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Evaluation of *In Vitro* Activity of Ceftolozane/Tazobactam and Ceftazidime/Avibactam Against Carbapenem-Resistant *Pseudomonas aeruginosa* Strains and Mechanisms of Carbapenem Resistance: Data from Tertiary Care Hospital

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Abstract

Background: Carbapenem-resistant *Pseudomonas aeruginosa* is an endemic problem in several countries, notably Turkey.
Objectives: This study aimed to investigate the underlying mechanisms contributing to the carbapenem resistance phenotype and enhance the *in-vitro* activity of ceftolozane-tazobactam (C/T) and ceftazidime-avibactam (C/A) against carbapenem-resistant *P. aeruginosa* strains.
Methods: A total of 114 carbapenem-resistant *P. aeruginosa* strains were isolated from different types of clinical specimens. The tested antibiotics were evaluated using the antibiotic disk diffusion method. Additionally, C/T and C/A were tested using the gradient test method. The efficacy of phenylalanine-arginine-β-naphthylamide (PAβN) as efflux pump inhibitors was assessed to determine their ability to reduce meropenem minimum inhibitory concentrations. Polymerase chain reaction (PCR) assays were conducted to identify bla_{IMP}, bla_{VIM}, and bla_{NDM-1}.
Results: Among 114 strains of carbapenem-resistant *P. aeruginosa*, overall resistance rates for C/T and C/A were 10.7% and 8.8%, respectively. Efflux pump inhibitor-based antibiotic susceptibility testing indicated that 35.08% of strains showed resistance modulated by PAβN. Among the strains, 27 (24.5%) were found to produce metallo-beta-lactamase (MBL), with bla_{VIM} (17 strains, 14.91%) being the most common, followed by bla_{IMP} (12 strains, 10.53%).
Conclusions: Emerging carbapenem resistance in *P. aeruginosa* strains is a serious therapeutic challenge for clinicians. Carbapenem resistance can be influenced by various factors, some of which were not assessed in our study. Nonetheless, our results revealed that the main mechanism associated with carbapenem-resistant *P. aeruginosa* strains is a PAβN-sensitive efflux pump. Among acquired MBLs, VIM-type enzymes were found to be the most prevalent.

Keywords: Carbapenems, Efflux Pumps, PaβN, *Pseudomonas aeruginosa*, Ceftolozane/Tazobactam, Ceftazidime/Avibactam

1. Background

Pseudomonas aeruginosa is a non-fermenting Gram-negative bacillus that can cause nosocomial infections resulting in sepsis, urinary tract infections, endocarditis, and pneumonia (1). *Pseudomonas aeruginosa* exhibits resistance to various antibiotics, including cefixime, penicillin G, kanamycin, aminopenicillins (alone or in combination with inhibitors), ceftriaxone, first- and second-generation cephalosporins, cefotaxime, ertapenem, and trimethoprim. This bacterium has the ability to rapidly acquire additional resistances, leading to treatment failures (2, 3). Antipseudomonal carbapenems are commonly used to manage infections caused by multidrug-resistant (MDR) *P. aeruginosa*. However, their increased utilization has resulted in the development of carbapenem resistance, which hampers the effective treatment of *P. aeruginosa* infections (4). Carbapenem resistance in *P. aeruginosa* can arise from various mechanisms, including the production of carbapenemases, over-expression of efflux pumps, loss of outer membrane porins, and the generation of extended-spectrum AmpC β-lactamase or β-lactamase (4).

The Ambler class B metallo-β-lactamases (MBLs) are the primary carbapenemases found in PA. MBL-encoding genes can be present on plasmids and transferred to other strains, leading to antibiotic resistance. *Pseudomonas aeruginosa* can harbor various types of MBLs, including

IMP, NDM-1, VIM, GIM, SPM, FIM-1, SIM, and HMB-1 (5). Overexpression of MexAB-OprM, a member of the resistance nodulation-cell division (RND) family and a multidrug efflux pump, contributes to carbapenem resistance in *P. aeruginosa* (6). Ceftolozane-tazobactam (C/T) and ceftazidime-avibactam (C/A) are novel β-lactam/β-lactamase inhibitor combinations effective against Gram negative bacteria, including *P. aeruginosa*. They are potential therapeutic options for infections caused by carbapenem-resistant *P. aeruginosa* strains (2, 7). C/T exhibits stability against chromosomal AmpC β-lactamases, deleted OprD porins, and overexpressed MexAB-OprM efflux pumps. However, both C/T and C/A are ineffective against MBL-producing *P. aeruginosa* (8).

