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Research Article

Antimicrobial Susceptibility Testing and Biofilm Production of *Burkholderia cepacia* Complex Organisms from Ultrasound Gels in India

Ravichandran Karthikeyan, Bhoj R Singh*, Akanksha Yadav, Himani Agri, Varsha Jayakumar, Vinodhkumar OR, Dharmendra K Sinha

Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Izatnagar, India

***Corresponding Author:** Bhoj R Singh, Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Izatnagar, India.

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Abstract

Burkholderia cepacia complex (Bcc) bacteria are versatile difficult to treat pathogens with extraordinary metabolic diversity. They are important nosocomial pathogens having multi-drug resistant potential. Contamination and biofilm formation on medical devices and equipment are the important reasons for the multiple outbreaks in recent decades. In this study, we subjected 32 isolates of Bcc to the two important virulent traits such as antimicrobial susceptibility and biofilm producing ability with and without different stress conditions. All the isolates were susceptible to co-trimoxazole (TMP-SMX) (100%) suggesting it as the preferred drug or the drug of the first choice for treating Bcc from ultrasound gels infections. The isolates showed the varied degree of susceptibility to meropenem (91%), doxycycline (85%), gatifloxacin (85%), piperacillin+ tazobactam (82%), ceftazidime (79%), and levofloxacin (71%). Multiple antibiotic resistance (MAR) indices for all the isolates were >0.2 and three isolates had >0.5. Most of the Bcc isolates (81.25%) were weak biofilm producers, while three strains each of *B.cepacia, B. cenocepacia* and *B. pseudomultivorans* produced moderate and strong types of biofilms under standard laboratory conditions. The combination of change in pH with other stress conditions significantly increased the biofilm formation. This study found the difference in the antimicrobial susceptibility pattern of Bcc isolates from ultrasound gels and the adaptability to in-vitro stress conditions explaining the variability of virulence among Bcc species.

Keywords: *Burkholderia cepacia* Complex (Bcc); Ultrasound Gels; Antimicrobial Susceptibility Testing; Multiple Antibiotic Resistance

Introduction

Burkholderia cepacia complex (Bcc) organisms are important nosocomial pathogens causing severe disease, especially in immunocompromised individuals with cystic fibrosis and chronic granulomatous disease [1]. It causes "cepacia syndrome", characterized by sepsis and necrotizing pneumonia with an overall negative prognosis [2]. Bcc bacteria can be found in a variety of natural environments, including water, plants, soil, and food [3]. While they do not grow well in dry environments, they can survive in moist environment for months [4]. They do not exhibit a specific colonisation in nosocomial infections and may appear in immunocompromised patients as asymptomatic colonisers, causing pneumonia, septicemia, urinary tract infections, and post-operative wound infections [5]. The main route for Bcc infections is the use of contaminated medical products. A total of 14 Bcc outbreaks were reported through use of the contaminated ultrasound gels [6]. They either develop resistance to various antibiotics, toxins and preservatives or are inherently resistant to them. Several mechanisms contribute to the bacteria's pan-resistance, including inducible chromosomal-lactamases, altered

penicillin-binding proteins, restricted membrane permeability, function of porins, drug target modifications, presence of multiple multidrug efflux pumps, and changes in lipopolysaccharide structure. Several members of this complex are frequently reported in patients, mainly *B. cenocepacia*, *B. multivorans* and *B. vietnamiensis*. The Bcc bacteria are intrinsically resistant to high-end antibiotics including polymyxins, making these drugs, often used for treating infections caused by most of the other drug-resistant Gram-negative bacteria, useless. The resistance mechanism is complex and is thought to be partially attributable to a unique LPS structure [7]. Clinical and Laboratory Standards Institute (CLSI) recommends strict criteria for antibiotic susceptibility testing of Bcc. They provided the interpretative standards for ticarcillin–clavulanate, levofloxacin, ceftazidime, meropenem, minocycline and trimethoprim-sulfamethoxazole [8]. Unfortunately, there is a lack of studies on therapeutic options for Bcc infections in humans and animals. Currently, treatment options are based on the antibiotic susceptibility testing of the infecting strains on a case-by-case approach [9].

