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Evaluation of Biofilm Formations of ESKAPE Group Bacteria Isolated from Clinical Samples: Experience with Different Methods

Klinik Örneklerden İzole Edilen ESKAPE Grup Bakterilerin Biyofilm Formasyonlarının Degerlendirilmesi: İki Farklı Yöntem Deneyimi

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ABSTRACT

Introduction: *The "ESKAPE" group of bacteria is a group of pathogens that poses a global health threat due to their ability to escape the biocidal effects of antibiotics. This group includes Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp. This study aimed to determine the biofilm-forming activities of ESKAPE group bacteria from different clinical specimens using two different methodologies and to compare the results of these two approaches.*

Materials and Methods: *In cultures obtained from various clinical specimens collected from patients in Sivas Cumhuriyet University Faculty of Medicine Practice and Research Hospital services and intensive care units, 27 isolates of E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and 26 isolates of Enterobacter spp. were identified. The biofilm production capacities of these microorganisms were assessed using the Congo red agar method and the microtiter plate assay. Statistical analyses were conducted using the McNemar test and Cohen's kappa test.*

Results: *No concordance was observed between the methods in S. aureus, A. baumannii, and P. aeruginosa isolates. In A. baumannii and P. aeruginosa isolates, the microtiter plate assay appears to be more sensitive than other methods. Using this method, biofilm formation was detected at a higher frequency. For E. faecium and K. pneumoniae, a moderate level of concordance was observed between the methods, whereas for Enterobacter spp., both methods demonstrated perfect agreement. The p-values determined for S. aureus, E. faecium, A. baumannii, P. aeruginosa, K. pneumoniae, and Enterobacter spp. were found to be 0.023, 0.025, <0.001, <0.001, and 0.366, respectively. The corresponding kappa values were calculated as -0.068, 0.501, 0, 0.13, 0.375, and 1.*

Conclusion: *Since different methods used for biofilm detection may yield varying results, it is of paramount importance to select the most appropriate research method. This study demonstrated the consistency of the methods in biofilm research. It showed that the microtiter plate method provides more consistent results, especially in biofilm detection in non-fermenting bacteria such as P. aeruginosa and A. baumannii.*

Key Words: *ESKAPE; Biofilm; Congo red agar method; Microtiter plate test method*

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ÖZ

Klinik Örneklerden İzole Edilen ESKAPE Grup Bakterilerin Biyofilm Formasyonlarının Degerlendirilmesi: İki Farklı Yöntem Deneyimi

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Giriş: *"ESKAPE" bakteri grubu, antibiyotiklerin biyosidal etkilerinden kaçabilme yetenekleri nedeniyle küresel sağlık tehdidi oluşturan bir grup patojendir. Bu grupta Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp. bulunur. Bu çalışmada çeşitli klinik örneklerden üretilen ESKAPE grubu bakterilerin biyofilm formasyon aktivitelerinin iki farklı yöntem ile belirlenmesi ve bu iki yöntemin sonuçlarının karşılıklı olarak değerlendirilmesi amaçlanmıştır.*

Materyal ve Metod: *Sivas Cumhuriyet Üniversitesi Tıp Fakültesi Uygulama ve Araştırma Hastanesi servislerinde ve yoğun bakım ünitelerinde yatan hastalardan gönderilen çeşitli klinik örneklerden yapılan kültürlerde üreyen 27 tane E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa ve 26 tane Enterobacter spp.'nin biyofilm üretim kapasiteleri Kongo red agar yöntemi ve mikrotitre plaka test yöntemleri kullanılarak tespit edilmiştir. İstatistiksel analiz için McNemar testi ve Cohen's kappa testi yapılmıştır.*

Bulgular: *S. aureus, A. baumannii ve P. aeruginosa izolatlarında yöntemler arasında uyum gözlemlenmemiştir. Özellikle A. baumannii ve P. aeruginosa izolatlarında mikrotitre plaka test yöntemi daha duyarlı görünmektedir. Mikrotitre plaka test yöntemiyle biyofilm varlığı daha fazla tespit edilmiştir. E. faecium ve K. pneumoniae'da yöntemler arasında orta seviyede uyum bulunmuş, Enterobacter spp. için ise her iki yöntem mükemmel uyum göstermiştir. S. aureus, E. faecium, A. baumannii, P. aeruginosa, K. pneumoniae ve Enterobacter spp. için belirlenen p değerleri sırasıyla 0.023, 0.025, <0.001, <0.001 ve 0.366 olarak bulunmuştur. Kappa değerleri ise aynı sırayla -0.068, 0.501, 0, 0.13, 0.375 ve 1 olarak hesaplanmıştır.*

Sonuç: *Biyofilm saptamada kullanılan farklı yöntemler farklı sonuçlar verebildiği için en uygun yöntemin seçilmesi önemlidir. Mikrotitre plaka test yöntemi, laboratuvar uygulamalarında daha hassas olabilirken, Kongo red agar yöntemi daha hızlı ve pratik bir yöntem olarak kullanılabilir. Bu çalışma ile yöntemlerin biyofilm araştırılmasındaki tutarlılığı ve özellikle P. aeruginosa ve A. baumannii gibi nonfermenter bakterilerde biyofilm tespitinde Mikrotitre plaka test yönteminin daha tutarlı sonuç verdiği gösterilmiştir.*

Anahtar Kelimeler: *ESKAPE; Biyofilm; Kongo red agar yöntemi; Microtitre plate test yöntemi*

INTRODUCTION

The "ESKAPE" group of bacteria poses a global health threat due to their ability to evade the biocidal effects of antibiotics. This group of pathogens includes six organisms, both gram-positive and gram-negative, that are often multidrug-resistant and commonly associated with nosocomial infections. These are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.[1,2]. These pathogens are also called "super microbes". Due to their resistance to multiple antibiotics and their ability to persist within biofilms, these bacteria contribute to a perfect storm, further exacerbating the infection[3].

Studies have established that *S. aureus*, a member of the ESKAPE group of bacteria, possesses a biofilm formation capacity that enables the bacterium to survive in hostile environments within the host, thereby facilitating the development of chronic infections[4-6]. Similarly, *E. faecium* is known to cause serious infections in humans. These bacteria stabilize by forming biofilms on biotic and abiotic surfaces and exhibit higher resistance to physical and chemical treatments compared to planktonic cells[7]. Among the ESKAPE pathogens, *A. baumannii* is a gram-negative, non-fermenting opportunistic bacterium capable of forming biofilms. It is frequently responsible for various device-associated infections in humans, including sepsis, meningitis, peritonitis, urinary tract infections, soft tissue infections, and ventilator-associated pneumonia[8]. Likewise, *P. aeruginosa* poses a significant risk, particularly for hospitalized patients with compromised immune

systems. Furthermore, the biofilm production by these bacteria presents a serious challenge in the treatment of infections[9,10]. Within the ESKAPE group, *K. pneumoniae* exhibits biofilm formation as a key virulence factor. Biofilm-producing strains are associated with high morbidity and mortality, especially in patients hospitalized in intensive care units (ICUs)[11]. Similarly, *Enterobacter* spp. belongs to the ESKAPE group, which includes highly resistant bacterial pathogens. It causes severe infections, particularly in immunocompromised patients and those receiving intensive care[12].

Biofilm is defined as an organized community of microorganisms that is irreversibly attached to a living or non-living surface. These microorganisms live in an extracellular polymeric matrix that they produce[13-15]. Biofilm is clinically very important. The ability of bacteria to escape host defense and develop resistance to antibiotics and many disinfectants increases the potential for chronic infection. Therefore, biofilm formation is recognized as one of the main causes of nosocomial infections[16-19].

The determination of biofilm formation is of significant importance, yet the availability of tests that offer rapid and precise results remains limited. Therefore, there may be challenges in the diagnosis and treatment of infections associated with biofilm-forming bacteria. Today, different in vitro and in vivo methods are used to detect biofilms. Congo red agar method (CRA), modified tube adherence method (modified Christensen method), and microtiter plate method (MtM) are the most commonly used methods to detect biofilm formation. Some quantitative methods based on the detection of metabolic activity and methods using fluorescence microscopy and electron microscopy are also used[20,21].

