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Posted Date: 13 December 2024

doi: 10.20944/preprints202412.1056.v1

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Article

Combating Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa*: The Fractional Inhibitory Concentration Index as a Tool to Evaluate Antibiotic Synergy

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Abstract: Background: Multi-drug-resistant Gram negative bacteria producing metallo-β-lactamase are an increasing concern. Here we describe three cases of infection due to difficult to treat resistant *P. aeruginosa* producing metallo-β-lactamases, successfully treated with antibiotic combination of cefiderocol plus imipenem-relebactam and report on the molecular and epidemiological features of the isolates and the in vitro synergistic effects of different antibiotic combinations to guide the antibiotic treatment. **Patients and methods**: Three *P. aeruginosa* strains were isolated from respiratory or blood cultures of three different patients. Minimum inhibitory concentrations breackpoints were interpreted according to EUCAST recommendations. Next Generation Sequencing data were used for in silico identifying resistance genes, sequence types and for core genome multi-locus sequence typing analysis. The fractional inhibitory concentration index was performed as a measure of synergy of cefiderocol plus imipenem and imipenem-relebactam. **Results**: The three isolates exhibited different multi-drug resistant and molecular profiles, carrying blaIMP-13 (isolates named Pse-1 and Pse-3) and blaVIM-2 carbapenemases (isolate Pse-2). Typing showed that the isolates did not cluster and belonged to different sequence types. The E-test method showed the presence of synergy of cefiderocol in combination with imipenem-relebactam in the two *P. aeruginosa* isolates producing IMP-13 (Pse-1 and Pse-3). No synergy was observed in the isolate producing VIM-2 (Pse-2). **Conclusion**: Cefiderocol in association with imipenem-relebactam exhibited a synergistic effect against IMP-producing *P. aeruginosa* isolates. Further studies with a range of drugs and an expanded number of isolates are required to ascertain potential novel synergistic associations and the clinical utility of the fractional inhibitory concentration index.

Keywords: Carbapenem-resistant *Pseudomonas aeruginosa*; Metallo-β-Lactamase; IMP-13 carbapenemases; antimicrobial synergy; Cefiderocol; Imipenem-relebactam; bloodstream infection; antimicrobial stewardship; antibiotic treatment; Verona integron–encoded metallo-β-lactamase; VIM

1. Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major cause of hospital-acquired infections. Frail hospitalized patients such as those who have suffered burns, require ventilation, or have neutropenia or chronic debility are at higher risk to develop infections due to *P. aeruginosa*. *P. aeruginosa* possesses intrinsic resistance and may acquire mechanism conferring resistance to a variety of antibiotics, including extended-spectrum-lactamases (ESBLs) and carbapenemases.

 doi:10.20944/preprints202412.1056.v1

Worldwide, difficult-to-treat (DTR) *P. aeruginosa* causes a wide range of severe infections, including pneumonia, bloodstream infections (BSI), endophthalmitis, endocarditis, meningitis and is frequently associated with high mortality and morbidity rates [1,2].

A common feature of DTR and pan-drug-resistant *P. aeruginosa* isolates is the production of metallo-β-lactamase (MBL) such as Verona integron–encoded (VIM) and active on imipenem (IMP) metallo-β-lactamase. A great variety of ESBLs is also reported, such as oxacillinase (OXA), Pseudomonas extended resistance β-lactamase (PER), Guiana extended spectrum β-lactamase (GES), Vietnam extender spectrum β-lactamase (VEB) and polyamine oxidase (PAO) β-lactamase.

Nowadays, β-lactamase enzymes are widely disseminated among different *P. aeruginosa* sequence types (STs) and are often associated to other resistance mechanisms, including decreased membrane permeability or active efflux pump systems [3–5]. Since the year 2000, a persistent circulation of DTR *P. aeruginosa* strains producing VIM and IMP has been reported in Italian hospitals, frequently resulting in outbreaks with limited treatment options [6–8]. As novel antimicrobial compounds such as ceftolozane-tazobactam, ceftazidime-avibactam and imipenem-relebactam are useful against non-MBL producer *P. aeruginosa*, cefiderocol might be considered a last resort antibiotic for MBL producing strains [2,8].

Here, we report on the management of three cases of severe infections caused by DTR, MBL producer *P. aeruginosa* strains and investigate the molecular and epidemiological features of the isolates as well as the *in vitro* synergistic effects of different antibiotic combinations.