Biofilm cells are typically immersed inside a self-produced matrix of extracellular polymeric substances (EPSs) that are attracted to one another and a surface in complex forms of sessile microbial communities [10]. The matrix EPSs include lipids, nucleic acids, polysaccharides, and proteins; they play a role in maintaining the structural integrity of the biofilm, facilitating adhesion to surfaces and forming a network of cohesive polymers that ensure biofilm cells remain stationary [10]. The Bcc bacteria can form biofilms on abiotic (e.g., plastics and glass) and biotic (e.g., epithelial cells) surfaces. They can form interspecies sessile cells with *Pseudomonas aeruginosa* [11]. The Bcc biofilms are highly resistant to multiple antibiotics much more than the resistance in the planktonic cells. The delayed penetration of antimicrobials, biofilm-specific adaptive stress response, biofilm heterogeneity, and the presence of persister cells are regarded to be some of the factors behind resistance in biofilms. Sessile Bcc is relatively insensitive for Dettol (5%, 5–30 min), acetic acid (1.25%, 15–60 min), hot water (70°C, 15–60 min), ethanol (70%, 2–10 min), NaOCl (0.05%–0.3%, 5 min), hydrogen peroxide (0.5–3%, 30 min), and cetrimide (0.15%, 15 min) treatment but they are shown to reduce at least 99.99% in planktonic cultures [12]. The ability of the *B. cepacia* complex to produce biofilm under stress conditions is seen in the lungs of CF patients where oxygen is limited and anaerobic conditions prevail [13,14]. Keeping this in mind, we performed antimicrobial susceptibility testing and assessment of biofilm formation ability under different stress conditions of Bcc isolates from ultrasound gels (USGs) of various veterinary clinical settings in India.

Materials and Methods

- **Bcc isolates:** A total of 32 Bcc strains isolated from different states of India were revived from the clinical epidemiology laboratory, ICAR- Indian Veterinary Research Institute for this study. They were confirmed up to species level with MALDI-TOF analysis and *rec*A gene partial sequencing.
- **Antimicrobial susceptibility testing:** Antimicrobial susceptibility testing (AST) was carried out by disk diffusion assay as per the CLSI guidelines [8]. Additionally, we have tested the isolates for antibiotics for which the Bcc members are said to be intrinsically resistant by the CLSI. The antibiotics tested are listed in table 1. The AST was performed with Kirby - Bauer disk diffusion test using commercially available disks (HiMedia, Mumbai). Briefly, isolates were grown overnight in Muller Hinton Broth (MHB) at 37°C, adjusted to 0.5 McFarland standard, and inoculated on the MHA plate with a sterile cotton swab. Sterile antibiotic discs were placed over it. After 24 hours, the diameter of clear zones of growth inhibition around each of the antibiotic discs was measured. The interpretation of the AST was based on the CLSI guidelines. The antibiotics which did not have CLSI standards for Bcc were interpreted using standards for *Pseudomonas aeruginosa*.
- **Multiple antibiotic resistance (MAR) index:** The resistance rates of all the Bcc isolates against the total number of antimicrobials tested were analysed to calculate the MAR index (the ratio of the number of antimicrobials to which the isolate was resistant and the total number of antimicrobials to those the isolate was exposed). A MAR Index < 0.2 indicated low risk, while a MAR Index ≥ 0.2 indicates a high risk of antimicrobial resistance [15,16].