2. Objectives

We examined (1) the presence of efflux pumps using a phenotypic method, (2) the distribution of bla_{IMP}, bla_{VIM}, and $bla_{\text{NDM-1}}$ genes among carbapenem-resistant *P. aeruginosa* strains, and (3) the *in-vitro* activity of C/A and C/T against carbapenem-resistant *P. aeruginosa* strains. These antibiotics are recognized for their high efficacy against carbapenem-resistant *P. aeruginosa* but have been insufficiently studied in Turkey.

3. Methods

3.1. Bacterial Strains

In the present study, a total of 114 non-duplicate clinical strains of meropenem-non-susceptible *P. aeruginosa* were examined. These strains were obtained from Hacettepe University Hospitals between 2019 and 2020. The specimens originated from various sources, including blood (n = 14), lower respiratory samples such as tracheal aspirate and sputum (n = 39), pus (n = 12), sterile body fluids such as peritoneal, pleural, and bile fluids (n = 13), tissue (n = 6), and urine (n = 30). Prior to the assessment, all strains were stored at 80°C and subcultured twice on 5% sheep blood agar.

3.2. Antimicrobial Susceptibility Testing

In vitro, antibiotic susceptibility testing was performed using the Phoenix™ Automated Microbiology System (BD Becton-Dickinson, Sparks, USA) to assess the susceptibility of ceftazidime (CAZ), amikacin (AMK), piperacillin/tazobactam (TZP), cefepime (FEP), and ciprofloxacin (CIP) (9). The minimum inhibitory concentration (MIC) of meropenem was determined using the micro broth dilution technique. Gradient test strips (biomerieux, France) were utilized to measure the MICs of C/T and C/A. The interpretation of the antibiotic susceptibility test results was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables (version 9.0) (10). *Pseudomonas aeruginosa* ATCC 27853 was used as a quality control strain.

3.3. Efflux Pump Inhibition

To investigate the interaction between meropenem (Sigma-Aldrich, USA) and phenylalanine arginine β-naphthylamide (PAβN, Sigma-Aldrich, Germany), the MICs of the antibiotics were measured in the presence and absence of PAβN at a concentration of 25 mg/L (11, 12). The strains overexpressing efflux pumps exhibited at least a four-fold decrease in MICs in the presence of PAβN compared to the MICs in the absence of PAβN (12). The strains were assessed in duplicate, and the results were screened 24 hours after incubation at 35°C.

3.4. Detection of Carbapenemase-Producing Genes

All carbapenem-resistant *P. aeruginosa* strains were examined for the presence of carbapenemase-encoding genes, including bla_{IMP}, bla_{VIM}, and $bla_{\text{NDM-1}}$, using the polymerase chain reaction (PCR) method with minor modifications as previously described (13-15). For DNA isolation, 4 - 5 single colonies were picked from fresh cultures and incubated at 95°C for 15 min in PCR-grade water. Following centrifugation at 10,000 rpm for 10 min, 2 μL of the supernatant was used as the DNA template in a final volume of 20 μL, which included 1X PCR Buffer, 2.5 mM MgCl2, 0.5 μM of each primer, 200 μM of each dNTP, and 1U Taq DNA polymerase. The specific primer sequences are presented in Table 1. The cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 50 s, with a final elongation step at 72°C for 5 min (see Appendix 1).

