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Investigating biofilm formation, *cblA* and *cblC* genes prevalence and antibiotic resistance profiles in *Burkholderia cenocepacia*

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ABSTRACT

Background: *Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (Bcc), a group of closely related and phenotypically similar species. In the present study, we aimed to determine the biofilm formation capacity of *Burkholderia cenocepacia* and the impact of clove oil on their biofilm formation capability with the detection of two genes that are involved in pili formation. **Methods:** In this study 10 bacterial strains belonging to *Burkhorderia cenocepacia* were used to investigate biofilm formation by Micro Titer Plate Method (MTP) with the detection of two genes that were involved in pili formation by PCR and antibiotic susceptibility profile were determined by Kirby and Bauer method. **Results:** Our findings indicated that 60% of *B. cenocepacia* strains exhibited strong biofilm formation which then lowered to 30% after treatment with Clove oil whereas 40% of the strains exhibited moderate biofilm formers which then lowered to 30% with 40% weak biofilm formers after treatment with Clove oil. The results of PCR amplification showed that all 7 strains were positive for *cblA* gene at 238bp whereas only 2 of 7 strains were positive for *cblC* genes at 220bp. The bacteria exhibited varying susceptibilities to antibiotics with 100% sensitivity toward meropenem and 90% to resistance toward trimethoprim-sulphamethoxazole. **Conclusion:** Our results indicate that clove oil has the potential to be used as a therapeutic agent since it dramatically lowers the formation of biofilms, especially in strong biofilm producers. According to molecular analysis the *cblA* gene was consistently present in all strains while the *cblC* gene was detected in just a portion of the strains, indicating genetic diversity. *B. cenocepacia* strains also showed significant resistance to trimethoprim-sulfamethoxazole, despite being extremely sensitive to meropenem. These findings highlight how complicated antibiotic resistance is in *B. cenocepacia*.

Introduction

Burkholderia cenocepacia is a Gram-negative, rod-shaped bacterium that is commonly found in soil and water environments and may also be associated with plants and animals, particularly as a human pathogen [1].

It is one of over 20 species in the *Burkholderia cepacia* complex (BCC) and is notable due to its virulence factors that render it a prominent opportunistic pathogen responsible for life-threatening, nosocomial infections in immunocompromised patients, such as those with cystic fibrosis or chronic granulomatous disease [2].

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The genome of *B. cenocepacia* strains contains more than a dozen putative virulence-enhancing factors in genomic islands and metabolism-associated genes that may be involved in all virulence-enhancing factors [3]. Unfortunately, BCC organisms are difficult to eradicate because of their capacity to form biofilms and their innate resistance to a wide range of antibiotics [4].

BCC members, including *B. cenocepacia*, produce biofilms on abiotic and biotic surfaces [5]. The process of biofilm formation is advantageous as it offers protection to the producing organisms from antibiotics, disinfectants, or dynamic environmental conditions. It also helps them to survive in nutrient-deficient or oligotrophic conditions [6].

Clove oil is a kind of aromatic oil extracted from the buds and leaves of clove trees which seems promising for its anti-adhesion and its anti-biofilm effects against many bacterial pathogens. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [7]. CEO has been used as natural food preservative and colorant based on its antibacterial and health promoting activities[8,9].Generally, eugenol (4-allyl-2-methoxyphenol) accounts for 60%–90% of the total composition of CEO and is the source of the antiseptic property of CEO[10]. Eugenol and CEO have been confirmed to be effective in combating some pathogenic bacteria including S. aureus, E. coli, Proteus mirabilis, Pseudomonas aeruginosa and Streptococcus mutans[11].

The cable pilus is a well-established virulence factor associated with increased binding to epithelial cells, persistence, and an increased proinflammatory response [12]. The *cblA* gene encodes the major subunit protein of the pilus from an epidemic *Burkholderia cenocepacia* strain highly transmissible among CF patients [13] whereas *cblC* encode outer membrane usher which form a pore in the membrane for the transport of assembled pili [14].

