Red	+Ve
21	Strong black	Decreased blackness become white in color	+Ve
22	Strong black	Decreased blackness become white in color	+Ve
23	Strong black	Decreased blackness become white in color	+Ve

On MCRA 20 isolates appeared strong blackness colored colonies 85% blackness, after 48 h incubation at 37 C^0 and remain as such blackness for 2-4 days after that at room temperature, 2 were non biofilm producer (red), and 1 appeared pink colored moderate biofilm producer.

There was no significant difference between CRA method and MCRA for detection of biofilm among MDR *K. pneumonaie* as Kappa agreement coefficient is lesser than 1 and *p* value > 0.05. Although the strength and consistency of blackness among strong biofilm producer was much higher by MCRA than CRA and considered less cost effective than CRA method. Phenotypic characteristics of biofilm production on MCRA is shown in **Table 6**.

Table 6: Phenotypic characterization of biofilm generation was conducted using Modified Congo Red Agar

Isolate No.	Biofilm formation on MCRA after 24h incubation at 37 C^0	Biofilm formation on MCRA after 2 to 4 days at room temperature	*fimH*
1	Strong black (85% Blackness)	Remain same blackness strength	+Ve
2	Strong black (85% Blackness)	Remain same blackness strength	+Ve
3	Strong black (85% Blackness)	Remain same blackness strength	+Ve
4	Non biofilm producer Red	Red	+Ve
5	Moderate pink	Remain same color	+Ve
6	Strong black (85% Blackness)	Remain same blackness strength	+Ve
7	Strong black (85% Blackness)	Remain same blackness strength	+Ve
8	Strong black (85% Blackness)	Remain same blackness strength	+Ve
9	Strong black (85% Blackness)	Remain same blackness strength	+Ve
10	Strong black (85% Blackness)	Remain same blackness strength	+Ve
11	Strong black (85 % Blackness)	Remain same blackness strength	+Ve
12	Strong black (85% Blackness)	Remain same blackness strength	+Ve
13	Strong black (85% Blackness)	Remain same blackness strength	+Ve
14	Strong black (85% Blackness)	Remain same blackness strength	+Ve
15	Strong black (85% Blackness)	Remain same blackness strength	+Ve
16	Strong black (85% Blackness)	Remain same blackness strength	+Ve
17	Strong black (85% Blackness)	Remain same blackness strength	+Ve
18	Strong black (85% Blackness)	Remain same blackness strength	+Ve
19	Strong black (85% Blackness)	Remain same blackness strength	+Ve

20	Non biofilm producer Red	Red	+Ve
21	Strong black (85% Blackness)	Remain same blackness strength	+Ve
22	Strong black (85% Blackness)	Remain same blackness strength	+Ve
23	Strong black (85% Blackness)	Remain same blackness strength	+Ve

Correlation of biofilm formation and antibiotic susceptibility test

There was strong +Ve correlation between biofilm production and antibiotic resistance pattern of *K. pneumonaie* as shown in **Table 6**

Isolate number	**Biofilm production (Sstrong, M moderate N Non) biofilm**	**Antibiotic resistance**
1	+ ve (S)	+ ve
2	+ ve (S)	+ ve
3	+ ve (S)	+ ve
4	+ ve (S)	+ ve
5	+ ve (M)	+ ve
6	+ ve (S)	+ ve
7	+ ve (M)	+ ve
8	+ ve (S)	+ ve
9	+ ve (S)	+ ve
10	+ ve (W)	+ ve
11	+ ve (M)	+ ve
12	+ ve (S)	+ ve
13	+ ve (S)	+ ve
14	+ ve (S)	+ ve
15	+ ve (S)	+ ve
16	+ ve (S)	+ ve
17	+ ve (S)	+ ve
18	+ ve (M)	+ ve
19	+ ve (S)	+ ve
20	- ve (N)	+ ve
21	+ ve (S)	+ ve
22	+ ve (S)	+ ve
23	+ ve (S)	+ ve

Pearson correlation factor r factor = 0.6
***p*-Value = 0.0151**
Highly significant