4. Results

4.1. Antimicrobial Susceptibility Patterns in Carbapenem-Resistant Pseudomonas aeruginosa Strains

Among the 114 carbapenem-resistant *P. aeruginosa* strains, the overall resistance rates were 10.7% for C/T and 8.8% for C/A. The MIC50 values for C/A and C/T were 1 mg/mL and 0.75 mg/mL, respectively. The MIC90 values for C/T and C/A were 48 mg/mL and 16 mg/mL, respectively. Based on the MIC90 values, C/A demonstrated greater potency than C/T. The majority of strains (94.7%, n = 108) showed categorical agreement with both C/T and C/A. However, six strains were susceptible to C/A but resistant to C/T. Among the five antibiotics tested on carbapenem-resistant *P. aeruginosa*

Table 1. Primers Used for PCR Amplification of Metallo-β-Lactamase Genes

Target	Primer Sequences (5' - 3')	Amplicon Size (bp)	References
***bla*$_{IMP}$**		232	(14)
IMP-F	GGAATAGAGTGGCTTAAYTCTC		
IMP-R	GGTTTAAYAAAACAACCACC		
***bla*$_{VIM}$**		390	(14)
VIM-F	GATGGTGTTTGGTCGCATA		
VIM-R	CGAATGCGCAGCACCAG		
***bla*$_{NDM-1}$**		621	(15)
NDM-F	GGTTTGGCGATCTGGTTTTC		
NDM-R	CGGAATGGCTCATCACGATC		

strains, amikacin exhibited the highest potency (19.5% resistance), followed by FEP (44.4% resistance), CAZ (46.4% resistance), and TZP (51.8% resistance). The highest resistance was observed against CIP (62.2%) (Figure 1A). Table 2 presents the susceptibility test results for other antimicrobial agents. Four strains were classified as MDR and harbored both MBLs, exhibiting a four-fold reduction in meropenem MIC in the presence of the efflux pump inhibitor PAßN.

4.2. Efflux Pump Inhibition

In carbapenem-resistant *P. aeruginosa* strains, 35.08% of the strains have become susceptible to 25 mg/L of PAβN, resulting in a decrease in MICs of meropenem in 40 *P. aeruginosa* strains. The bacteria cultured in Mueller-Hinton broth containing PAßN (25 mg/L), with or without meropenem, exhibited robust growth, indicating that PAßN (25 mg/L) itself did not have an antibacterial effect. Among the 40 strains with PAβN-sensitive efflux pumps, five strains were found to have MBLs (Figure 1B).

4.3. Detection of Carbapenemase-Producing Genes

Among the 114 strains of carbapenem-resistant *P. aeruginosa*, 27 (24.5%) strains were found to produce MBL. The most common carbapenemase gene was *bla*$_{VIM}$, detected in 17 strains (14.91%), followed by *bla*$_{IMP}$ in 12 strains (10.53%). No positive strains for *bla*$_{NDM-1}$ were identified. Two strains were positive for both *bla*$_{IMP}$ and *bla*$_{VIM}$ simultaneously (Figure 1B). The overall distribution of carbapenemase genes, efflux pump, resistance rates to tested antibiotics, and coexistence of *bla*$_{IMP}$, *bla*$_{VIM}$, and phenotypic efflux pump test-positive strains are depicted in Figure 1A and B. A heat map in Figure 1C represents the percentage distribution of resistant strains for all tested antibiotics, the prevalence of MBLs, and the presence of 4 phenotypic efflux pump inhibitors based on sample origin.

5. Discussion

Carbapenem resistance in *P. aeruginosa* strains poses a significant clinical challenge, as carbapenems have been widely considered the most potent and effective agents against MDR *P. aeruginosa* strains. However, there has been a recent rise in the prevalence of carbapenem resistance among *P. aeruginosa* strains globally, including in Turkey (13, 16, 17). According to the Antimicrobial Testing Leadership and Surveillance program (18), the lowest rate of carbapenem-resistant *P. aeruginosa* was observed in Oceania (5.1% - 7.1%), while the highest rates were found in the Middle East (27.9% - 19.5%). Resistance rates were 30.7% in South America, 28.0% in Europe, 24.4% in North America, 22.8% in Africa, and 18.1% in Asia (19). A meta-analysis conducted in Turkey reported a pooled prevalence of 30.1% for resistance against meropenem and 28.0% for imipenem (17). Among carbapenem-resistant *P. aeruginosa* clinical strains, MBLs are the most common type of carbapenemases. VIMs are the most widely distributed (Middle East, South America, Africa), followed by IMPs and NDMs (20).