2. Patients and Methods

In January-February 2024, three cases of infection due to MBL-producing *P. aeruginosa* were diagnosed in three different Intensive Care Units in the San Camillo Hospital in Rome. The patients were treated with a combination of cefiderocol and imipenem/relebactam. Samples collected before the commencement of combination antibiotic therapy were sent to the Microbiology Laboratory of the National Institute for Infectious Diseases "L. Spallanzani", IRCCS Microbiology Laboratory for further phenotypic and molecular characterization of the isolates and to evaluate in vitro antibiotic synergy.

2.1. Phenotypic and Molecular Characterization of Isolates

Antibiotic susceptibility and species identification were determined by the Vitek-2 System (bioMérieux, Marcy l'Étoile, France), AST-438 plus XZ26 and MALDI-TOF MS Biotyper Sirius (Bruker Daltonics) respectively. Minimum inhibitory concentrations (MICs) for cefiderocol was performed by broth microdilution and synergy test by gradient stripes method (Liofilchem, Roseto degli Abruzzi, Italy). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing [9]. First detection and identification of the most diffused carbapenemases (KPC, OXA-48-like, IMP, VIM and NDM) was achieved using immunochromatographic assay (NG-Test CARBA 5, Biotech, France) and confirmed by Whole Genome Sequencing (WGS) performed by Illumina Miseq (San Diego, United States). All raw reads generated were submitted to the Sequence Read Archive (SRA) under the BioProject ID PRJNA1161848.

The resistance profile and Sequence types (STs) of isolates, were identified by *in silico* analysis using the ResFinder v3.0 web server [10]. Bacterial epidemiological typing was performed by the WGS-based core genome MLST (cgMLST) scheme v1.0, using the Ridom SeqSphere+ software (Ridom GmbH, Münster, Germany) with default settings. Based on the defined cgMLST for P. aeruginosa, a gene-by-gene approach with 3867 target genes, was used to compare the genomes [11]. According to the manufacture instruction, compared to the reference strain (GenBank accession no. NC_002516.2), the resulting set of target genes was then used for interpreting the clonal relationship displayed in a minimum spanning tree (MST) using a complex type (CT) distance of 12 alleles [12].

 doi:10.20944/preprints202412.1056.v1

2.2. The Fractional Inhibitory Concentration (FIC) Index as a Measure of Antibiotic Synergy

An inoculum equal to 0.5 McFarland turbidity was prepared from each isolate. Determination of MICs by E-test was first performed for the two drugs separately, and the MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip. For synergy testing, the two E-test strips were placed on the same culture plate in a cross formation, so that they intersected each other at their respective MICs with a 90° angle or at the highest concentration present on the E-test strip, when the MIC exceeded this value (e.g., > 256 mg/L). The plates were then incubated at 37°C for 24 hours. The Fractional Inhibitory Concentration Index (FICI) was calculated on the basis of the resulting zone of inhibition as follows: FICI = FIC A+FIC B, where FIC A is the MIC of the combination/MIC of drug A alone, FIC B is the MIC of the combination/MIC of drug B alone [13]. interpretation of the FIC results, according to accepted criteria, were as follows: <= 0.5, synergy; 0.5 to 1.0, additivity; >1.0 to 4.0, indifference; and >4, antagonism [13].

3. Results

3.1. Clinical Cases Presentation

Three patients were included in the study, receiving combination therapy with cefiderocol and imipenem/relebactam. All three patients had prompt clinical improvement and microbial eradication, without the occurrence of relapses. This resulted in their discharge from the Intensive Care Units where they had been admitted.

Case 1 - isolate Pse-1

The initial case was that of a 65-year-old patient who had undergone cardiac surgery to replace the ascending aorta due to a diagnosis of type A dissection. This patient was diagnosed with mechanical ventilator-associated pneumonia. The patient was receiving intensive care and was undergoing continuous renal replacement therapy. The microorganism isolated from bronchoalveolar lavage was a P. aeruginosa producing IMP-13 MBL (Pse-1). The patient was treated with combination therapy with cefiderocol, 2 grams every 8 hours as a continuous infusion, in association with imipenem/relebactam, 0,625 grams every 6 hours for a total duration of 7 days. This resulted in a rapid resolution of the clinical picture, allowing a rapid respiratory weaning with early extubation four days after the start of combination antimicrobial therapy.