ESKAPE pathogens are among the most prevalent and antimicrobial-resistant bacteria, making them a priority group for antimicrobial resistance development. In addition to antimicrobial resistance, their ability to form biofilms presents significant clinical challenges. Biofilm formation is challenging to assess in practice, making the identification of biofilm-forming capacity in this group of microorganisms particularly valuable[2,16,22]. This study aims to determine the proportion of biofilm-forming microorganisms among these problematic pathogens in a single-center setting through in vitro analysis and to compare the two most commonly used methods, MtM and CRA, for biofilm detection.

MATERIALS and METHODS

Sample

Based on this result, 161 isolates were collected. The study included a total of 27 bacterial isolates from the species *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, as well as 26 isolates from *Enterobacter* spp. *E. aerogenes* is classified as *K. aerogenes* in the literature and European Committee on Antimicrobial Susceptibility Testing[12,23]. Therefore, *E. aerogenes* was not included in the study. Of the 26 Enterobacter isolates used in the study, 19 were *Enterobacter cloacae*, three were *Enterobacter hormaechei*, three were *Enterobacter kobei*, and one was *Enterobacter xiangfangensis*.

Blood and cerebrospinal fluid samples submitted to the Microbiology Laboratory were cultured on 5% sheep blood agar (HiMedia, India), eosin methylene blue agar (HiMedia, India), and chocolate agar (HiMedia, India) in accordance with standard procedures. The cultures were incubated at 37 °C for 24 hours. Following incubation, microorganisms were identified based on colony morphology, Gram staining, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the Microflex LT device (Bruker Daltonics, Germany), according to the manufacturer's protocols. The identified isolates were stored at -20 °C for two years for potential use in future studies. The bacterial isolates used in this study were selected through a haphazard sampling approach from the available stock strains. No randomization protocol was applied during the selection process. Biofilm analysis was performed on the regrown ESKAPE group bacteria. Biofilm analyses of these isolates were conducted in July, August, and September 2024.

Microtiter Plate Test Method

The microtiter plate test method, as described by Christensen and colleagues, was employed to examine biofilm structures of bacteria. The bacteria were inoculated into a blood agar medium[4].

The inoculated bacteria were obtained as fresh and pure cultures following a 24-hour incubation period at 37 °C. The isolates were suspended in Tryptic Soy Broth (TSB) (Merck, United States of America) containing 0.25% glucose (Merck, USA) and incubated overnight at 37 °C. The bacterial density in the suspensions was adjusted to 0.5 McFarland, and 200 µL of each suspension was transferred to 96-well U-bottom microplates. A negative control was established by adding 200 µL of TSB to the well and inoculating it with no microorganisms. The microplates were incubated at 37 °C for 24 hours. After the 24-hour incubation period, the wells were gently emptied and washed twice with phosphate buffer solution (PBS) (Pars, Türkiye). Two hundred microliters of 0.1% crystal violet solution (Rokim, Türkiye) were added to the wells and allowed to dry at room temperature for 30 minutes. The wells were then washed twice with PBS. Following a drying period at room temperature, 95% ethanol was added to the wells, and the absorbance values at 550 nm were read Triturus micro ELISA device (Grifols, Spain). The study was conducted in triplicate. As reported by Chusri et al., each microorganism's biofilm formation activities were classified according to a four-point scale: no biofilm, weak, moderate, and strong[24]. The formation of biofilm structures is illustrated in Figure 1.

Congo Red Agar Method

The study was conducted using the Congo red agar method as described by Freeman et al.[25]. Congo red medium was prepared containing 10 g agar (Rokim, Türkiye), 50 g sucrose (Merck, USA), 37 g brain-heart infusion broth (Merck, USA), and 0.8 g Congo red (Rokim, Türkiye) per liter and poured into petri dishes. Bacteria seeded onto the medium using the single colony seeding technique were incubated at 37 °C for 24 and 48 hours. Bacteria forming dark red-black colonies were evaluated as biofilm-positive, and bacteria forming red-pink colonies were evaluated as biofilm-negative (as shown in Figure 1).

Statistical Analysis

The data obtained from the study were entered into IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA) for analysis. Cohen's Kappa test and the McNemar test were used for statistical evaluation. In the Cohen's Kappa test, the

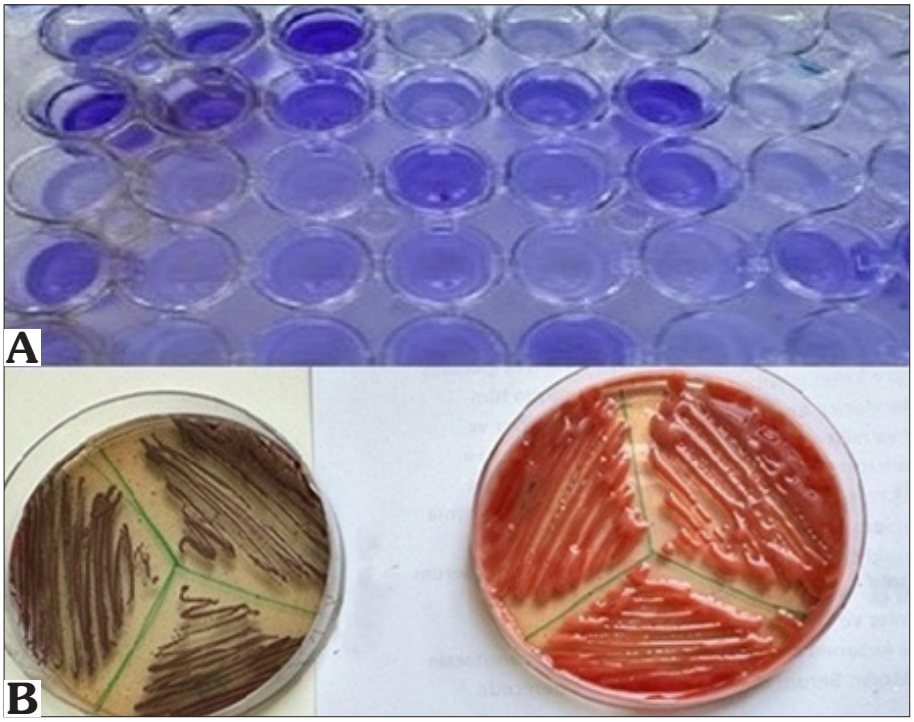

Figure 1. The demonstration of biofilm structure was conducted using the MtM **(A)** and CRA **(B)** methods.

results are interpreted as follows: ≤0 indicates no agreement, 0.01-0.20 indicates little or no agreement, 0.21-0.40 indicates fair agreement, 0.41-0.60 indicates moderate agreement, 0.61-0.80 indicates substantial agreement, and 0.81-1.00 indicates almost perfect agreement[26]. The level of statistical significance was set at $p< 0.05$. The graphs were generated using GraphPad Prism 8 software.

RESULTS

In the present study, biofilm examination was conducted on 27 *S. aureus* isolates, employing the CRA and MtM methods. The MtM method revealed that 96.3% of the bacteria exhibited weak, moderate, or strong biofilm formation. The CRA method revealed the presence of biofilm in 87.8% of the bacteria. Statistical analysis using the McNemar test demonstrated a significant difference between the two methods ($p= 0.023$). Additionally, Cohen's Kappa test yielded a kappa value of zero, indicating a lack of agreement between the methods (as shown in Table 7). The results of the tests conducted with *S. aureus* are summarized in Table 1 and Figure 2, with additional details provided in Table 1.