In general, *Burkholderia* manifests innate resistance to aminoglycoside antibiotics and widespread resistance to many beta-lactam agents, including extended-spectrum penicillins such as piperacillin [15]. Antibiotic resistance of *Burkholderia cenocepacia* could be intrinsic or acquired. The first one is independent of antibiotic selective pressure and horizontal gene transfer; instead, it is the result of inherent structural or functional characteristics. On the other hand, bacteria can acquire resistance to antibiotics, such as mutations in drug targets or transfer of resistance genes through phage-mediated transduction and mobile plasmids [16]. In the present study we aimed to determine the biofilm formation capacity of *B. cenocepacia* and effect of Clove oil on their biofilm formation capability with detection of two genes that involved in pili formation. In addition the antibiotic susceptibility profile of *B. cenocepacia* was determined.

Materials and Methods

Bacterial strains

In the current study, ten bacterial strains belonging to *Burkholderia cenocepacia* that were isolated from cystic fibrosis patients were used. All bacterial strains were obtained from the Department of Biology /College of Science/University of Mosul, and they were diagnosed with universal 16S rRNA and *hisA* genes sequencing.

Biofilm formation detection by Micro Titer Plate method (MTP)

The microtiter plate assay was used to quantitatively detect and measure biofilm formation. Bacterial strains were cultured on nutrient agar (LAB, England) at 37°C for 24h. After incubation, bacterial suspensions were prepared in Brain Heart Infusion (BHI) broth (Merck, Germany), adjusting to McFarland standard (No. 0.5). Negative controls (sterile BHI broth) were used as blanks. Then, 180 µl of BHI broth with 1% glucose was added to 10 triplicates of a 96-well plate, followed by 20 µl of bacterial suspension. The plate was incubated at 37°C for 24h. After incubation, the culture medium was discarded, and each well was rinsed with 200 µl PBS, then dried at 60°C for 1h. Each well was stained with 200 µl crystal violet for 20 minutes, washed twice with PBS (pH 7.2), and dried at 60°C for 1h. The dye in the biofilm was solubilized with 200 µl of 96% ethanol, and the absorbance was measured at 570 nm. Wells with OD values higher than the blank were considered biofilm formers [17,18]. The strains were classified into four categories, according to the mean optical densities (ODi) in relation to the ODc results. If ODi ≤ ODc; considered non-biofilm former, ODc≤ODi≤2*ODc; considered moderately biofilm former and if 2*ODc≤ODi which considered strongly biofilm former [18].

Antibiofilm formation test using Clove oil

The procedure of the biofilm formation test was repeated as in (2.2) in addition of adding 40 µl of Clove oil to each well [17,18].

Molecular detection of *cblA* and *cblC* genes

Genomic DNA Presto TM mini kit (Geneaid Company, Taiwan) was used for the DNA extraction from 7 strains following the manufacturer's instructions. The Master Reaction Mixture was prepared for each PCR reaction, by mixing the 2µl of DNA sample and the primer (1µl for each primer at a concentration 10 pmol/µl) with the Master-mix (10µl) inside an Eppendorf tube with a capacity of 0.2 ml, where the volume of the reaction was completed to 20µl with nuclease-free water. The mixture was centrifuged with a microfuge device for a period of (3-5) seconds to ensure that the reaction components were mixed and collected at the bottom of the tube. The PCR was conducted at 94 C for 5 min, followed by 35 cycles of 94 C for 30s, 50 C for 15s, and 72 C for 30s, and a final extension at 72 C for 10 min. Moreover, agarose gel electrophoresis was applied at 2X concentration to visualize the PCR products, 238bp for cable A and 220bp for cable C genes that were compared with 1000bp ladder. For *cblC* gene primer was designed by using the Primer-Blast program from NCBI whereas for *cblA gene* the primer sequence obtained from research article as shown in table (1) and to estimate an appropriate annealing temperature the NEB Tm calculator program was used.

