



Detection of Carbapenemase Encoding Gene and Resistance to Cefiderocol in Hospital and Community eXtensive Drug Resistance and Carbapenem-Resistant *Pseudomonas aeruginosa* Strains in Morocco

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Abstract

Pseudomonas aeruginosa (Pa) remains among clinically-significant Gram-negative species. The carbapenems are often the last resort for treating infections due to multidrug resistant isolates such as Pa. The carbapenems' efficacy is increasingly compromised by the emergence and the rapid spread of Pa carrying carbapenemases which represent a serious threat to public health. This study aimed to establish the resistance profile and to identify carbapenemase genes in isolates with imipenem resistant phenotypes. Among 134 Pa isolates collected both in the community (46) and hospital (88) from January 2021 to December 2021 in Morocco, 18 (8 were from the community and 10 from the hospital settings) were carbapenem resistant. The identification of these strains has been confirmed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF). The antibiotic susceptibility testing against 16 antibiotics was carried out and interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (2021). The worrying antibiotics resistance profiles, which spread to cefiderocol for two isolates, were obtained for all isolates, which were eXtensive Drug Resistance showing highly resistant to all antibiotic categories tested, even to ceftolozane–tazobactam. Colistin (100% susceptible) and cefiderocol (88.88%) were the most active agents against carbapenem-resistant Pa (CRPa). Phenotypic detection by NP-CARBA and NG-CARBA tests of metallo- β -lactamase (M β L) production was confirmed by PCR amplification and sequencing. Three CRPa isolates coharboring *bla*_{VIM-2}-*bla*_{NDM-1} (two isolates) and *bla*_{VIM-2}-*bla*_{IMP-8} (one isolate) genes were detected. In this study, we describe the coexistence of these M β L genes and the cefiderocol resistance in CRPa strains in Morocco. The alarming antibiotic resistance patterns of all these CRPa isolates and their resistance genes emphasize the importance of antimicrobial susceptibility testing in the choice of antibiotics for treating Pa infections.

Keywords: *Pseudomonas aeruginosa*, community, hospital, antibiotic resistance, antibiotic susceptibility, carbapenem-resistance genes, cefiderocol

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Introduction

PSEUDOMONAS AERUGINOSA (Pa) has been recognized as an opportunistic pathogen and a common cause of nosocomial infections. The worryingly increased dissemination of multi and extensively drug-resistant (MDR and XDR) Pa strains, especially in carbapenem resistant strains, is a major health challenge in clinical practice (Tacconelli et al., 2018). The continuous increase of MDR isolates presents a complicated situation for antimicrobial therapy since it limits the therapeutic choice (Pang et al., 2019). World Health Organization (WHO) has recently listed carbapenem-resistant Pa (CRPa) as one of six bacterial “ESKAPE” (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) species for which there is a critical need for the development of new antibiotics to treat infections (WHO, 2017).

Carbapenems are commonly used to treat Pa infections (Shaaban et al., 2017). Carbapenem resistance is complex; it can be mediated by three major mechanisms: enzyme production, efflux pumps, and porin alteration by mutations. The production of carbapenemases remains the most important mechanism. A high number of carbapenemases were reported in Pa isolates; they include mainly class B enzymes «Metallo- β -lactamases» (M β LS) (IMP, VIM, SPM, GIM, SIM, AIM, FIM, and NDM) (Elsherif et al., 2016), but also, to a lesser extent, class A and class D β -lactamases. The M β LS are considered the most clinically threatening enzymes because of their efficient hydrolysis of carbapenems and high levels of resistance in producers' bacteria. In Morocco, most studies focus mainly on carbapenem resistance in *Enterobacteriaceae* (*Escherichia coli* and *Klebsiella* spp). Therefore, this study was undertaken to establish the susceptibility profile of Pa carbapenem resistant isolates, highlight the production, and determine the prevalence of carbapenemase enzymes in these isolates from community and hospital settings in Casablanca (Morocco).