Table 1. Results of tests performed for biofilm detection in *S. aureus* isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*S. aureus*)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
S-1	0.156 ± 0.074	0.457 ± 0.221	Moderate	Red	Red
S-2	0.156 ± 0.074	0.465 ± 0.122	Moderate	Red	Red
S-3	0.156 ± 0.074	0.625 ± 0.171	Strong	Red	Red
S-4	0.156 ± 0.074	0.553 ± 0.317	Moderate	Burgundy -Black	Burgundy -Black
S-5	0.156 ± 0.074	0.38 ± 0.044	Moderate	Burgundy -Black	Burgundy -Black
S-6	0.156 ± 0.074	0.664 ± 0.057	Strong	Burgundy -Black	Burgundy -Black
S-7	0.156 ± 0.074	0.698 ± 0.069	Strong	Red	Red
S-8	0.156 ± 0.074	0.888 ± 0.21	Strong	Red	Red
S-9	0.156 ± 0.074	0.937 ± 0.036	Strong	Burgundy -Black	Burgundy -Black
S-10	0.156 ± 0.074	0.401 ± 0.114	Moderate	Burgundy -Black	Burgundy -Black
S-11	0.156 ± 0.074	0.112 ± 0.015	No	Burgundy -Black	Burgundy -Black
S-12	0.156 ± 0.074	0.374 ± 0.019	Moderate	Burgundy -Black	Burgundy -Black
S-13	0.156 ± 0.074	0.246 ± 0.024	Weak	Burgundy -Black	Burgundy -Black
S-14	0.156 ± 0.074	0.367 ± 0.142	Moderate	Burgundy -Black	Burgundy -Black
S-15	0.156 ± 0.074	0.243 ± 0.026	Weak	Burgundy -Black	Burgundy -Black
S-16	0.156 ± 0.074	0.334 ± 0.124	Moderate	Burgundy -Black	Burgundy -Black
S-17	0.156 ± 0.074	0.371 ± 0.013	Moderate	Burgundy -Black	Burgundy -Black
S-18	0.156 ± 0.074	0.34 ± 0.026	Moderate	Burgundy -Black	Burgundy -Black
S-19	0.156 ± 0.074	0.219 ± 0.042	Weak	Burgundy -Black	Burgundy -Black
S-20	0.156 ± 0.074	0.231 ± 0.059	Weak	Burgundy -Black	Burgundy -Black
S-21	0.156 ± 0.074	0.239 ± 0.014	Weak	Burgundy -Black	Burgundy -Black
S-22	0.156 ± 0.074	0.215 ± 0.033	Weak	Burgundy -Black	Burgundy -Black
S-23	0.156 ± 0.074	0.249 ± 0.024	Weak	Burgundy -Black	Burgundy -Black
S-24	0.156 ± 0.074	0.284 ± 0.019	Weak	Burgundy -Black	Burgundy -Black
S-25	0.156 ± 0.074	0.317 ± 0.017	Moderate	Burgundy -Black	Burgundy -Black
S-26	0.156 ± 0.074	0.27 ± 0.039	Weak	Burgundy -Black	Burgundy -Black
S-27	0.156 ± 0.074	0.192 ± 0.004	Weak	Red	Red

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

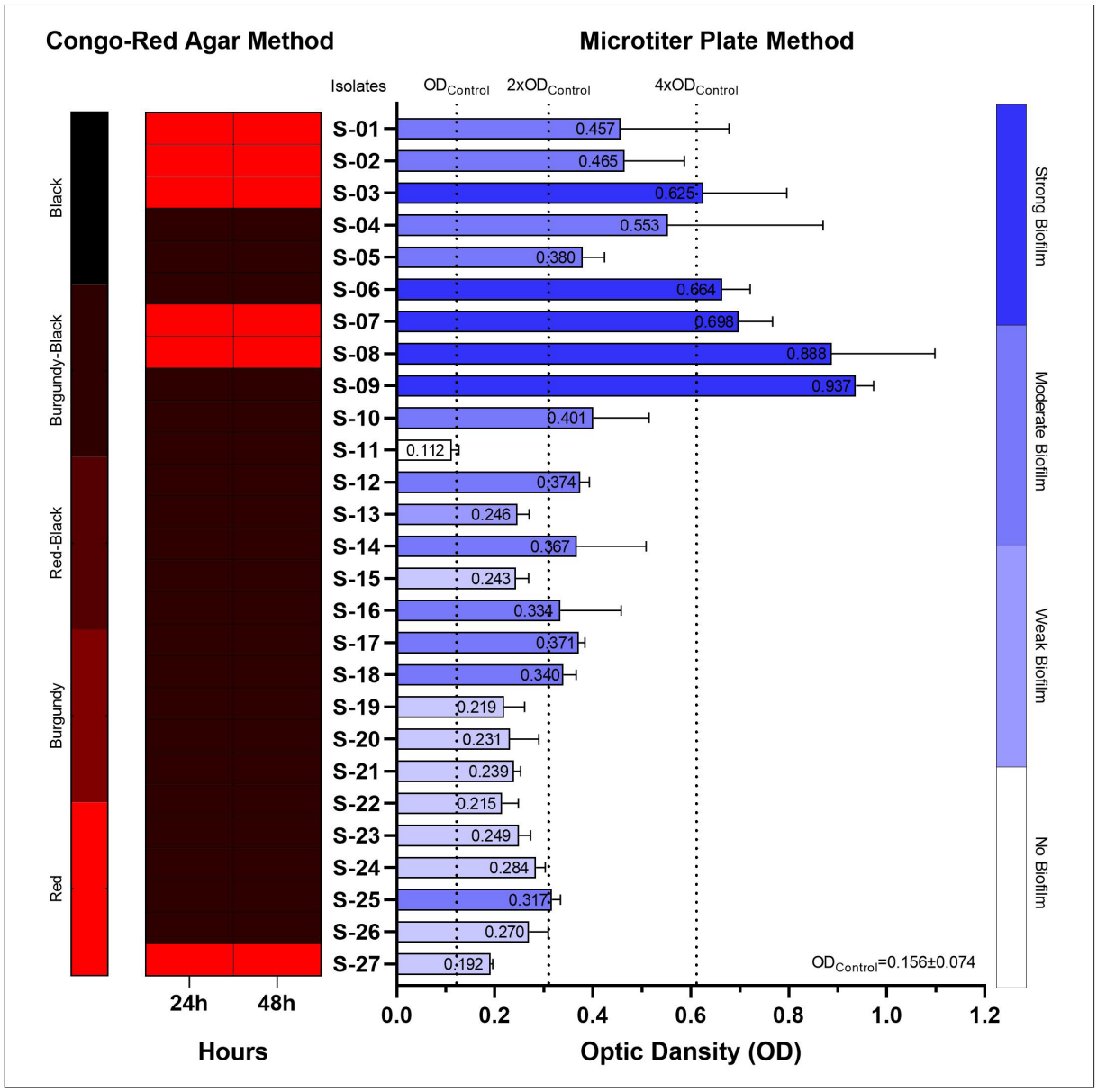

Figure 2. A graphical representation of the test results performed for the detection of biofilms in *S. aureus* isolates.

Biofilm analysis was conducted on 27 *E. faecium* isolates using the MtM and CRA methods. The MtM method identified biofilm formation in 87.2% of bacteria, while the CRA method showed 96.3% of bacteria. The McNemar test revealed a statistically significant difference between the two methods in terms of biofilm detection (p= 0.025). Furthermore, the kappa value was 0.583, as determined by Cohen's Kappa test, indicating a moderate level of agreement between the methods. The results of the tests conducted with *E. faecium* are summarized in Table 2 and Figure 3, with additional details provided in Table 2.

Biofilm analysis was conducted on 27 *A. baumannii* isolates employing the MtM and CRA methods. Biofilm formation was detected in 100% of the isolates using the MtM method, compared to 14.8% using the CRA method. The McNemar test results obtained in the present study revealed a statistically significant difference

Table 2. Results of tests conducted to identify biofilm formation in *E. faecium* isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*E. faecium*)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
Ef-1	0.156 ± 0.074	0.529 ± 0.241	Moderate	Black	Black
Ef-2	0.156 ± 0.074	0.359 ± 0.012	Moderate	Black	Black
Ef-3	0.156 ± 0.074	0.48 ± 0.119	Moderate	Black	Black
Ef-4	0.156 ± 0.074	0.395 ± 0.076	Moderate	Black	Black
Ef-5	0.156 ± 0.074	0.605 ± 0.209	Moderate	Black	Black
Ef-6	0.156 ± 0.074	0.462 ± 0.153	Moderate	Black	Black
Ef-7	0.156 ± 0.074	0.531 ± 0.066	Moderate	Black	Black
Ef-8	0.156 ± 0.074	0.551 ± 0.078	Moderate	Black	Black
Ef-9	0.156 ± 0.074	0.426 ± 0.151	Moderate	Black	Black
Ef-10	0.156 ± 0.074	0.484 ± 0.134	Moderate	Black	Black
Ef-11	0.156 ± 0.074	0.578 ± 0.062	Moderate	Red	Red
Ef-12	0.156 ± 0.074	0.495 ± 0.135	Moderate	Black	Black
Ef-13	0.156 ± 0.074	0.562 ± 0.098	Moderate	Black	Black
Ef-14	0.156 ± 0.074	0.554 ± 0.194	Moderate	Black	Black
Ef-15	0.156 ± 0.074	0.589 ± 0.191	Moderate	Black	Black
Ef-16	0.156 ± 0.074	0.614 ± 0.207	Moderate	Black	Black
Ef-17	0.156 ± 0.074	0.351 ± 0.023	Moderate	Black	Black
Ef-18	0.156 ± 0.074	0.423 ± 0.087	Moderate	Black	Black
Ef-19	0.156 ± 0.074	0.471 ± 0.069	Moderate	Black	Black
Ef-20	0.156 ± 0.074	0.455 ± 0.188	Moderate	Black	Black
Ef-21	0.156 ± 0.074	0.128 ± 0.006	No	Black	Black
Ef-22	0.156 ± 0.074	0.129 ± 0.01	No	Black	Black
Ef-23	0.156 ± 0.074	0.141 ± 0.008	No	Black	Black
Ef-24	0.156 ± 0.074	0.162 ± 0.027	Weak	Black	Black
Ef-25	0.156 ± 0.074	0.107 ± 0.012	No	Black	Black
Ef-26	0.156 ± 0.074	0.104 ± 0.005	No	Red	Red
Ef-27	0.156 ± 0.074	0.109 ± 0.007	No	Red	Red