Antibiotic sensitivity test

A test of bacterial sensitivity to antibiotics was carried out on 10 strains by the disc diffusion method on Mueller-Hinton agar medium (HiMedia, India) by the modified Kirby 1966 and Bauer method [19]. Using antibiotic discs shown in table (4) (Bioanalyze/Turkey) and following the recommendations of the Clinical Laboratory Standard Institute [20] as follows: Bacterial suspensions were prepared by transferring 2-3 colonies from a culture grown for 24 hours on nutrient agar medium into 5 ml of sterile physiological saline and the turbidity of the suspension was controlled in comparison with the turbidity of McFarland's standard constant turbidity solution (NO. 0.5). An amount of the suspension prepared above was spread on Mueller-Hinton agar plates, left for 2-3 minutes to get impregnation, and then the antibiotic discs were placed using sterile forceps at the rate of 7 discs per plate, and incubated at 37 °C for 24 hours. After incubating the inoculated agar plate with antibiotic discs, the zone of inhibition was examined. The size of the zone of inhibition correlates with the effectiveness of the antibiotic. Larger zones indicate greater sensitivity of the bacteria to the antibiotic, while smaller zones or the absence of a zone suggest resistance.

Results

Detection of biofilm formation in *Burkholderia cenocepacia strains*

The quantification result of biofilm formation by the microtiter plate method revealed that all *Burkholderia cenocepacia* strains produced biofilm in microtiter plates at different ranges (60%) of strains were characterized as strong biofilm producers, and (40%) of strains were moderate biofilm production as shown in Table (2) and Figure (1-A).

After treatment of biofilm with clove oil, the formation of biofilm reduced from 60% to 30% for those strains that produced strong biofilm and from 40% to 30% for those strains that produced moderate biofilm with 40% of the strains produced weak biofilm as shown in figure (1-B) and Table (2).

Ampification of *cblA* and *cblC* genes

The results of PCR amplification showed that all 7 strains were positive for *the cblA* gene at 238bp whereas only 2 of 7 strains were positive for *cblC* genes at 220bp as shown in Figures (2),(3), and table (3).

Antibiotic Susceptibility of *Burkholderia cenocepacia* strains

A sensitivity test was conducted for the ten *Burkholderia cenocepacia* strains towards 7 types of various antibiotics. Table (4), and Figures (4) and (5) show the results of this test. It was observed that there was a variation in the patterns of sensitivity and resistance toward the studied antibiotics according to different bacterial strains. The results of the study showed that *Burkholderia cenocepacia* strains were given high resistance (90%) to trimethoprim-sulphamethoxazole, while (80%) of strains appeared resistant to tetracycline and (60%) to chloramphenicol and ceftazidime. The resistance of strains to levofloxacin appears in (40%) of strains, but the resistance of bacterial strains to piperacillin was recorded in (20%). Our data showed that the resistance to meropenem was 0% (100% sensitive) which is the lowest resistance rate among all applied antibiotics in this study.

Table 1. Primers used in this study.

Target Gene	Primers Sequences		Size (bp)	Reference
cblA	GCAGCTGTAGTGAACACG TCTGACCGATCGACAGCG	F R	238	Tomich *et al* (2004)
cblC	AAGGCGAAGGGAACATCGAG AAATT CCAGTTCCCGCCGAT	F R	220	In the current study

Table 2. Biofilm formation ability of *Burkholderia cenocepacia* strains.

Biofilm formation ability	*Burkholderia cenocepacia* strains										Control
	ES14	ES68	ES8	ES11	ES12	ES23	ES3	ES4	ES50	ES7	
Without Clove oil	+++	+++	+++	+++	++	++	+++	+++	++	++	-
With Clove oil	+++	+	++	++	+++	+	++	+	+	+++	-

Biofilm formation: +++ strong, ++ moderate, + weak, - not produced.