Kazmierczak et al. studied carbapenemases in *P. aeruginosa* strains collected from 96 medical centers across 18 European countries. They found that 13.4% of the strains carried MBLs, with VIM being the most prevalent (21). In Turkey, *P. aeruginosa* strains exhibit various types of MBLs, including VIM-1, VIM-2, VIM-5, and IMP (22-25). The first isolation of NDM-1-producing *P. aeruginosa* was reported in 2011 in Serbia, and subsequent cases of NDM-positive strains have been identified in Italy, France, Egypt, and Slovakia (26-30). In Turkey, a few *P. aeruginosa* strains producing NDM-1 have been identified (24, 31). However, in our study, no NDM-1-positive strains were detected, suggesting that the presence of NDM-1 may be limited to a localized area. In the current study, the detection rate of MBLs was 24.5%, with VIM being the most common type. These findings highlight the high prevalence of carbapenemase

Table 2. Phenotypic Resistance in Clinical Strains of Carbapenem-Resistant *Pseudomonas aeruginosa*

Antimicrobial Class	Antimicrobial Agents	MIC_{50}	MIC_{90}	Resistance (%)
β-lactam (carbapenem)	Meropenem	> 8	> 8	88.50
β-lactam (carbapenem)	Imipenem	> 8	> 8	97.3
β-lactam (cephalosporin)	Ceftazidime	4	> 16	46.4
β-lactam (cephalosporin)	Cefepime	4	> 8	44.4
β-lactam (penicillin)/β-lactamase inhibitor	Piperacillin/tazobactam	4	32	51.8
β-lactam (cephalosporin)/ β-lactamase inhibitor	Ceftazidim/avibactam	1	16	8.8
β-lactam (cephalosporin)/β-lactamase inhibitor	Ceftolozane/tazobactam	0.75	48	10.7
Aminoglycoside	Amikacin	< 8	> 32	19.5
Quinolone	Ciprofloxacin	0.5	> 1	62.2