Biofilm formation assay

Biofilm formation assays were done as per standard methodology with defined modifications [17,18]. Briefly, the overnight liquid culture of each Bcc isolate was transferred to Luria

S. No	CLSI recommended antimicrobials	S. No	Other classes of antimicrobials
1.	Ticarcillin-Clavulanate (75/10 mcg)	1.	Tetracycline (30 mcg)
2.	Ceftazidime (30 mcg)	2.	Doxycycline (10 mcg)
3.	Meropenem (10 mcg)	3.	Gatifloxacin (5 mcg)
4.	Minocycline (30 mcg)	4.	Ciprofloxacin (5 mcg)
5.	Levofloxacin (5 mcg)	5.	Fusidic acid (30 mcg)
6.	Sulfamethoxazole – Trimethoprim (Co-trimoxazole) (TMP-SMX) (23.75/1.25 mcg)		
7.	Chloramphenicol (50 mcg)		
S. No	**Intrinsically resistant antibiotics**	**S. No**	**Intrinsically resistant antibiotics**
1.	Ampicillin (10 mcg)	8.	Polymixin B (300 Units)
2.	Amoxicillin (30 mcg)	9.	Cefepime (30 mcg)
3.	Amoxicillin+clavulanic acid (20/10 mcg)	10.	Aztreonam (30 mcg)
4.	Piperacillin (100 mcg)	11.	Colistin (10 mcg)
5.	Piperacillin+tazobactam (100/10 mcg)	12.	Imipenem (10 mcg)
6.	Cefotaxime (30 mcg)	13.	Ceftriaxone
7.	Amikacin (30 mcg)		

Table 1: Antibiotics tested for the susceptibility of Bcc isolates from ultrasound gels.

Broth (LB) medium and grown at 37°C and adjusted to OD_{640} of 0.5, and 20 µl of this cell suspension was used to inoculate the wells of a 96-well polystyrene microtiter plate containing 180 µl of LB medium. Un-inoculated wells containing LB medium were used as negative controls. Plates were incubated at 37°C for 24 h. For biofilm quantification, the culture media and unattached bacterial cells were removed from the wells by careful inverting to empty wells and then rinsing with sterile physiological saline solution by vigorous shaking (three times, 250 µl for each rinse). Then 200µl of 99% methanol was added to the well for cell fixation and kept at room temperature for 15 minutes. The wells were emptied and left to stand outside for drying. Adherent bacteria were stained with 200 µl of a 1% crystal violet solution for 15 min at room temperature (50 ml of the solution was prepared by adding 1% [wt/vol] crystal violet in 10 ml of 95% ethanol and then adding 40 ml of water). After three gentle rinses with 200 µl of water each time, the dye associated with the attached cells was solubilized in 160 µl of 33% glacial acetic acid and OD was measured at 590 nm with a microplate reader. The OD values were compared to the control, and an interpretation was made (Table 2) [19].

Biofilm ability	Comparison with OD	Classification
Non-adherent	OD ≤ ODc	0 (Non-biofilm-former (NBF))
Weakly-adherent	ODc < OD ≤ 2x ODc	+
Moderately-adherent	2x ODc < OD ≤ 4x ODc	++
Strongly adherent	4x ODc < OD	+++

Table 2: Interpretation of Biofilm formation.

ODc = OD_{avg} of negative control + 3 × standard deviation (SD) of ODs of the negative control.

Biofilm production under stress conditions

The biofilm formation ability was investigated under different stress conditions (Table 3) like pH, temperature, dynamic stress and change in oxygen levels [20,21]. The statistical significance of the difference in biofilm production due to each stressor and without stressor (pH 6.7, Normal) was determined by one way ANOVA test using SPSS software (IBM SPSS Version 20.0).

Stress Conditions	pH	5.8	6.7	7.4	8
CO_2		5% CO_2			
Oxygen levels		Microaerophilic			
Dynamic		Revolutions Per Minute (RPM) 100			

Table 3: Biofilm formation under stress conditions.

Results

A total of 32 Bcc isolates from USGs were tested for their antimicrobial susceptibility and biofilm formation with and without stressors. The AST results showed that all the isolates were completely resistant to fusidic acid, polymyxin B, colistin, ampicillin, amoxicillin, and amoxicillin + clavulanic acid (Figure 1). Most of the isolates were resistant to ticarcillin + clavulanic acid (88%). For intrinsically resisted antibiotics such as piperacillin, piperacillin+ tazobactam, amikacin, cefepime, imipenem, cefotaxime, ceftriaxone and aztreonam isolates were not equally resistant. All the isolates were susceptible to co-trimoxazole (TMP-SMX) (100%). The isolates showed a varying degree of susceptibility to meropenem (91%), doxycycline (85%), gatifloxacin (85%), piperacillin+ tazobactam (82%), ceftazidime (79%), and levofloxacin (71%) (Figure 2).