Table 3. Prevalence of *cblA and cblC* genes among *Burkholderia cenocepacia* strains.

NO	*Burkholderia cenocepacia* strains	PCR result for *cblA* gene	PCR result for *cblC* gene
1	ES14	+	+
2	ES68	+	-
3	ES8	+	-
4	ES11	+	-
5	ES12	+	-
6	ES23	+	+
7	ES3	+	-

Table 4. Antibiotic Susceptibility pattern of *Burkholderia cenocepacia* strains.

No.	Antibiotics	Concentration µl/disk	Results of ten strains			
			R	I	S	Resistant%
1.	Levofloxacin	5	4	6	0	%40
2.	Trimethoprim-Sulfamethoxazole	25	9	1	0	%90
3.	Tetracycline	10	8	2	0	%80
4.	Meropenem	10	0	0	10	0
5.	Chloramphenicol	10	6	3	1	%60
6.	Ceftazidime	30	6	2	2	%60
7.	Piperacillin	100	2	1	7	%20

Figure 1. A. The ability of *Burkholderia cenocepacia* strains to form biofilm. B. Effect of Clove oil on biofilm formation.

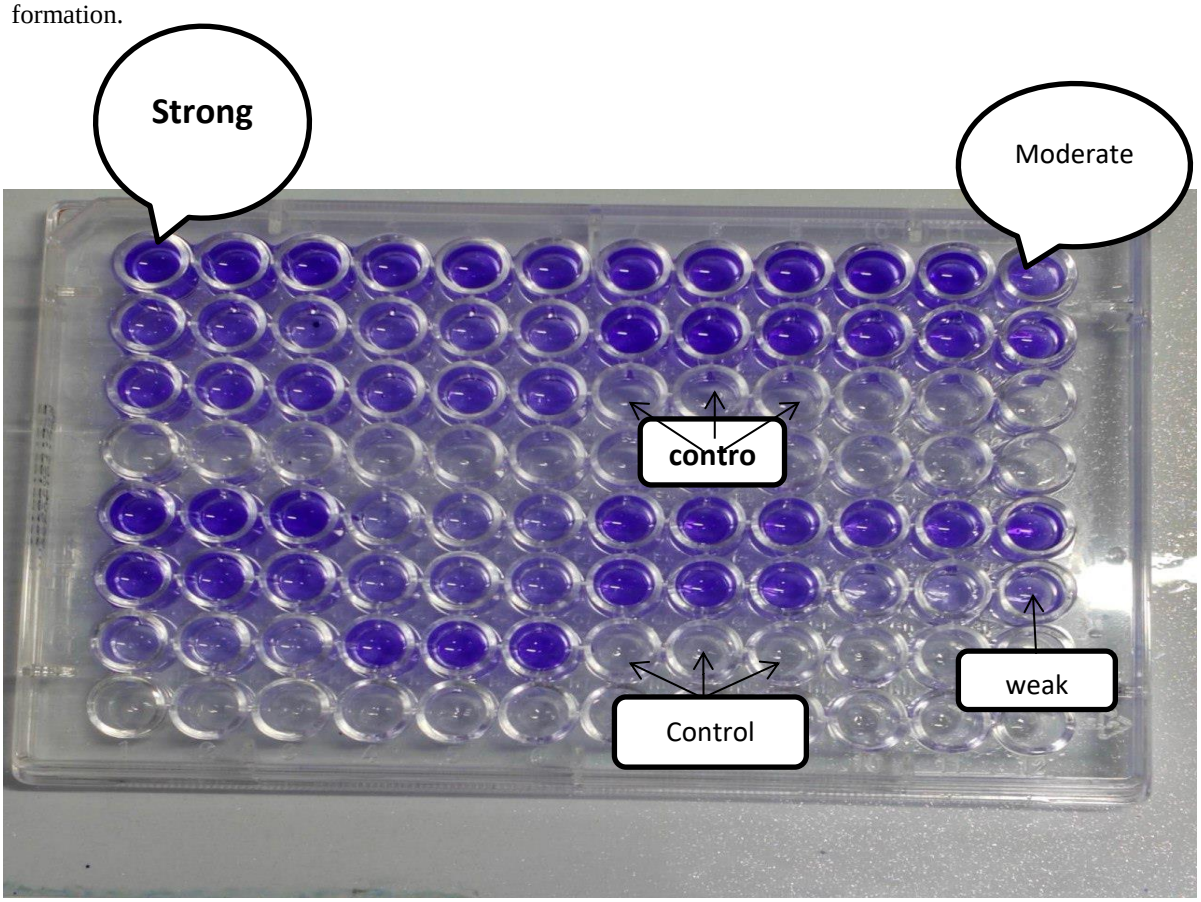


Figure 2. *cblA* genetic segment at 238 bp in 2% agarose gel electrophoresis at 50 volt for 1 h, the bands visualized under U.V light.

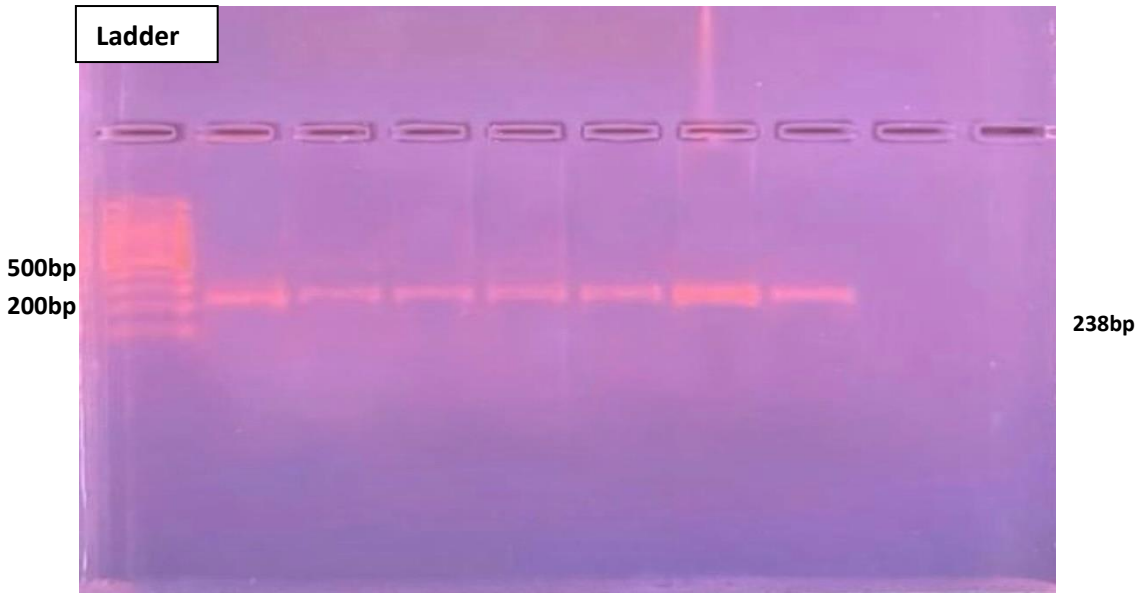
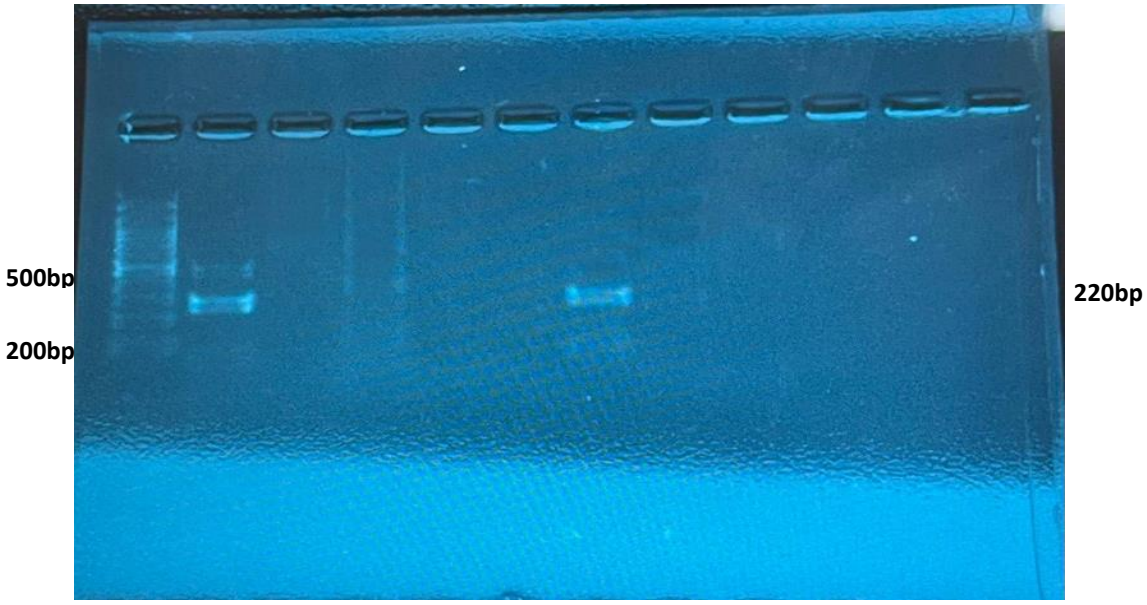