Materials and Methods

Bacterial isolates

From January to December 2021, a total of 134 non-duplicate Pa strains isolates recovered from urine (48), sputum (47), pus (22), and other specimens (17) were collected in Casablanca (Morocco). These isolates were from the community (88) (obtained from private medical analysis laboratories) and nosocomial (46) origin (obtained from hospitals). To check their purity and to verify the bright green color characteristic of Pa species, a bacterial suspension obtained after incubation in the Brain-Heart Infusion medium (BHI) (Bio-rad, Marnes-la-Coquette, France) at 37°C for 20 to 24 h was subcultured in cetrimide agar medium (Bio-rad).

Furthermore, they were presumably identified as belonging to Pa species by the standard microbiological and biochemical tests (colony morphology, Gram staining, oxidase test, blue-green pigmentation, and fluorescence under UV light). The identification of isolates was further confirmed accurately by matrix assisted laser desorption ionization-time of flight (MALDI-ToF) Biotyper IVD 2.2. (BRUKER; Microflex) at the National Reference Center for Gram-Negative bacilli (CNRBGN) coordinated by the University Hospital

Center (UHC-UCL) Namur (Mont-Godinne). Pa ATCC 27853 strain was used as a positive control. All isolates were stored in the BHI supplemented with 15% glycerol at -20°C for further use.

All isolates were screened for their ability to produce carbapenemases first by disc diffusion method using Mueller-Hinton (MH) (Bio-Rad) agar and imipenem discs (10 μ g) (Bio-Rad). The inhibition zones were measured (in millimeters) and interpreted following the EUCAST 2021 guidelines (<https://www.sfm-microbiologie.org/2021/04/23/casfm-avril-2021-v1-0>). Pa ATCC® 27853 and Pa UKA2237 strains were used as negative and positive control, respectively. Only these CRPa (as detected by disk diffusion method using imipenem) isolates were included in the rest of the study.

Antibiotic susceptibility testing of CRPa

The disk diffusion method on MH was used to determine the antibiotic susceptibility patterns of CRPa isolates according to the EUCAST 2021 guidelines. A panel of 16 clinically important antibiotics was used: piperacillin (30 μ g), piperacillin-tazobactam (30/6 μ g), ticarcillin (75 μ g), ticarcillin-acid-clavulanic (75 μ g/10 μ g), meropenem (10 μ g), aztreonam (30 μ g), cefepime (30 μ g), ceftazidime (10 μ g), amikacin (30 μ g), gentamicin (10 μ g), ciprofloxacin (5 μ g), ceftazidime-avibactam (10/4 μ g), tobramycin (10 μ g), and ceftolozane-tazobactam (30/10 μ g) (Bio-Rad). For colistin and cefiderocol, the minimum inhibitory concentration (MIC) was determined by broth microdilution assay (Sensititre™ Vizion, Manual Viewer). Antimicrobial susceptibility results were interpreted according to EUCAST clinical breakpoints 2021. Pa ATCC 27853 strain was used as negative control.

Multiresistance was established as resistance to carbapenem and to at least one antibiotic representative of two or more classes.

Phenotypic detection of carbapenemase activity in CRPa

According to the manufacturer's instructions, carbapenemase production was examined phenotypically among CRPa using the Biochemical Test Carba NP® rapid diagnostic technique (Rapidec®; BIOMERIEUX). The immunological Carba NG® test (NG.BIOTECH®, LABORATORIES) was used to confirm the production and differentiation of the five most common carbapenemase families (KPC, OXA-48-like, VIM, IMP, and NDM). Pa ATCC 27853 strain was used as negative control, and the results were interpreted according to Dortet et al. (2012) interpretative criteria.

Molecular detection of carbapenemases in CRPa

Preparation of DNA template for PCR. DNA extraction was performed by PureLink™ Genomic DNA Kit according to the manufacturer's instructions. The DNA extracts were quantified using NanoDrop (Thermo Fisher Scientific, Wilmington) and stored in a freezer at -20°C until to be used as templates in PCRs.