MAR indices of Bcc isolates exposed to 10 different classes of antimicrobials (β-lactams, carbapenems, aminoglycosides, sulphonamide + dihydrofolate reductase inhibitor, chloramphenicol, tetracyclines, quinolones, macrolides, monobactams, and polypeptides were >0.2 (Figure 3). Three isolates had MAR indices >0.5.

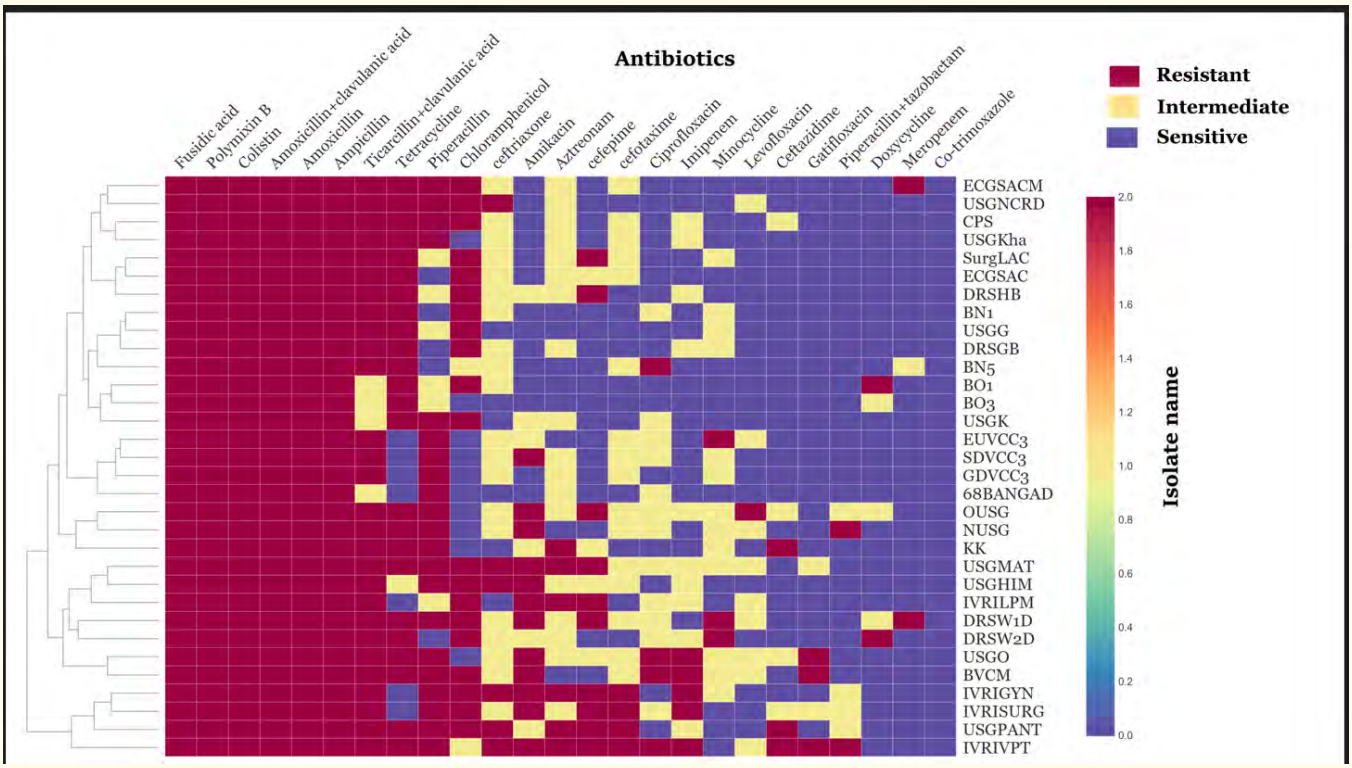

Figure 1: Heatmap of susceptibility pattern of all Bcc isolates against the tested antimicrobials.