Figure 3. *cblC* genetic segment at 220 bp in 2% agarose gel electrophoresis at 50 volt for 1 h, the bands visualized under U.V light.



PCR result for *cblA and cblC* genes: + Present, - Absent

Figure 4. Sensitivity test results of *Burkholderia cenocepacia ES8.*

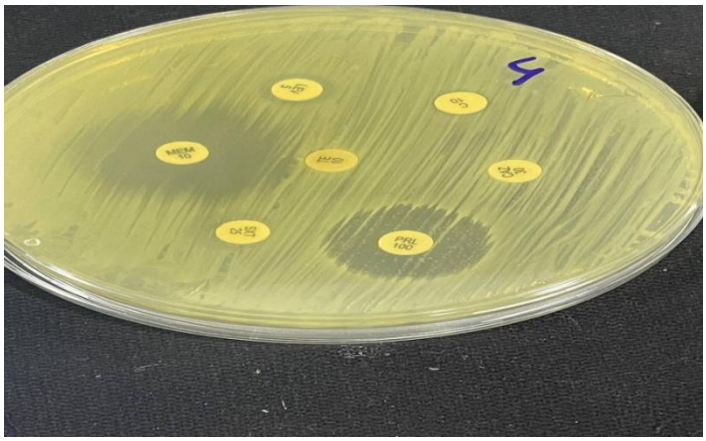
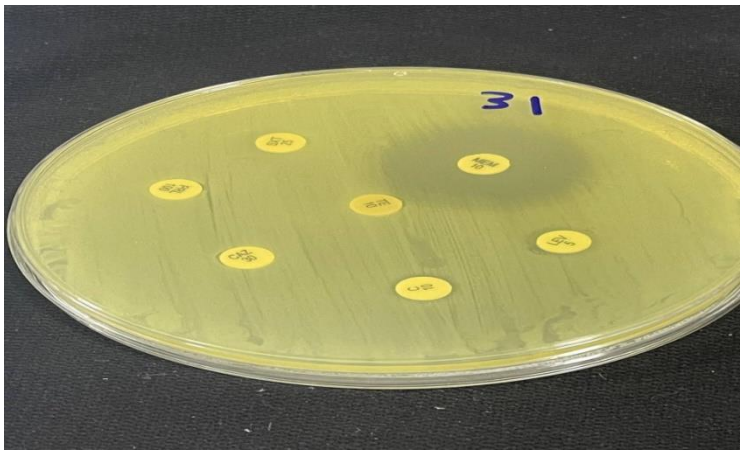


Figure 5. Sensitivity test results of *Burkholderia cenocepacia ES11*.