Discussion

Bacterial infections present a significant risk to public health and, due to the growing problem of antibiotic resistance, are a pressing concern in healthcare (15). Bacteria's tendency to survive in biofilm has been noted as a significant factor contributing to persistent human infections and various forms of antibiotic resistance (16-18). Although the medical importance of biofilm identification is well-established, low- and middle-income countries (LMICs), such as Iraq, have inadequate methods for routine microbiological diagnosis of biofilms. Hence, it is crucial to ensure that doctors, microbiologists, and researchers involved in the scientific goal of improving the diagnosis and treatment of biofilm-related infections are well-informed on the present status of biofilm diagnostics.

The largest occurrence of *K. pneumoniae* was found in urine samples, which aligns with several local research. For instance, a study conducted in Baghdad city revealed that out of 108 isolates, 37 (37%) were Klebsiella spp. and were predominantly found in urine samples (19). Additionally, investigations conducted in Duhok and Erbil cities corroborated the findings of the current study (20,21). Conversely, studies conducted in various countries such as China, Iran, and Indonesia revealed that upper respiratory tract samples were the most commonly affected locations in *K. pneumoniae* infections (22,23). The variation in results among several research on the primary clinical sample, which includes *K. pneumoniae*, may be attributed to disparities in sample collection, study design, sample population, and environmental conditions (20).

The highest levels of resistance were found against tetracycline, colistin, and ceftriaxone, all at a rate of 100%. Resistance rates to penicillin and amikacin were 78.26% and 65.21%, respectively. All isolates showed 100% sensitivity to imipenem. This may be attributed to the excessive use of certain drugs, particularly in Mosul city, where imipenem is less commonly used compared to other drugs (26,27). As a result, there is lower resistance to imipenem due to its high cost, making it less accessible to the general population [(3)]. This finding aligned with similar studies conducted in Iraq and other developing countries (20,28,29).

In this study, two in vitro phenotypic tests, namely CRA, and MCRA, were employed to identify biofilm formation in the isolated strains. These tests were chosen over genotypic methods due to their cost-effectiveness, time efficiency, ease of use, and widespread availability in laboratory settings (30).

Both CRA and MCRA identify 20 colony out of 23 were strong biofilm producer where as the remaining 2 were in CRA as moderate biofilm producer and only one as non biofilm producer while in MCRA 2 were non biofilm producer and only one as moderate biofilm producer, this mild difference was non-significant at *p* value more than 0.05 by Kappa agreement coefficient test. This was in alignment other studies (31,32,33).

Basne *et al* in their study studied the difference between four method for detection of biofilm namely Tissue Culture Plate (TCP), Tube Method (TM), CRA and MCRA and found that ube method is the most accurate method after TCP and unlike MCRA, black pigmentation in colonies formed on CRA declined with time the mild difference may be due to difference in studied bacterial types, in sample collection, and study design (31).

On another hand The phenotypic pigmentation on agar was enhanced through the change of agar components and this enhance the efficacy of detection of biofilm. The decrease in the concentration of agar components led to the persistent development of a strong black pigment in isolates containing the *fimH* gene, with no drop in pigmentation seen over time. The alteration of the agar composition facilitated the synthesis of black pigment while also decreasing the cost of

agar manufacturing. The presence of a stable black pigment enhances the precision of identifying biofilm agents. Identifying the specific agents present in a biofilm will enhance the accuracy of medication or antibiotic prescriptions, leading to more effective and efficient eradication of pathogens. Additional research will be undertaken in order to determine the actual economic benefits resulting from the creation of a modified Congo Red Agar.

Conclusion

There is a correlation between a consistent black colony-forming characteristic on modified Congo red agar and the presence of fimH gene. These findings suggest that using both phenotypic and genotypic approaches is necessary for accurately and quickly identifying bacterial types, specifically MDR K. pneumonaie in a biofilm matrix. In contrast, the identification efficacy is limited when using only genotypic or phenotypic methods on published Congo Red Agar.

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