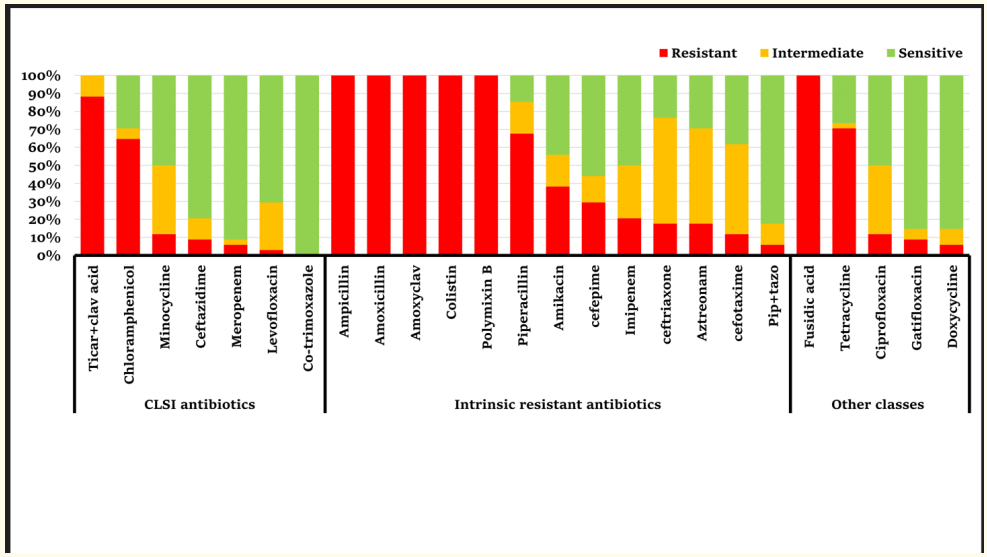

Figure 2: Resistant, intermediate and susceptible percentage of all Bcc isolates against the tested antimicrobials.

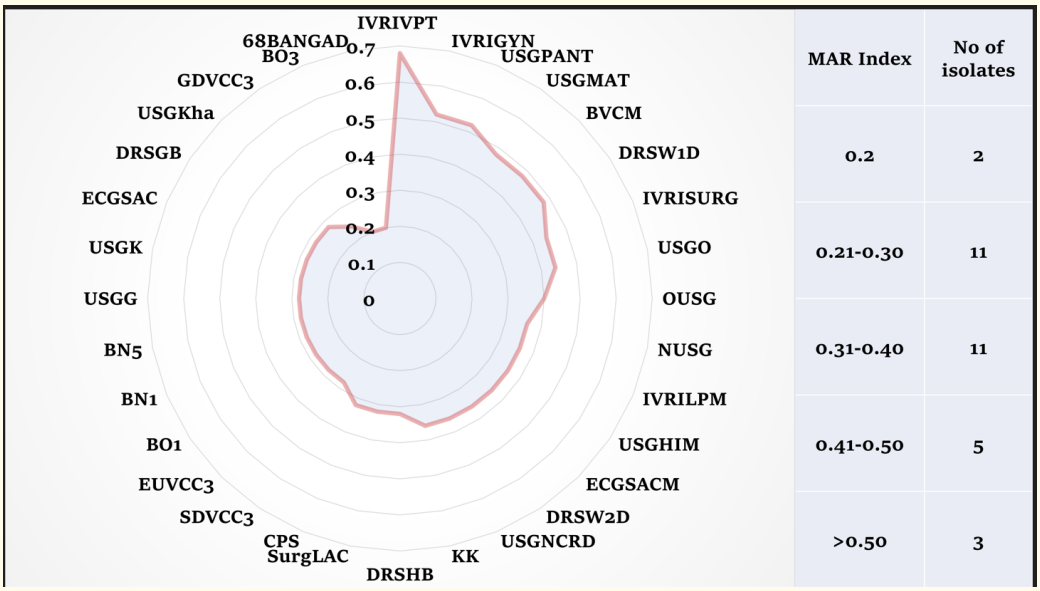

Figure 3: Multiple antibiotic resistant (MAR) indexes of Bcc isolates.