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

between the two methods in terms of biofilm detection ($p< 0.001$). Cohen's Kappa test yielded a kappa value of 0, indicating that there was no agreement between the two methods. The results of the tests conducted with *A. baumannii* are summarized in Table 3 and Figure 4, with additional details provided in Table 3.

A markedly higher biofilm detection rate was observed with the MtM method, while 37% exhibited this capacity using the CRA method. The McNemar test results obtained in the present study revealed a statistically significant difference between the two methods in terms of biofilm detection ($p< 0.001$). Cohen's Kappa test yielded a kappa value of 0.13, indicating that there was no agreement between the two methods. The results of the tests conducted with *P. aeruginosa* are summarized in Table 4 and Figure 5, with additional details provided in Table 4.

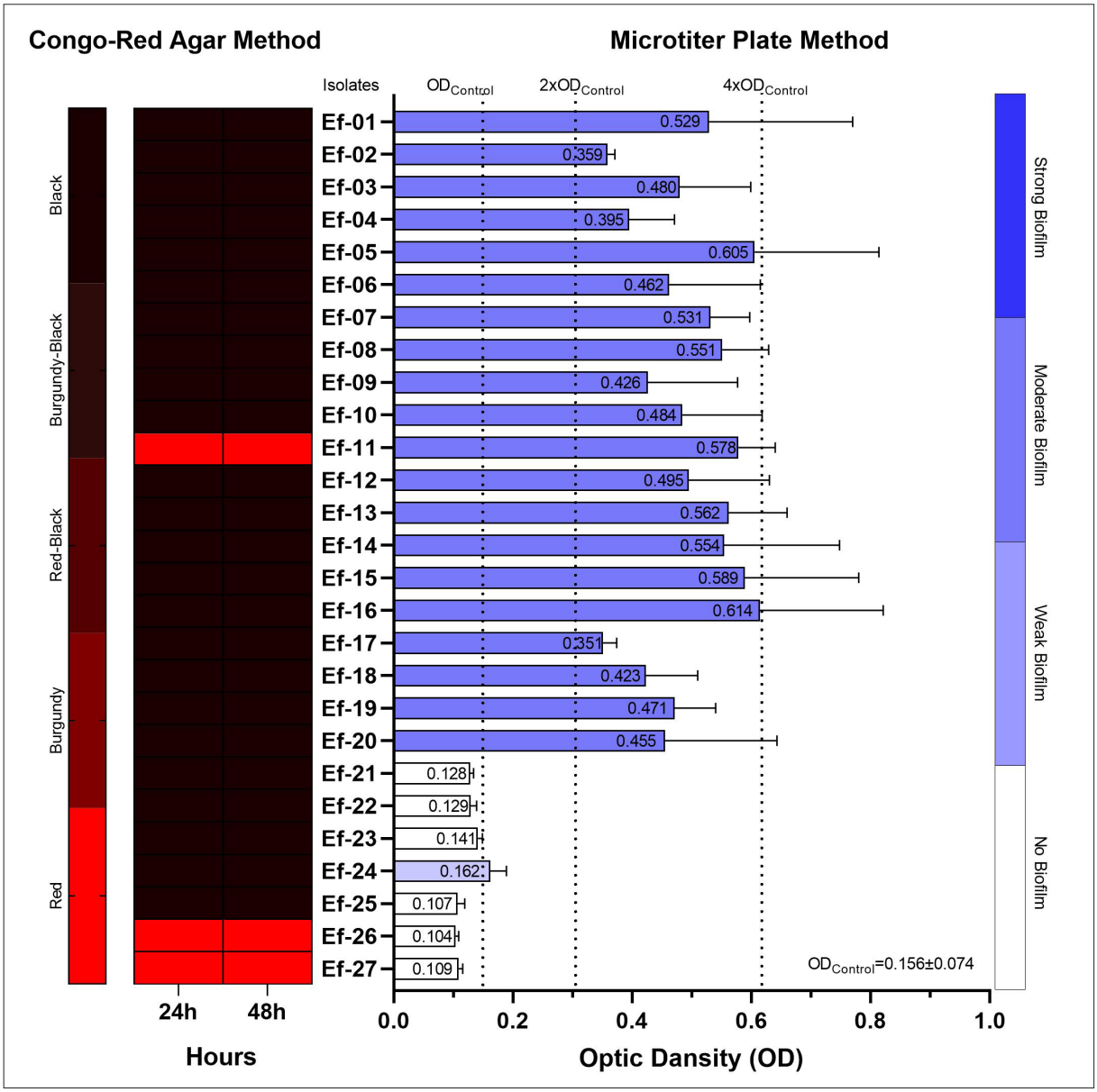

Figure 3. Graphical representation of test results for biofilm detection in *E. faecium* isolates.

Biofilm analysis was conducted on 27 *K. pneumoniae* isolates employing the MtM and CRA methods. The results demonstrated that biofilm formation was detected in 78% of the isolates using the MtM method and in 85% using the CRA method. The results of the McNemar test indicated that there was no statistically significant difference between the two methods in terms of detecting the presence of biofilm (p= 0.366). The results of Cohen's Kappa test yielded a kappa value of 0.583, indicating a moderate level of agreement between the two methods. The results of the tests conducted with *K. pneumoniae* are summarized in Table 5 and Figure 6, with additional details provided in Table 5.

Biofilm analysis was conducted on 26 *Enterobacter* spp. isolates using the CRA and MtM methods. The results demonstrated that biofilm formation was detected in 100% of the

Table 3. Results of tests conducted to identify biofilm formation in *A. baumannii* isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*A. baumannii*)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
A-1	0.156 ± 0.074	0.429 ± 0.107	Moderate	Red	Red
A-2	0.156 ± 0.074	0.625 ± 0.069	Strong	Red	Red
A-3	0.156 ± 0.074	0.467 ± 0.036	Moderate	Burgundy	Burgundy
A-4	0.156 ± 0.074	0.585 ± 0.207	Moderate	Red	Red
A-5	0.156 ± 0.074	0.529 ± 0.059	Moderate	Burgundy	Burgundy
A-6	0.156 ± 0.074	0.745 ± 0.12	Strong	Red	Red
A-7	0.156 ± 0.074	0.551 ± 0.038	Moderate	Red	Red
A-8	0.156 ± 0.074	0.74 ± 0.283	Strong	Red	Red
A-9	0.156 ± 0.074	0.621 ± 0.451	Moderate	Red	Red
A-10	0.156 ± 0.074	0.428 ± 0.016	Moderate	Red	Red
A-11	0.156 ± 0.074	0.595 ± 0.098	Moderate	Burgundy	Burgundy
A-12	0.156 ± 0.074	0.46 ± 0.255	Moderate	Red	Red
A-13	0.156 ± 0.074	0.647 ± 0.256	Strong	Red	Red
A-14	0.156 ± 0.074	0.454 ± 0.066	Moderate	Red	Red
A-15	0.156 ± 0.074	0.635 ± 0.165	Strong	Red	Red
A-16	0.156 ± 0.074	0.665 ± 0.285	Strong	Red	Red
A-17	0.156 ± 0.074	0.548 ± 0.128	Moderate	Red	Red
A-18	0.156 ± 0.074	0.383 ± 0.005	Moderate	Burgundy	Burgundy
A-19	0.156 ± 0.074	0.514 ± 0.105	Moderate	Red	Red
A-20	0.156 ± 0.074	0.425 ± 0.057	Moderate	Red	Red
A-21	0.156 ± 0.074	0.865 ± 0.187	Strong	Red	Red
A-22	0.156 ± 0.074	0.443 ± 0.053	Moderate	Burgundy	Burgundy
A-23	0.156 ± 0.074	0.424 ± 0.091	Moderate	Red	Red
A-24	0.156 ± 0.074	0.75 ± 0.231	Strong	Red	Red
A-25	0.156 ± 0.074	0.576 ± 0.083	Moderate	Red	Red
A-26	0.156 ± 0.074	0.435 ± 0.026	Moderate	Red	Red
A-27	0.156 ± 0.074	0.645 ± 0.229	Strong	Red	Red

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

isolates by both methods. Cohen's Kappa value was found to be 1, indicating excellent agreement between the CRA and MtM methods. The results of the tests conducted with *Enterobacter* spp are summarized in Table 6 and Figure 7, with additional details provided in Table 6.