Detection of carbapenemase-encoding genes. All CRPa isolates were screened for carbapenemase genes (*bla*_{GES}, *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP}, and *bla*_{OXA-48-like}) by

TABLE 1. PRIMERS AND THEIR SEQUENCES USED TO DETECT CARBAPENEMASE GENES IN CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATES

Genes	Primers	Sequences	Size (bp)	Reference
<i>bla</i> _{GES}	GES (F) GES (R)	5'-ATGCGCTTCATTACGCAC-3' 3'-CTATTTGTCCTGCTCAGGA-5'	863	(UHC-UCL-NAMUR)
<i>bla</i> _{IMP}	IMP (F) IMP (R)	5'-ACAYGGYTTRGTDGKCTTG-3' 3'-GGTTTAAAYAAARCAACCACC-5'	387	
<i>bla</i> _{KPC}	KPC (F) KPC (R)	5'-TCGCCGTCTAGTTCTGCTGTCTTG-3' 3'-ACAGCTCCGCCACCGTCAT-5'	353	
<i>bla</i> _{OXA-48}	OXA-48 (F) OXA-48 (R)	5'-ATGCGTGTATTAGCCTTATCG-3' 3'-CATCCTTAACCACGCCCAAATC-5'	265	
<i>bla</i> _{VIM}	VIM (F) VIM (R)	5'-TGTCCGTGATGGTGATGAGT-3' 3'-ATTCAGCCAGATCGGCATC-5'	437	
<i>bla</i> _{NDM}	NDM (F) NDM (R)	5'-ACTTGGCCTTGCTGTCCTT-3' 3'-CATTAGCCGCTGCATTGAT-5'	603	

bp, base pairs; F, forward primer; R, reverse primer; UHC-UCL-NAMUR, University Hospital Center-Université Catholique de Louvain-Namur.

multiplex PCR using specific primers (Table 1). PCR amplifications for carbapenem resistance genes were performed in a volume of 25 μ L containing: 2 μ L of the bacterial DNA template and 23 μ L of the Taq PCR Master Mix provided by the manufacturer. "QIAGEN 57 ISO" PCR amplification conditions were carried according to the preregistered program "QIAGEN 57 ISO" as follows: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min 30 s, and extension at 72°C for 1 min 30 s, ending with a final extension period of 72°C for 10 min (Thermocycler ABI 2720). The primers and protocol were adopted according to the ISO15189 procedure (QIAXcel) validated at the Belgian National Reference Center for Antimicrobial Resistance in Gram-negative. Pa ATCC 27853 and Pa UKA2237 strains were used as negative and positive control, respectively.

The products were visualized by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and viewed using ultraviolet illumination.

Sequencing. The PCR products were purified using enzymatic method (ExS Pure™; Cleanup Kit/NmaGen BV, The Netherlands). The program of the enzymatic reaction is: one cycle (20 min at 37°C, 20 min at 85°C). The amplified products obtained were sequenced to validate their identities. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130×1 sequencer (Applied Biosystems, Foster City, CA), with the same primers used for PCR amplification.

The nucleotide and deduced protein sequences were analyzed with software available over the internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Results

Bacterial strains

Regardless of their origin, out of 134 Pa isolates, 18 (13.43%) were carbapenem resistant according to EUCAST 2021 guidelines and predominantly recovered from urine (13/18; 72.22%) followed by pus (2/18; 11.11%), sputum

(1/18; 5.5%), nasal sample (1/18; 5.5%), and bronchial sample (1/18; 5.5%). Among these 18 isolates, 8/18 (44%) were of community setting and 10/18 (55.55%) were of hospital setting.

Antibiotic resistance patterns of CRPa

The susceptibility to 16 antibiotics belonging to different classes of 18 CRPa isolates is presented in Table 2. Colistin is the only active drug on all Pa isolates. Two isolates, CSPa57 and CSPa84, were resistant to cefiderocol, with MIC values of 4 and 8 mg/L, respectively. All (100%) Pa isolates were resistant to antibiotics belonging to penicillins, monobactams, fluoroquinolones, and carbapenems. The inclusion criteria for 18 CRPa strains were imipenem resistance; in addition, this collection was also resistant to meropenem. A high percentage (90–95%) of isolates were resistant to cephalosporins and aminoglycosides. Only one strain was susceptible to ceftolozan–tazobactam. Globally, at least 90% of isolates exhibited nonsusceptibility to at least one agent in ≥ 6 antimicrobial categories and were therefore considered XDR and Difficult-to-Treat Resistance isolates (Kadri et al., 2018). They include isolates that were mainly collected from urine samples 13/18 (72.22%); 7/13 (53.85%) and 6/13 (46.15%) strains were from the community and clinical origin, respectively.