Most of the Bcc isolates (81.25%) were weak biofilm producers, while three strains each of *B. cepacia, B. cenocepacia* and *B. pseudomultivorans* species produced moderate and strong types of biofilms under standard laboratory conditions. Heatmap demonstrating biofilm formation under various stress conditions was constructed to summarize the biofilm potential (Figure 4). The pH at 7.4, enhanced the biofilm formation ($p < 0.05$). But other conditions such as 5% CO_2, microaerophilic conditions and shaking at 100 RPM not significantly affected biofilm formation at neutral pH. However, elevated CO_2 level (5% CO_2) enhanced the formation of biofilm at pH 5.8 and 7.4. The combination of change in pH with other stress conditions significantly increased the biofilm formation (Table 4).

pH, Stress conditions	df	Mean Square	F	Significance (p)
5.8, 5% CO_2	1	7.182	6.593	.013
5.8, Microaerophilic#	1	.021	.037	.848
5.8, Normal*	1	4.328	3.900	.053
5.8 RPM 100	1	1.901	1.415	.239
6.7, 5% CO_2	1	.015	.021	.886
6.7, Microaerophilic#	1	.829	1.414	.239
6.7, RPM 100	1	.092	.095	.758
7.4, 5% CO_2	1	16.763	11.537	.001
7.4, Microaerophilic#	1	19.024	9.767	.003
7.4, Normal*	1	21.801	4.673	.035
7.4, RPM 100	1	9.443	4.665	.035
8, 5% CO_2	1	.281	.335	.565
8, Microaerophilic#	1	4.682	11.496	.001
8, Normal*	1	2.618	5.164	.027
8, RPM 100	1	3.582	4.645	.035

Table 4: One way ANOVA results of comparison of biofilm formation between normal and different stress conditions.

*Normal – at 37°C for 24h without any stressors, #Microaerophilic - atmosphere with approximately 5–15% oxygen.