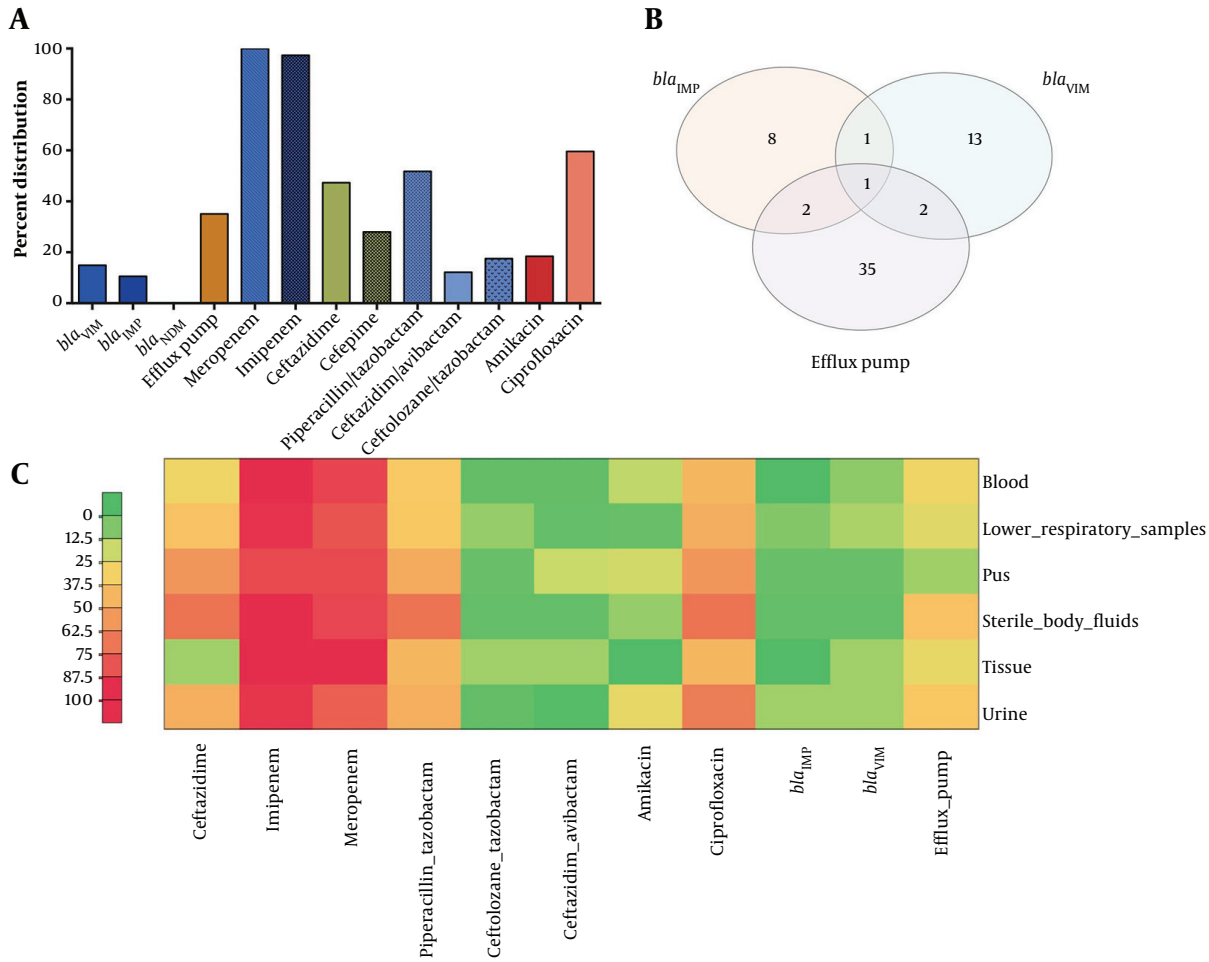

Figure 1. A, Percentage distribution of carbapenemase genes, efflux pump-positive strains based on phenotypic tests, and resistance rates to tested antibiotics. B, Coexistence of bla_{IMP}, bla_{VIM}, and phenotypic efflux pump test-positive strains. C, Heat map displaying the percentage distribution of resistant isolates for all tested antibiotics, the prevalence of MBLs, and phenotypic efflux pump inhibitors according to sample origin. The color gradient ranges from green to red, representing a linear scale of percent distribution from low to high. The red box indicates a higher prevalence compared to the green box.

genes among the strains, emphasizing the importance of their detection as they can easily disseminate among bacterial strains. Ongoing surveillance is necessary to identify potential outbreaks.

Efflux pump overexpression, particularly MexXY, and MexAB-OprM, contributes to increased resistance to carbapenems in *P. aeruginosa* strains (6). The reported prevalence rates of efflux pump overexpression in carbapenem-resistant *P. aeruginosa* strains range from 37.5% to 80.9% (13, 32, 33). In a Turkish study, efflux pump overexpression was found in 47.6% of carbapenem-resistant *P. aeruginosa* strains (34). It is worth noting that meropenem is a substrate for efflux pumps (35). In the presence of PaβN, approximately 35.9% of the strains under investigation exhibited a significant decrease in meropenem MICs, irrespective of the presence of any MBL genes. The efflux pump plays a crucial role in conferring carbapenem resistance in the strains examined. However, it is important to acknowledge that our study had limitations, including the lack of information regarding the expression of AmpC, other class C beta-lactamases, efflux pumps, and oprD gene mutations.

Treating infections caused by carbapenem-resistant *P. aeruginosa* strains poses a challenge due to their increased antibiotic resistance. Consistent with previous studies, these strains exhibit elevated resistance to various antibiotic groups, including quinolones, cephalosporins, and penicillins (16, 17). In our study, we assessed the *in vitro* activity of C/A against carbapenem-resistant *P. aeruginosa* strains and observed that the MICs of C/A were 2 - 4 times lower than the MICs for CAZ alone. This aligns with other *in vitro* studies that have demonstrated a smaller MIC for CZA compared to CAZ alone, indicating increased susceptibility of *P. aeruginosa* to C/A (36).

The susceptibility rates of carbapenem-resistant *P. aeruginosa* strains to C/T and C/A were 89.3% and 91.2%, respectively. In previous reports, carbapenem-resistant *P. aeruginosa* strains demonstrated susceptibility rates ranging from 51.8% to 92% for C/A and 65.4% to 94% for C/T (7, 37-40). Many studies have consistently shown higher susceptibility rates for C/T compared to C/A among carbapenem-resistant *P. aeruginosa* strains (7, 37, 38, 41). However, in contrast to these previous studies, our findings indicate that C/A exhibited greater activity than C/T against carbapenem-resistant *P. aeruginosa* strains.