The results of Cohen's Kappa test indicate that no agreement was observed between the methods for species *S. aureus*, *A. baumannii*, and *P. aeruginosa*. The results of the MtM method are particularly striking for *A. baumannii* and *P. aeruginosa*. Furthermore, the MtM method has identified a greater amount of biofilm. For the species *E. faecium* and *K. pneumoniae*, there is moderate agreement between the two methods, while for *Enterobacter* spp., both methods show excellent agreement.

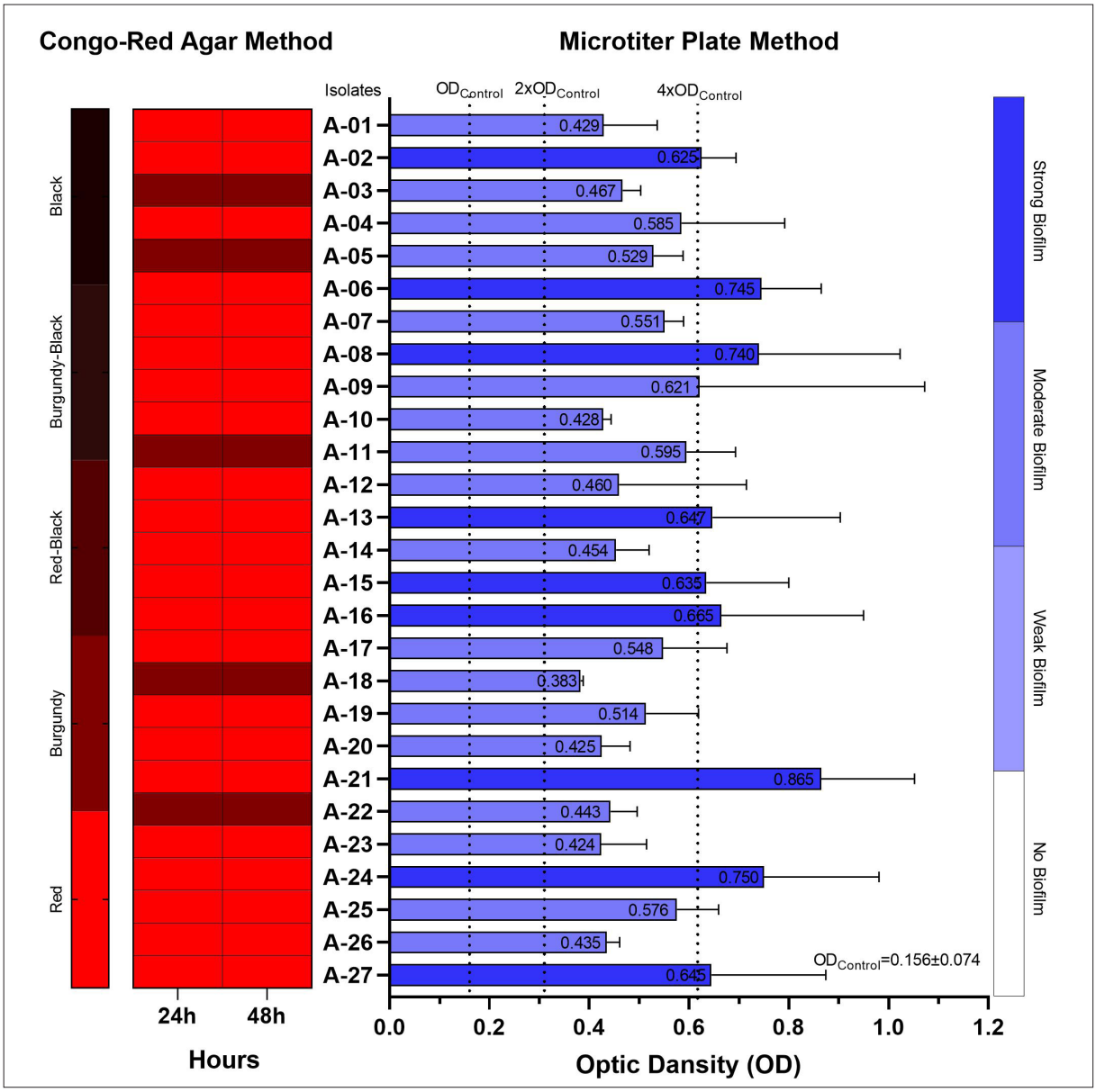

Figure 4. Graphical representation of the test results for biofilm detection in *A. baumannii* isolates.

DISCUSSION

The ESKAPE group of bacteria has been the subject of considerable research interest due to their potential for biofilm production[2,22]. The biofilm structure represents a significant virulence factor that contributes to the development of antibiotic-resistant hospital infections. This structure impedes the penetration of antimicrobial agents, thereby facilitating the development of resistance[27]. The correct application of methods to determine biofilm formation and the development of new techniques and treatment strategies is of great importance for the prevention of biofilm formation, the understanding of resistance mechanisms, and the development of new techniques and treatment strategies for biofilm infections[21].

A statistical analysis of the study results demonstrated a discrepancy between the two methods. From a proportional perspective, the

Table 4. Results of tests performed for biofilm detection in *P. aeruginosa* isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*P. aeruginosa*)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
P-1	0.156 ± 0.074	1.758 ± 0.615	Strong	Red	Red
P-2	0.156 ± 0.074	1.415 ± 0.844	Strong	Red	Red
P-3	0.156 ± 0.074	0.494 ± 0.061	Moderate	Red-Black	Black
P-4	0.156 ± 0.074	0.723 ± 0.256	Strong	Red	Red
P-5	0.156 ± 0.074	0.604 ± 0.087	Moderate	Red-Black	Black
P-6	0.156 ± 0.074	1.148 ± 0.134	Strong	Red	Red
P-7	0.156 ± 0.074	1.537 ± 0.976	Strong	Red	Red
P-8	0.156 ± 0.074	3.028 ± 0.439	Strong	Red	Red
P-9	0.156 ± 0.074	0.516 ± 0.065	Moderate	Red-Black	Black
P-10	0.156 ± 0.074	1.066 ± 0.463	Strong	Red-Black	Black
P-11	0.156 ± 0.074	0.824 ± 0.184	Strong	Red-Black	Black
P-12	0.156 ± 0.074	0.568 ± 0.084	Moderate	Red	Red
P-13	0.156 ± 0.074	0.527 ± 0.11	Moderate	Red-Black	Black
P-14	0.156 ± 0.074	0.709 ± 0.341	Strong	Red-Black	Black
P-15	0.156 ± 0.074	0.612 ± 0.046	Moderate	Red	Red
P-16	0.156 ± 0.074	1.042 ± 0.213	Strong	Red	Red
P-17	0.156 ± 0.074	0.381 ± 0.006	Moderate	Red	Red
P-18	0.156 ± 0.074	0.624 ± 0.243	Strong	Red	Red
P-19	0.156 ± 0.074	0.582 ± 0.12	Moderate	Red-Black	Black
P-20	0.156 ± 0.074	0.964 ± 0.306	Strong	Red	Red
P-21	0.156 ± 0.074	0.503 ± 0.184	Moderate	Red-Black	Black
P-22	0.156 ± 0.074	1.209 ± 0.129	Strong	Red	Red
P-23	0.156 ± 0.074	0.827 ± 0.026	Strong	Red	Red
P-24	0.156 ± 0.074	0.622 ± 0.129	Moderate	Red-Black	Black
P-25	0.156 ± 0.074	1.946 ± 1.057	Strong	Red	Red
P-26	0.156 ± 0.074	0.432 ± 0.043	Moderate	Red	Red
P-27	0.156 ± 0.074	0.49 ± 0.093	Moderate	Red	Red