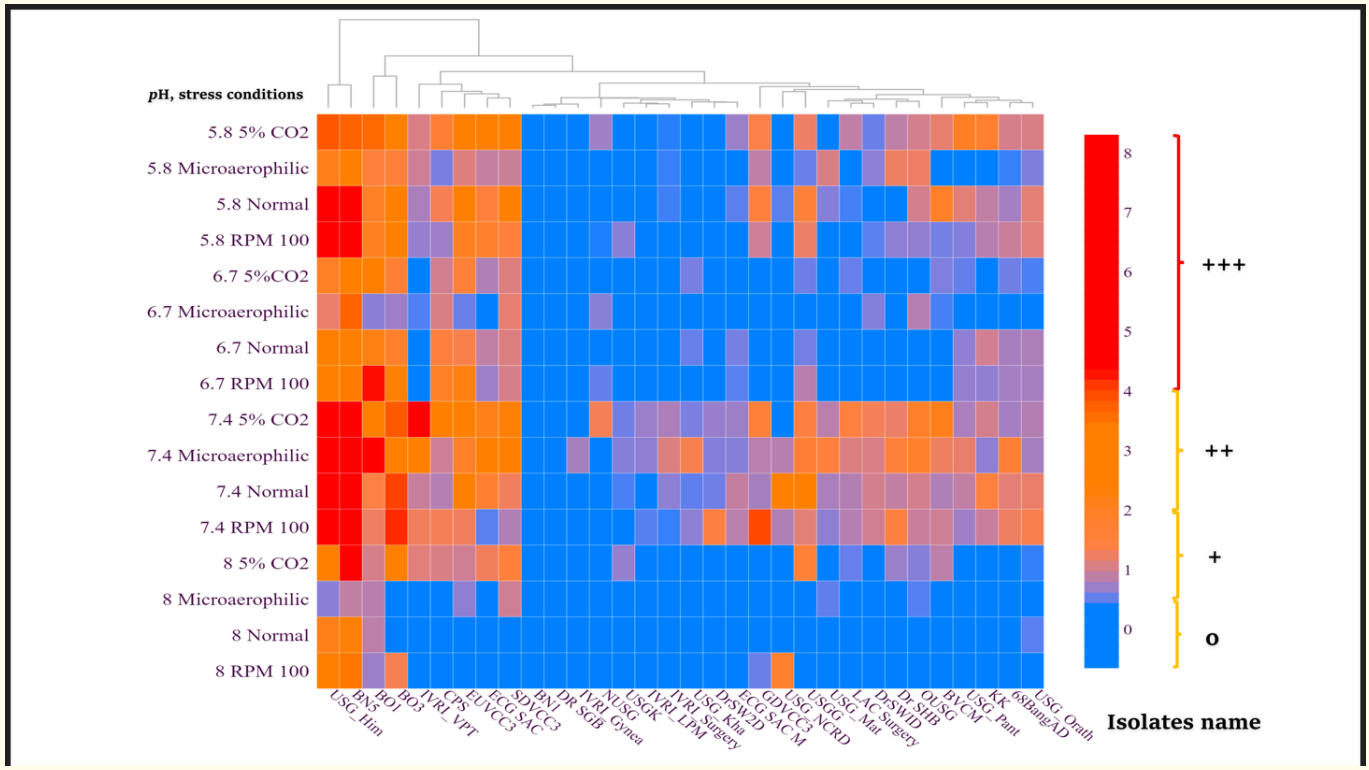

Figure 4: Heatmap of Biofilm forming ability under different stress conditions. O – Non adherent, + - Weakly adherent, ++ - Moderately adherent, +++ - Strongly adherent. Normal – at 37°C for 24h without any stressors, Microaerophilic - atmosphere with approximately 5–15% oxygen.

Discussion

Burkholderia cepacia complex (Bcc), a group of important nosocomial pathogens, has emerged as the cause of multiple healthcare-associated outbreaks linked to contaminated medical products, and devices used in the hospital environment. A systematic review revealed that over half of the outbreaks were due to contaminated medical solutions and medications including 28.2% of intrinsically contaminated ones [22]. In this study, the isolation of 32 Bcc isolates from 67 USGs (48%) indicated the level of contamination of USGs in India. The antimicrobial susceptibility pattern of the Bcc isolates was almost similar to earlier studies with some exceptions [23]. All isolates were susceptible to TMP-SMX, which concurred with previous studies reporting 83% to >95% isolates of Bcc susceptible to TMP-SMX [24,25]. Similar to our findings of susceptibility of Bcc isolates to meropenem (91%) Fehlberg and co-workers (2016) and Chien and co-workers (2018) reported 94% and 87% meropenem susceptible Bccs, respectively [26,27]. Earlier [26], higher susceptibility among Bccs were documented to levofloxacin (96.3%) and minocycline (94%) but it was quite low in the present study. Susceptibility to ceftazidime in Bcc isolates was observed as 79% and combination with avibactam with ceftazidime can increase the ceftazidime susceptibility. However, observations in previous studies highlight that the resistance among Bcc is not due to β-lactamase production alone [28,29]. In our study, adding tazobactam to piperacillin increased the susceptibility of Bcc strains from 15% to 82% much higher than in previous studies [30-32]. Zhou and co-workers (2007) after an extensive study on the Bcc isolates from CF patients reported that >50% of the isolates were resistant to rifampicin, co-trimoxazole, chloramphenicol, tetracycline, ciprofloxacin, amoxicillin + clavulanic acid, and avibactam [30]. Based on the findings of this study, TMP-SMX, meropenem, and ceftazidime with β-lactamase inhibitors can be considered as feasible treatment options in Bcc infections as suggested earlier [33]. In our study, many of the Bcc isolates were susceptible or intermediately susceptible to antibiotics reported as intrinsically resistant by CLSI. Though Bcc has chromosomal genes required for intrinsic resistance, those genes need mutational changes before leading to resistance [34]. Intrinsic resistance refers to the presence of resistance mechanisms in natural or wild-type strains, resulting in phenotypic resistance in all or nearly all strains [35]. However, environmental Bcc strains from the USGs, as in our study, may lack mutations and do not express mechanisms of resistance, resulting in low MICs to many such antimicrobial agents. Clinical strains that express resistance genes, on the other hand, have high MIC values to such antimicrobial agents. Inadequate clinical evidence suggests that strains that test susceptible in vitro will respond in vivo, despite intrinsic resistance mechanisms. Therefore, intrinsic resistance stated by CLSI (2020) could not be confirmed in the present study.