Our results align with those of Sader et al., who observed higher susceptibility rates for C/A (86.5%) compared to C/T (66.2%) among meropenem non-susceptible *P. aeruginosa* strains (40). This difference in susceptibility between C/A and C/T may be attributed to the ability of avibactam to suppress class A β-lactamases, class C β-lactamases, and class D β-lactamases, which is not seen with tazobactam (42). Moreover, variations in the activity of C/A compared to C/T among *P. aeruginosa* strains reflect the diverse resistance mechanisms expressed by these organisms, highlighting how these mechanisms can have different impacts on the efficacy of these two combinations.

Carbapenem-resistant *P. aeruginosa* strains were isolated from 2019 to 2021 across 17 centers in 12 countries as part of the ERACE-PA Global Surveillance Program. Among these strains, the most commonly identified carbapenemase genotype was VIM. The observed susceptibility rates of VIM-positive strains to C/T and C/A were 1% and 4%, respectively (20). In the ATLAS global surveillance program, which collected 214 MBL-positive *P. aeruginosa* strains from 2017 to 2019, 4.2% of the strains were found to be susceptible to C/A (18). Lomovskaya et al. determined that the susceptibility rates of MBLs-producing *P. aeruginosa* strains to C/T and C/A were 1.6% and 4.9%, respectively (43). Within our collection, VIM is the predominant carbapenemase produced by carbapenem-resistant *P. aeruginosa* strains.

The susceptibility rates to C/T and C/A among MBL-positive strains were 55.1% and 62.4%, respectively. Adam and Elhag previously reported the presence of MBL genes in carbapenem-sensitive Gram-negative strains, suggesting the potential presence of hidden MBL genes (44). In our study, we identified MBL genes in strains that were reported as susceptible to C/T and C/A, indicating that these strains may serve as reservoirs for these resistance genes, posing a potential risk for silent dissemination in both community and hospital settings. The emergence of meropenem resistance in our clinical strains of *P. aeruginosa*, where efflux pump activity was not detected, may be attributed to constitutive overproduction of the cephalosporinase AmpC or non-enzymatic mechanisms such as outer membrane impermeability. However, it is essential to note that our study did not analyze these resistance mechanisms in meropenem-resistant *P. aeruginosa* strains, which is a limitation of our findings.

5.1. Conclusions

In this study, the main mechanism associated with carbapenem-resistant *P. aeruginosa* strains is the PAβN-sensitive efflux pump. Among the acquired carbapenemases in *P. aeruginosa* strains, VIM-type enzymes are the most commonly observed with a worldwide distribution. C/T and C/A have demonstrated their retained *in vitro* potency against clinical strains of carbapenem-resistant *P. aeruginosa* collected from hospitalized patients in Turkey. These findings suggest that C/T and C/A may play a potentially important role in the management of *P. aeruginosa* infections, including those caused by carbapenem-resistant strains. However, it is crucial to consider local

susceptibility patterns and antibiotic susceptibility test results when deciding on the usage of C/T and C/A, as is the case with all antimicrobials.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: G.H. and C.Ö. were involved in the conceptualization and design of the study. G.H. and C.Ö. also participated in the review, critical revision of the article, and approval of the final version.

Conflict of Interests: We have no employment, personal financial interests, stocks or shares in companies, consultation fees, patents, or personal or professional relations with organizations and individuals (parents and children, wife and husband, and family relationships). We also do not hold unpaid membership in a government or non-governmental organization. Furthermore, we are not affiliated with the editorial board or serving as a reviewer for this journal.

Data Reproducibility: The data presented in this study are openly available in a repository or can be obtained upon request from the corresponding author through this journal's representative. Any inquiries regarding data availability can be addressed to the corresponding author. In the absence of access to the data or any related issues, the corresponding author will bear the responsibility for any potential withdrawal or future retraction.

Ethical Approval: The Ethics Committee of the Hospital granted an exemption from review for this study, as it primarily focused on bacteria. All procedures conducted in this study adhered to the ethical standards outlined by the institutional and/or national research committee, as well as the principles stated in the 1964 Helsinki Declaration and its subsequent amendments.

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