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

MtM method was deemed to be more sensitive. The present study's findings are consistent with those reported in previous studies. A separate study identified biofilm-forming capacity in 92% of *S. aureus* strains isolated from patients using the MtM method[28]. De Castro Melo et al., in their study on *S. aureus* isolates from cases of mastitis, reported biofilm detection in 98.9% of cases using the MtM method and in 85% using the KR method. The MtM test demonstrated higher sensitivity (100%) in detecting positive strains, indicating superior performance compared to the CRA test in identifying in vitro biofilm formation[6]. In a separate study, the capacity of *S. aureus* to form biofilms on diverse surfaces was assessed. The findings indicated that the MtM method exhibited greater sensitivity than the CRA method in detecting biofilms[29]. Another study demonstrated inconsistency between the Microplate and CRA methods in detecting biofilm production[30]. In contrast to the aforementioned findings, several studies have reported that the MtM and KR methods yield comparable results in detecting biofilm formation[31-33].

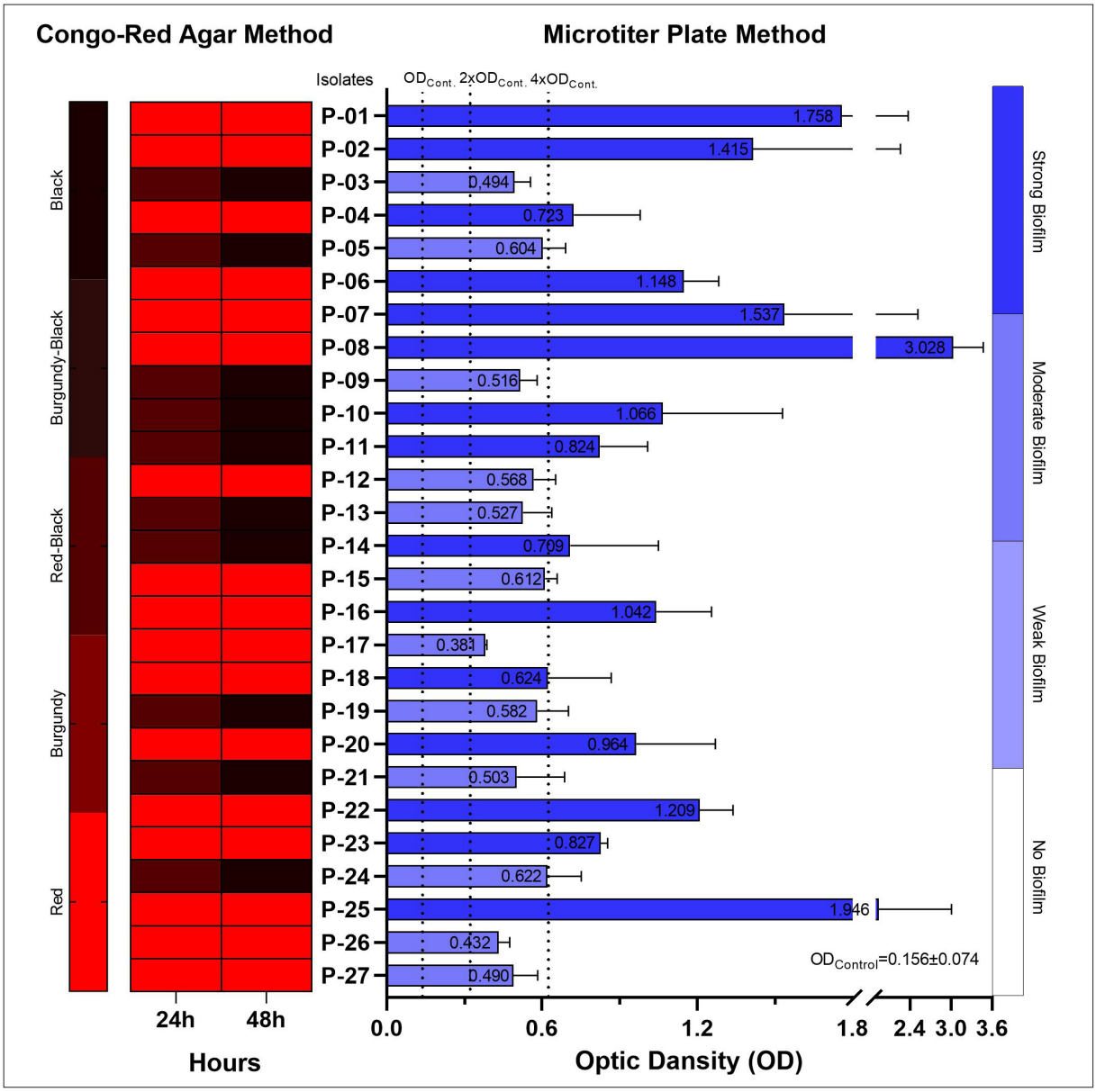

Figure 5. A graphical representation of the test results performed for the detection of biofilms in *P. aeruginosa* isolates.

The present study's findings with *E. faecium* are consistent with those reported in some earlier studies. A comparable degree of concordance was noted in the study conducted by Gürkan et al.[34]. Contrary to the results of our study, lower biofilm formation rates have been reported in some studies. A study involving 45 isolates revealed that biofilm formation was present in 25% of 12 environmental isolates and 35% of 28 infectious agents[35]. In another study, the biofilm formation rate of *E. faecium* obtained from vaginal microbiota was determined to be 22% by the MtM method[36]. Another study indicated a biofilm formation rate of 56%[37]. In contrast to the aforementioned studies, we suggest that the elevated biofilm formation rate observed in our study may be attributed to the fact that the isolates were obtained from hospitalized patients.

This study demonstrates that the MtM method provides superior results in detecting

Table 5. Results of tests performed for biofilm detection in *K. pneumoniae* isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*K. pneumoniae*)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
K-1	0.156 ± 0.074	0.482 ± 0.115	Moderate	Black	Black
K-2	0.156 ± 0.074	0.318 ± 0.060	Moderate	Black	Black
K-3	0.156 ± 0.074	0.327 ± 0.079	Moderate	Red	Red
K-4	0.156 ± 0.074	0.172 ± 0.083	Weak	Red	Red
K-5	0.156 ± 0.074	0.439 ± 0.087	Moderate	Black	Black
K-6	0.156 ± 0.074	0.201 ± 0.056	Weak	Black	Black
K-7	0.156 ± 0.074	0.735 ± 0.254	Strong	Black	Black
K-8	0.156 ± 0.074	0.678 ± 0.169	Strong	Black	Black
K-9	0.156 ± 0.074	0.566 ± 0.223	Moderate	Black	Black
K-10	0.156 ± 0.074	0.17 ± 0.037	Weak	Black	Black
K-11	0.156 ± 0.074	0.094 ± 0.002	No	Black	Black
K-12	0.156 ± 0.074	0.146 ± 0.027	No	Black	Black
K-13	0.156 ± 0.074	0.218 ± 0.046	Weak	Black	Black
K-14	0.156 ± 0.074	0.287 ± 0.081	Weak	Black	Black
K-15	0.156 ± 0.074	0.805 ± 0.096	Strong	Black	Black
K-16	0.156 ± 0.074	0.158 ± 0.04	Weak	Black	Black
K-17	0.156 ± 0.074	0.349 ± 0.104	Moderate	Red	Red
K-18	0.156 ± 0.074	0.127 ± 0.021	No	Black	Black
K-19	0.156 ± 0.074	0.162 ± 0.016	Weak	Black	Black
K-20	0.156 ± 0.074	0.117 ± 0.014	No	Black	Black
K-21	0.156 ± 0.074	0.526 ± 0.042	Moderate	Black	Black
K-22	0.156 ± 0.074	0.11 ± 0.023	No	Black	Black
K-23	0.156 ± 0.074	0.171 ± 0.028	Weak	Red	Red
K-24	0.156 ± 0.074	0.376 ± 0.075	Moderate	Black	Black
K-25	0.156 ± 0.074	0.477 ± 0.301	Moderate	Black	Black
K-26	0.156 ± 0.074	0.304 ± 0.081	Weak	Black	Black
K-27	0.156 ± 0.074	0.143 ± 0.017	No	Black	Black

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

the biofilm formation ability of *A. baumannii* when compared to the CRA method. These findings are consistent with those reported in other studies. In two different studies utilizing the MtM method, the biofilm formation rate of *A. baumannii* was reported as 100% in one study and 77% in the other[28,38]. Alamri et al. employed the KR method to detect biofilms in 2.7% of 207 *A. baumannii* isolates[39]. In a separate study employing the microtiter plate and CRA method, biofilm analysis was conducted on 156 *A. baumannii* isolates. The biofilm formation rate was found to be 98% using the standard MtM method and 10.2% using the CRA method[40].