Phenotypic detection of carbapenemase in CRPa

The phenotypic detection of carbapenemase activity by the Biochemical test Carba NP was positive in 95% ($n = 17$) of 18 CRPa tested. The immunological Carba NG test detected the *bla*_{NDM} gene in 95% ($n = 17$) of these strains, as well as a combination of two carbapenemase genes, *bla*_{NDM}–*bla*_{VIM} and *bla*_{VIM}–*bla*_{IMP}, in two (CSPa33 and CSPa 84) and one (CSPa90) clinical isolates, respectively (Table 3). No CRPa isolate was found to possess *bla*_{GES}, *bla*_{KPC} neither *bla*_{OXA-48}.

PCRs and DNA sequence analysis results of CRPa

The PCR results with the primers tested confirmed the results obtained by the phenotypic detection of carbapenemase in the 17 strains. The only strain susceptible to

TABLE 2. ANTIMICROBIAL RESISTANCE IN CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* STRAINS

Antibiotic classes	Antibiotics	Number (%) of clinical resistant strains (n=10)	Number (%) of community resistant strains (n=8)	Number (%) of all resistant strains (n=18)
Penicillin	PIP	(10) 100%	(8) 100%	(18) 100%
	PTZ			
	TIC			
	TCC			
Cephalosporin	FEB	(10) 100%	(8) 100%	(18) 100%
	CAZ			
	CEF	(1) 10%	(1) 12.5%	(2) 11.1%
	CZA	(10) 100%	(8) 100%	(18) 100%
	CLT	(10) 100%	(7) 87.5%	(17) 95%
Monobactam	AZM	(10) 100%	(8) 100%	(18) 100%
Carbapenem	MEM			
Fluoroquinolone	CIP			
Aminoglycoside	AK	(10) 100%	(7) 87.5%	(17) 95%
	GEN	(9) 90%	(7) 87.5%	(16) 88.8%
	TM			
Polymyxin	COL	0%	0%	0%

AK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CEF, cefiderocol; CIP, ciprofloxacin; CLT, ceftolozan-tazobactam; COL, colistin; CZA, ceftazidime-avibactam; FEB, cefepime; GEN, gentamicin; MEM, meropenem; PIP, piperacillin; PTZ, piperacillin-tazobactam; TIC, ticarcillin, TCC, ticarcillin-acid-clavulanic; TM, tobramycin.

ceftolozan-tazobactam is the same one that showed resistance to imipenem by the disc diffusion method and does not produce carbapenemase. The characteristics of CRPa strains coharboring two M β L genes are shown in Table 3.

Sequencing

The three isolates, namely CSPa33, CSPa84, and CSPa90, coharboring two M β L genes, were sequenced to validate their identities. Their sequences were interpreted using software available over the internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The amplified product sequencing results confirmed (*bla*_{NDM-1}-*bla*_{VIM-2}) and (*bla*_{VIM-2}-*bla*_{IMP-8}) genes in the three CRPa MBL producer isolates and demonstrated that they were *bla*_{NDM-1}, *bla*_{VIM-2}, and *bla*_{IMP-8} variants (Table 3).