Discussion

The result of biofilm formation is consistent with several other studies that reported the great ability of clinical strains of *B. cenocepacia* to form biofilms in vitro and in vivo [21,22]. Similarly, strains phytopathogenic of *B. cenocepacia* also showed a high capability of forming biofilms in onion tissue [23].

Biofilm formation is a common trait of BCC strains and has been associated with the persistence of BCC infections and the increased resistance to antibiotics relative to planktonic cells [24]. The total (10) clinical *B. cenocepacia* strains that were isolated from cystic fibrosis patients exhibited great ability to form biofilm this result is consistent with previous studies, which have shown that clinical strains of *B. cepacia* complex exhibit a high propensity for biofilm formation in patients with cystic fibrosis [25]. *P. aeruginosa and B. cenocepacia* are opportunistic human pathogens capable of forming persistent biofilm infections, e.g. in the lungs of cystic fibrosis patients [21]. An important feature of *P. aeruginosa* and *B. cenocepacia* infection is their ability to form biofilms, which is one of the contributing factors to reduced antibiotic efficacy and poor patient prognosis [26].

Our results demonstrated that clove oil plays a significant role in reducing biofilm formation, as it effectively decreased the ability of *B. cenocepacia* to form biofilms. This finding is consistent with previous studies that highlighted the antimicrobial and biofilm-inhibitory properties of clove oil, further supporting its potential as an agent to disrupt biofilm formation in pathogenic bacteria. Clove Essential Oil (CEO) seems promising for its anti-adhesion and anti-biofilm effects against *Listeria monocytogenes* and *Salmonella enteritidis*. Therefore, EOs and their active compounds seem a good and natural strategy in food processing environments to reduce the impact of biofilms [7]. The anti-adhesion assets of clove phenolic compounds, such as eugenol, on *Salmonella* spp. and *L. monocytogenes* have been outlined in the literature. It was found that the EO of clove inhibited cell attachment of *Salmonella* spp. by 65.67% [27]. In another study clove EO at a concentration of 1 mg/ml inhibited the initial adhesion of two strains of *L. monocytogenes* by almost 30% [28]. It has also been reported that eugenol can suppress the biofilms of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Enterococcus faecali* [29]. Moreover, it was proved the main component of CEO may inhibit the adhesion of *S. mutansto* glass, and prevent the formation of biofilm [30]. It was observed that eugenol can inhibit the biofilm-forming ability by affecting the cytoplasmic membrane [27].

Regarding the *cblA* gene, the *cblA* gene encodes the major structural subunit of cable pili appears in *B. cenocepacia* due to its essential role in biofilm formation, which is crucial for the bacterium's survival in chronic infections, particularly in cystic fibrosis patients [14]. Our result is consistent with the result obtained by Malešević study, who reported that all identified *Burkholderia cenocepacia* strains carry *the cblA* gene, phenotypic differences were observed regarding other virulence factors [31]. Members of the *Burkholderia cepacia complex* (BCC) bacteria are equipped with five types of morphologically distinct pili, namely mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable pilin [32]. Cable pili are peritrichous surface-associated organelles elaborated by strains of *B. cenocepacia*, as well as other species of the BCC. The shape of cable pili resembles intertwined cables, from which these organelles derive their name. Expression of cable pili by *B. cenocepacia* has been correlated with increased transmissibility of strains and adverse clinical outcomes. Cable pili have been shown to facilitate bacterial binding to both mucin and CF respiratory epithelia, suggesting a direct role for cable pili in mediating colonization [33].