According to our study results, the MtM method detected higher biofilm presence than the CRA method in detecting biofilm in *P. aeruginosa*. These findings are by those of other studies. In a study conducted using the MtM method, the biofilm percentage of *P. aeruginosa* was found to be 88%[28]. The results of the

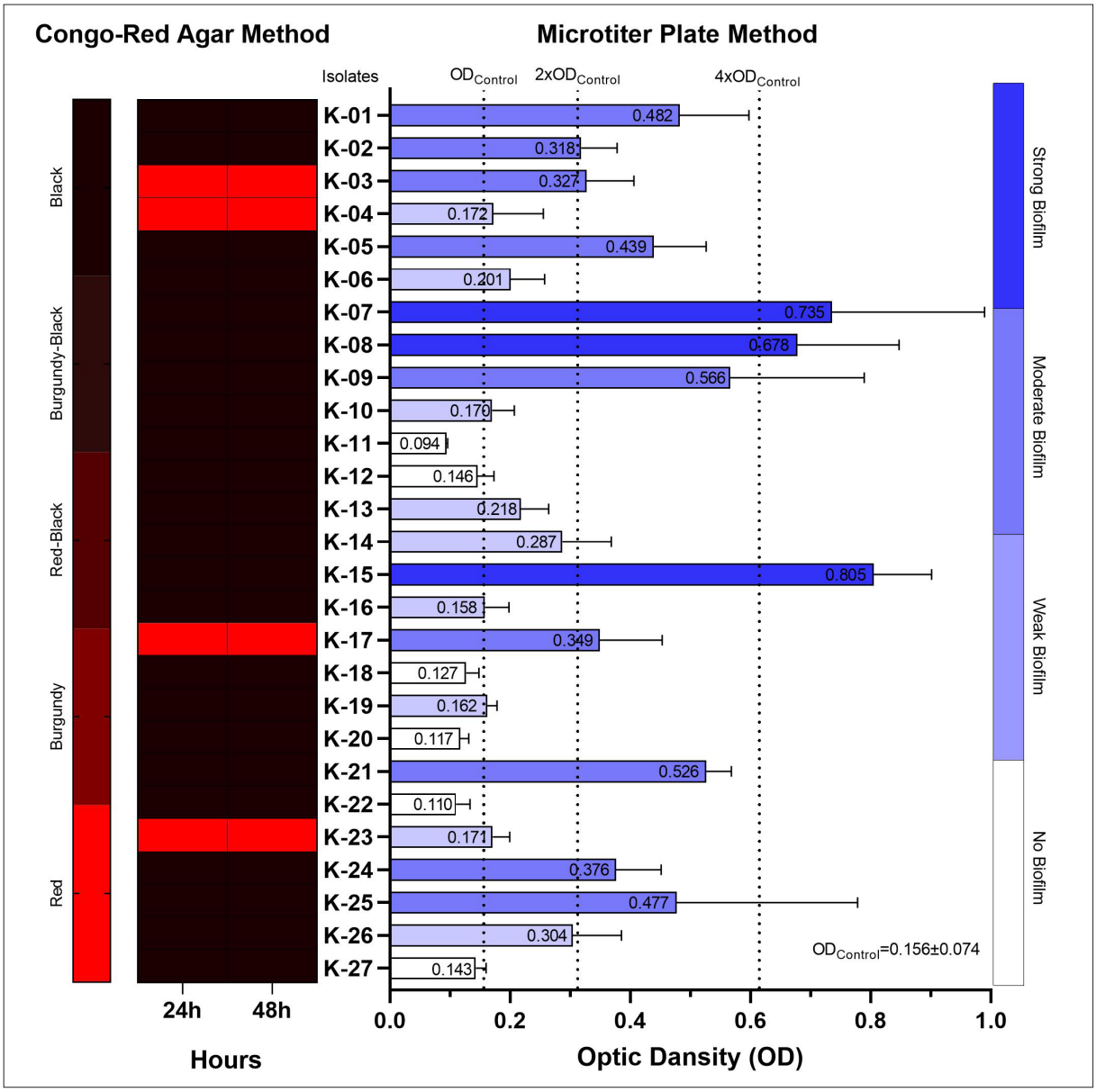

Figure 6. Graphical representation of test results performed for biofilm detection in *K. pneumoniae* isolates.

CRA agar method employed in the present study are consistent with those reported by Aladağ et al.[9]. The McNemar test revealed a statistically significant difference between the two methods in terms of biofilm detection ($p< 0.05$) when *P. aeruginosa* was used as the test subject. The data indicate that the MtM method is more sensitive than the CRA method in detecting biofilm formation. The results of Cohen's Kappa test indicated a kappa value of 0, suggesting that there was no agreement between the two methods. Current literature indicates that there is no consensus regarding the agreement between the two methods. In the study conducted by Nagaveni et al., biofilm analysis was performed with 25 *P. aeruginosa* isolates using the microtiter tube method and the CRA agar method. The study yielded a 75% detection rate for the microtiter tube method and a 25% detection rate for the CRA agar method[41]. In contrast to the findings of our study, a previous investigation utilizing bacteria isolated from contact lenses

Table 6. Results of tests performed for biofilm detection in *Enterobacter* spp. isolates

	Microtiter Plate Test Method			Congo Red Agar Method	
Isolate (*Enterobacter* spp.)	Control AM.OD ± SD	Biofilm AM.OD ± SD	Biofilm Degree	24 Hour Color Change	48 Hour Color Change
E-1	0.156 ± 0.074	0.348 ± 0.207	Moderate	Black	Black
E-2	0.156 ± 0.074	0.345 ± 0.045	Moderate	Black	Black
E-3	0.156 ± 0.074	0.628 ± 0.029	Strong	Black	Black
E-4	0.156 ± 0.074	0.53 ± 0.037	Moderate	Black	Black
E-5	0.156 ± 0.074	0.196 ± 0.005	Weak	Black	Black
E-6	0.156 ± 0.074	0.308 ± 0.066	Weak	Black	Black
E-7	0.156 ± 0.074	0.301 ± 0.038	Weak	Black	Black
E-8	0.156 ± 0.074	0.187 ± 0.011	Weak	Black	Black
E-9	0.156 ± 0.074	0.627 ± 0.048	Strong	Black	Black
E-10	0.156 ± 0.074	0.514 ± 0.098	Moderate	Black	Black
E-11	0.156 ± 0.074	0.49 ± 0.086	Moderate	Black	Black
E-12	0.156 ± 0.074	0.175 ± 0.034	Weak	Black	Black
E-13	0.156 ± 0.074	0.569 ± 0.084	Moderate	Black	Black
E-14	0.156 ± 0.074	0.853 ± 0.163	Strong	Black	Black
E-15	0.156 ± 0.074	0.656 ± 0.053	Strong	Black	Black
E-16	0.156 ± 0.074	0.458 ± 0.068	Moderate	Black	Black
E-17	0.156 ± 0.074	0.827 ± 0.026	Strong	Black	Black
E-18	0.156 ± 0.074	0.719 ± 0.064	Strong	Black	Black
E-19	0.156 ± 0.074	0.287 ± 0.081	Weak	Black	Black
E-20	0.156 ± 0.074	0.597 ± 0.227	Moderate	Black	Black
E-21	0.156 ± 0.074	0.582 ± 0.121	Moderate	Black	Black
E-22	0.156 ± 0.074	0.933 ± 0.003	Strong	Black	Black
E-23	0.156 ± 0.074	0.839 ± 0.151	Strong	Black	Black
E-24	0.156 ± 0.074	0.91 ± 0.176	Strong	Black	Black
E-25	0.156 ± 0.074	0.994 ± 0.034	Strong	Black	Black
E-26	0.156 ± 0.074	0.940 ± 0.063	Strong	Black	Black
S-27	0.156 ± 0.074	0.192 ± 0.004	Weak	Red	Red

OD: Optic density, AM: Arithmetic mean, SD: Standard deviation.

revealed the presence of 100% biofilm using the microtiter tube method and 91.6% using the CRA agar method. A significant statistical correlation was identified between the results of both methods (p= 0.006)[42]. It is hypothesized that this discrepancy may be attributed to the larger number of samples, the employment of 12 distinct bacterial species in the present study, along with the evaluation of the correlation across the entire sample set.