Discussion

Pa has emerged as one of the most common opportunistic pathogens in hospital-acquired infections, especially in infections caused by MDR/XDR isolates (Saderi and Owlia, 2015.). Carbapenems constitute the last-resort class of antibiotics for such infections because of their stability against β -lactamases and their broad spectrum of action. For these purposes, resistance to carbapenem should never be under-

estimated. Studies on CRPa in Morocco are scarce. Most of the studies in Morocco focus on carbapenem resistant enterobacteria, especially *E. coli* and *Klebsiella* sp. (Bonnin et al., 2020; Dilagui et al., 2022; Mahrach et al., 2019; Natoubi et al., 2020). In the present work, we aimed to assess the antimicrobial susceptibility, determine the prevalence of CRPa isolates, and detect the carbapenemase producer among the community and clinical Pa isolates in Morocco. The prevalence of CRPa was 13.43% (18/134) without considering their origin (hospital or community). Taken separately, this prevalence reached a percentage of 21.7 (10/46) in hospital isolates and dropped to 9.09 (8/88) in community isolates. This prevalence rate was higher than that reported in other studies. In a recent multicenter study from North Africa, South Africa, and the Middle East, the prevalence of CRPa among isolates collected from 2018 to 2020 in admitted patients in three Moroccan medical centers was 5.5% (Karlowsky et al., 2022). Carbapenem resistance in hospitals is on a rising trend and is alarming.

Our CRPa isolates were recovered from various pathological specimens, predominantly in urine samples (13/18, 72.22%). Urinary tract infections are one of the most prevalent diseases in the community and hospitalized patients, as pointed out in other studies (Lamas Ferreiro et al., 2017).

TABLE 3. CHARACTERISTICS OF CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* STRAINS COHARBORING TWO METALLO- β -LACTAMASE GENES

Strains code	Sample	Sex	Age	Resistance profile	Resistance genes detected
CSPa33	Nasal sample	Female	49	DTR/XDR	<i>bla</i> _{NDM-1} - <i>bla</i> _{VIM-2}
CSP 84	Sputum	Female	70	DTR/XDR	<i>bla</i> _{NDM-1} - <i>bla</i> _{VIM-2}
CSPa90	Urine	Male	62	DTR/XDR	<i>bla</i> _{VIM-2} - <i>bla</i> _{IMP-8}

CSPa, clinical strain *Pseudomonas aeruginosa*, DTR, difficult-treat resistance; XDR, eXtensive drug resistance.

In our study, only colistin was found to be susceptible in all Pa isolates. Around 95% of isolates showed resistance to all antibiotics tested, even to the new combination ceftolozan–tazobactam. In a Teo et al. (2021) study, whole-genome sequencing in 195 CRPa clinical isolates revealed that C/T resistance was largely mediated by the presence of horizontally acquired β -lactamases, especially M β L. The only CRPa ceftolozan–tazobactam susceptible does not produce carbapenemase according to carbapenemase tests and PCR results, which could explain its susceptibility to this cephalosporin/ β -lactamase inhibitor and confirms the results of Teo et al. (2021) study. The resistance to carbapenem of this imipenem-resistant carbapenemase negative strain could be due to another resistance mechanism. Pa may exhibit multidrug efflux machinery, mainly MexAB–OprM and MexXY–OprM (Li and Nikaido, 2009). The transport of carbapenems across the lipid bilayer membranes occurs through protein channels, porins, and especially OprD. Lack of OprD participates in Pa isolates resistant to carbapenems (Ocampo-Sosa et al., 2012; Quale et al., 2006).

To our knowledge, this study is the first in which these worrying resistance patterns of both community and hospital CRPa isolates are reported. More than 95% of clinical and community CRPa isolates were XDR, confirming the emergence of pan-drug resistant profiles in this species as reported in other studies conducted in Saudi Arabia (Shaaban et al., 2017), Egypt (El-Domany et al., 2017; Zafer et al., 2015), in Africa and the Middle East (Karlowsky et al., 2022), Italy (Pollini et al., 2013), Greece (Tsakris et al., 2000), Turkey (Bahar et al., 2004), and Iran (Ghasemian et al., 2018; Nikbin et al., 2007).

Cefiderocol received U.S. Food and Drug Administration approval in October 2019 for treating urinary tract infections and in September 2020 to include hospital-acquired pneumonia and ventilator-associated bacterial pneumonia. It is one of the best novel treatments against carbapenem-resistant Gram-negative bacteria (McCreary et al., 2021). The SCPa57 (from a 72-year-old Moroccan man patient with a urinary tract infection) and SCPa84 (from a 70-year-old Moroccan female patient in hospital) strains were surprisingly resistant to cefiderocol (MIC = 4 and 8 mg/L, respectively), especially, since this antibiotic is not yet available in Morocco; therefore, the patients were not exposed to cefiderocol. This is the first case report until today of cefiderocol resistance in Morocco.