Biofilm formation and intracellular localization favour Bcc, which allows them to avoid the antimicrobial actions of antiseptics in the outside environment, neutrophils in the body and resulting in infection persistence and treatment failure. It is an important behavioural trait frequently expressed in the *Burkholderia* strains isolated from CF patients and protects the bacteria from antibodies and the host immune system [36]. It is considered an important virulence factor involved in establishing and maintaining the infection because it has been proved that biofilm formation by *Burkholderia* leads to the destruction of the glycocalyx layer produced by epithelial cells and cell invasion [37]. In our study, at normal laboratory growth conditions, most of the isolates were weak/low-level biofilm producers. However, it may not reflect the actual biofilm-forming potential of these bacteria, as *in vivo* bacterial biofilms are formed under a complex interaction with surrounding tissues and the immune system of the host [38]. Because we noticed a change in biofilm formation ability under different stress conditions. The adaptability of the bacteria to survive in the acidic condition is necessary for successful colonization in infected lungs. The observation in the study that lower pH with higher CO_2 concentration enhanced biofilm forming capability explains why the Bcc colonize better in the lungs of CF patients having lower pH of exhaled breath condensate than healthy subjects [39,40]. The airway surface liquid (ASL) is an important defence barrier in the respiratory tract which contains several antimicrobial factors [41]. Usually, ASLs pH varies from 6.85 to 7.65. However, in disease conditions, the pH homeostasis is altered and varies from 4.5 to 8 [42]. which might be favourable for Bcc biofilm formation observed in the present study. According to Pessi and colleagues (2013), *B. cenocepacia* H111 grown under micro-oxic conditions (0.5%-5% O_2, i.e. conditions similar to those observed in CF lung infection) produced more biofilm and up-regulated genes involved in the synthesis of the exopolysaccharide (EPS) cepacian [43].

Transcriptomic profiling of nine different growth conditions revealed gene expression changes in *B. cenocepacia* across one-quarter of its genome. A gene cluster known as the low-oxygen-activated (lxa) locus was identified as the reason for persistence and a key determinant of this important ecophysiological trait under low oxygen concentrations (6%) [44].

Conclusion

In the current study, the antibiotic resistance pattern of the 32 Bcc isolates from USGs revealed that they had Multiple drug resistance and some of the isolates may be potentially pathogenic to humans and animals being strong biofilm formers. Based on the observations, co-trimoxazole (TMP-SMX) may be considered as the preferred drug or the drug of the first choice for treating Bcc infections. Alternatively, meropenem and ceftazidime with or without β-lactamase inhibitors can also be considered for the treatment options. However, for suspected Bcc infections clinicians should have antimicrobial susceptibility testing in mind before proceeding for the treatment. Biofilm formation is an important virulent factor for the establishment of disease by Bcc species and variation in expression of biofilm formation under different stress conditions has shown the versatility of the bacteria to adapt and invade the respiratory system of susceptible.

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Conflict of Interest

None to declare.

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