The *B. cenocepacia* cbl locus is comprised of at least five genes, designated *cblB, cblA, cblC, cblD, and cblS*. The first four genes encode the structural and accessory components of the cable pilus biogenesis pathway. The *cblA* gene encodes the major structural subunit of cable pili, while *cblB*, *cblC*, and *cblD* are predicted to encode the periplasmic chaperone, outer membrane usher, and minor structural subunit, respectively [14]. Usher proteins are outer-membrane porin-like proteins and create a membrane pore that allows formed pili to be transported. Thus, the features of *CblC* are consistent with the notion that this protein serves as an usher for cable pili assembly on the bacterial surface [34].

The results of antibiotic sensitivity test are somewhat consistent with results obtained in various other studies regarding BCC. It was found that the majority of *Burkholderia cenocepacia* strains were resistant to chloramphenicol (77%, 27/35) followed

by levofloxacin (34%, 12/35) and meropenem (8.5%, 3/35) [35]. The studies reported that 100%, 88.5%, 94%, and 87% of the *Burkholderia cenocepacia* strains were susceptible to meropenem [36, 37, 38,39]. However, in another study on BCC strains it was found that 85 % were resistant to sulfamethoxazole-trimethoprim, 76 % were resistant to chloramphenicol, 57% and 55% were resistant to ceftazidime and tetracyclines respectively [40]. Moreover, a local study showed that 20 BCC strains were resistant to vancomycin, neomycin, tetracycline, and chloramphenicol by 100%, 95%, 95% and 95%, respectively. While the resistance to trimethoprim/sulfamethoxazole was 67%, levofloxacin was 62%, gentamicin was 48%, ceftazidime was 38% and resistance to meropenem was 33% [41].

Moreover, a study by Bush and Bradford showed that all the strains were uniformly susceptible to piperacillin [42]. This result is somewhat in agreement with our reported result on piperacillin. The four main mechanisms of antibiotic resistance in *Burkholderia cenocepacia* are: prevention of access to target due to (1) reduced permeability of the cell envelope or (2) increased efflux activity; (3) mutation in antibiotic target; (4) enzymatic modification or inactivation of the drug by hydrolysis or transfer of a chemical group [43]. Resistance to clinically significant antibiotics, chloramphenicol, levofloxacin, and tetracycline correlates with the presence of RND efflux pumps and tetracycline resistance genes in these strains [16].

Conclusion

This study provides important insights into the biofilm-forming ability, genetic makeup, and antibiotic resistance of *Burkholderia cenocepacia* isolated from cystic fibrosis patients. The findings highlight that clove oil significantly reduces biofilm formation, especially in strong biofilm producers, supporting its potential as a natural therapeutic agent. Molecular analysis confirmed the consistent presence of the *cblA* gene and variable presence of the *cblC* gene, indicating genetic diversity among strains. The antibiotic susceptibility testing revealed a concerning level of resistance to several antibiotics, particularly trimethoprim-sulfamethoxazole and tetracycline, while meropenem remained highly effective. These results emphasize the need for continuous surveillance of resistance patterns and exploration of alternative treatments like essential oils to combat biofilm-related infections caused by *B. cenocepacia*.

Conflict of interest:

The authors confirm that they have no conflicts of interest to declare.

Data availability

The data supporting this study are available from the corresponding author upon reasonable request.

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The authors received no financial support or funding for this research.

Authors contribution

All authors made substantial contributions to the following:

(1) The conception and design of the study: Ahmed Nadhim Rashid, Essra Ghanim AlSammak.

(2) Acquisition, analysis, and interpretation of data: Ahmed Nadhim Rashid.

(3) Drafting or critical revision of the article: Both authors.

(4) Final approval of the version to be submitted: Both authors.

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