According to Karakonstantis et al. (2022), as well as Simner et al. (2021), the alterations in the target binding sites of Pa-derived AmpC β -lactamases have the potential to reduce the activity of three of four novel β -lactams (i.e., ceftolozan–tazobactam, ceftazidime–avibactam, and cefiderocol).

Cefiderocol is distinct from other β -lactam antibiotics because it enters the periplasmic region through siderophore iron transporters known as TonB-dependent receptors. In Pa, the genes PirA, PiuA, and PiuD encode for TonB-dependent receptors (Streling et al., 2021).

Loss of function for the TonB-dependent receptors necessary for cefiderocol import may result from mutations in these genes. It has been demonstrated that deletion of PiuA and PiuD increases the MIC of cefiderocol against Pa by 2 and 32 times, respectively. A Pa isolate

taken from a patient not exposed to cefiderocol has been found to have PiuD and PiuR mutations (Streling et al., 2021).

In the present study, the concordance rate between the disc diffusion method and phenotypic tests for carbapenemase detection was around 95% (17/18, 94.44%). All strains Carba NG test positive also gave a positive result in the PCR for tested carbapenemase encoding genes (17/17). The sensitivity of Carba NG test for carbapenemases detection was therefore shown to be 100%. *bla*_{NDM} gene is detected in all isolates. As confirmed by sequencing, the combination *bla*_{NDM-1}–*bla*_{VIM-2} genes were detected in CSPa33 isolate and CSPa 84 cefiderocol resistant isolate, whereas CSPa90 strain coharbored *bla*_{VIM-2} and *bla*_{IMP-8} genes. It must be emphasized that sequencing was carried out only for these three isolates.

These gene combinations in these three Moroccan isolates are submitted in a Letter to the editor as the first report of Pa strains coharboring these M β L genes in Morocco. Pa strains coharboring *bla*_{VIM} and *bla*_{IMP} were also identified in Egypt at a rate of 3.5% (4/114) El-Domany et al. (2017), whereas only one *bla*_{VIM-2} M β L gene was detected in clinical Pa isolates (2/25, 8%) collected in two major hospitals of Meknes-Tafilalet region in Morocco (Maroui et al., 2016).

More extensive studies of genomic variation in these Pa isolates are needed to determine strain-specific sequences and genetic typing, which may allow our understanding of their resistance genetic determinants.

Conclusion

This study reports a high rate of CRPa in Morocco, especially in the hospital, and the spread of strains coharboring two carbapenemase genes. The worrying antibiotics resistance profiles of all isolates are also a cause of concern highlighting the great threat to treating Pa infections. In addition, we report the first emergence case of Pa resistance to cefiderocol without patient exposure to this drug in Morocco. The spread of XDR strains is a major concern as it is associated with high death rates. A thorough understanding of molecular mechanisms of resistance and antibiotics prescription according to the antibiogram results is recommended for the optimal management of pseudomonas infections due to CRPa, given that delays in appropriate therapy increase mortality in Pa infections. Implementing multidisciplinary strategies and exploring other therapeutic strategies could contribute to infection control and minimize the dissemination of these strains.

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Authors' Contributions

Conceptualization: B.I.; Methodology: B.I., N.K.; Technical supports: B.P., H.T.-D., and K.A.; Acquisition of data

isolates: B.F.; validation: B.I., N.K., and E.O.F.; Formal analysis: B.I.; writing-original draft preparation: B. I.; writing-review and editing: B.I.; Visualization: E.O.F.; Supervision: E.O.F.; Project administration: N.K. and T.M. All authors have read and agreed to the published version of the article.

Institutional Review Board Statement

This study was approved by the Ethics Committee of Faculty of Medicine and Pharmacy Rabat, Morocco (Date: 23.12.2021, No: 85/21).

Disclosure Statement

No competing financial interests exist.

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