According to the results of our study, the two methods yielded similar results in detecting biofilm formation in *K. pneumoniae*. In two other studies that assessed biofilm presence using the MtM method, prevalence rates of 100% and 60% were reported, respectively[43,44]. In the study by Pradhan et al., it was determined that 65.45% of 110 *K. pneumoniae* isolates were identified as biofilm producers with the CRA agar method, and 73.59% produced biofilm quantitatively, with the MtM method[11].

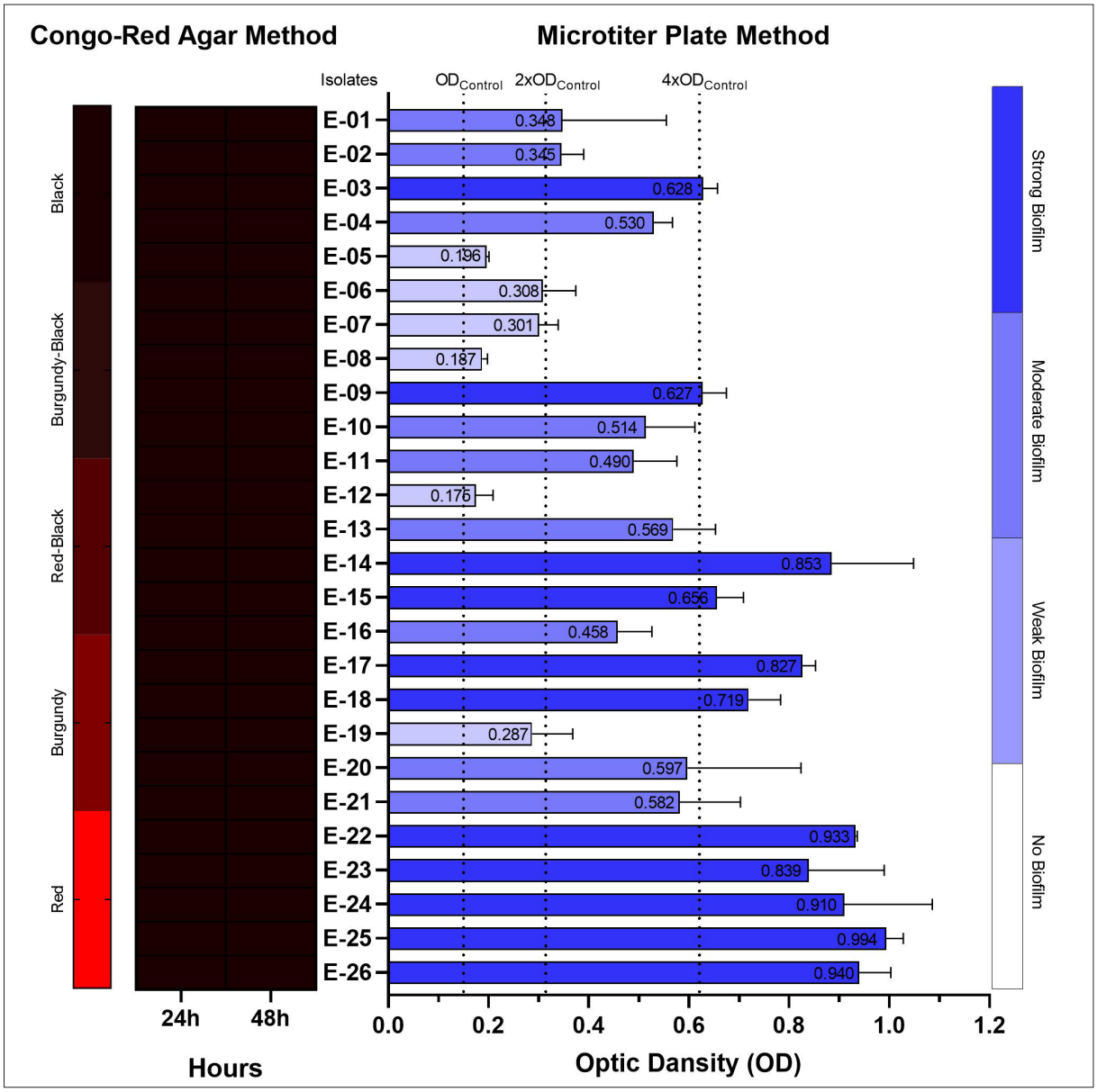

Figure 7. Graphical representation of test results performed for biofilm detection in *Enterobacter* spp. isolates.

Table 7. Comparative table of statistical agreement showing p-values from the McNemar test, kappa coefficient, and p-values from Cohen's analysis

Isolates	McNemar Test P-Values	Cohen's Kappa Test Kappa Values	Cohen's Kappa Test P-Values
S. aureus	0.023	-0.068	0.58
E. faecium	0.025	0.501	0.048
A. baumannii	<0.001	0	0.671
P. aeruginosa	<0.001	0.13	1
K. pneumonia	0.366	0.583	0.247
Enterobacter spp.	-	1	-

In contrast to the findings of our study, the biofilm percentage of 24 susceptible bacteria was found to be 45.8%, the biofilm percentage of 40 multidrug resistant (MDR) bacteria was found to be 47.5%, and the biofilm percentage of 56 extensive drug resistance (XDR) strains was found to be 78.6% with the CRA agar method. In studies conducted with the MtM method, 100% of susceptible, MDR, and XDR strains were found to be biofilm producers[45]. It is postulated that the discrepancy may be due to the CRA agar method being evaluated subjectively, with results dependent on the individual's interpretation.

The results of the present study demonstrate that both methods are entirely consistent in terms of detecting the presence of *Enterobacter* spp. biofilm. In a similar study, high rates of biofilm formation were observed in bacteria belonging to the *Enterobacter* spp. group, with the MtM method[44].

A key strength of our study is that it examines biofilm formation across the ESKAPE bacterial group rather than focusing on a single species, allowing for broader generalization across different bacterial strains, and thus a comparison of the two methods within a species. This approach provides a comprehensive perspective on biofilm formation. Additionally, the statistical analyses performed using McNemar's test and Cohen's Kappa test validate the agreement and discrepancies between the methods employed. However, the bacterial isolates used in this study were obtained from a single center. Furthermore, this study does not explore the relationship between biofilm formation and clinical outcomes, which could be an important aspect for future research.

In conclusion, given the clinical significance and multidrug resistance of ESKAPE pathogens, accurately determining their biofilm-forming capacity is essential for effective infection control and treatment planning. This single-center study demonstrated the consistency of the CRA and MtM methods, with the MtM method yielding more reliable results-particularly for non-fermenting species such as *P. aeruginosa* and *A. baumannii*. Since ESKAPE members can form biofilms on foreign materials, especially catheters, prostheses, heart valves, and urinary catheters, clinicians should prioritize early source control, select antibiotics with proven biofilm penetration, and adjust treatment durations accordingly. The findings emphasize the importance of selecting appropriate detection methods in clinical microbiology laboratories and integrating these results into comprehensive patient management strategies.

ETHICS COMMITTEE APPROVAL

This study was approved by the Sivas Cumhuriyet University Faculty of Medicine Non-Invasive Clinical Research Ethics Committee (Decision no: 2024/07-22, Date: 18.07.2024).

CONFLICT of INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: CÇ, MÖ

Analysis/Interpretation: CÇ, RA, MÖ

Data Collection or Processing: MH, ZS, CÇ

Writing: MÖ, AÇ, CÇ, RA

Review and Correction: AÇ, RA, MH, ZS

Final Approval: All of authors

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