Case 2 - isolate Pse-2

The second case concerned a central venous catheter-related bloodstream infection in a 37-year-old patient who had been hospitalized in the intensive care unit due to a recent road polytrauma. A strain of P. aeruginosa producing VIM was isolated from blood cultures (Pse-2). The patient underwent replacement of the central venous catheter and a combination antibiotic treatment was initiated, comprising cefiderocol (2 grams every 8 hours in extended infusion) and imipenem/relebactam (1,250 grams every 6 hours for a total duration of 14 days). During this period, the patient made a rapid recovery, with negative control blood cultures and echocardiogram.

Case 3 - isolate Pse-3

The third case was a 43-year-old woman with bacteremic pneumonia associated with mechanical ventilation. The patient was initially admitted to the intensive care unit for the treatment of pneumonia and severe acute respiratory failure caused by the influenza A H1N1 virus. A strain of P. aeruginosa producing IMP-13 was isolated from the blood (Pse-3). The patient was treated with a combination of cefiderocol (2 grams every 8 hours in prolonged infusion) and imipenem/relebactam (1,250 grams every 6 hours for a total duration of 14 days). The patient made a rapid recovery during treatment with early negativity of control blood cultures, rapid and progressive improvement of respiratory exchanges, allowing extubation three days after starting the combination therapy.

3.2. Phenotypic and Molecular Characterization of Isolates

As shown in Table 1, the three isolates (named Pse-1 Pse-2 and Pse-3) were obtained from different specimens: bronchoalveolar lavage for Pse-1, blood culture for Pse-2 and Pse-3. Pse-1 and Pse-2 were resistant to carbapenems, ceftazidime-avibactam, ceftolozane-tazobactam, meropenem-

 doi:10.20944/preprints202412.1056.v1

vaborbactam, imipenem-relebactam and ciprofloxacin. Isolate Pse-3 was resistant to ceftazidime-avibactam and ceftolozane-tazobactam. All the three isolates were susceptible to cefiderocol.

Table 1. Antimicrobial susceptibility results for clinical isolates described in this study.

Strain	Sample	Date	MBL	Antibiotic MIC values (mg/L)										
				CZA	FDC	C/T	M/V	I/REL	IPM	MEM	CAZ	CIP	FOS	PZT
Pse-1	BAS	02.01.24	IMP-13	>256, R	0.064, S	>256, R	32, R	>32, R	>32, R	≥16, R	≥ 64, R	≥ 4, R	16, S	8, SI
Pse-2	Blood	26.01.24	VIM-2	>256, R	0.19, S	>256, R	≥ 64, R	>32, R	>32, R	≥16, R	≥ 64, R	≥ 4, R	>256, SI	≥ 128, R
Pse-3	Blood	26.01.24	IMP-13	>256, R	0.047, S	>256, R	2, S	1.5, S	2, SI	4, SI	≥ 64, R	0.25, SI	6, S	8, SI

CZA, ceftazidime/avibactam; FDC, Cefiderocol; C/T, Ceftolozane/tazobactam; M/V, Meropenem/vaborbactam; I/REL, Imipenem/relebactam; IPM, imipenem; MEM, meropenem; CAZ, Ceftazidime; CIP, Ciprofloxacin; FOS, Fosfomycin; PZT, Piperacillin/tazobactam. S, susceptible; R, resistant, SI, susceptible high dosage.

Detection of acquired antimicrobial resistance genes and STs are recorded in **Table 2**.

Table 2. Molecular characterization of the clinical isolates.

Strain	Genetic determinants		Typing	
	Beta-lactam	Additional resistance genes	ST	CT
Pse-1	***bla*IMP-13**, *bla*OXA-50, *bla*PAO	*aph(3')-IIb, qacE, fosA, catB7, crpP, sul1*	621	
Pse-2	***bla*VIM-2**, *bla*OXA-486, *bla*PAO	*aac(6')-II, aph(3')-IIb, aac(3')-Id, qacE, fosA, catB7, cmlA1, floR, crpP, sul1, tet(G), dfrB5*	233	
Pse-3	***bla*IMP-13**, *bla*OXA-395, *bla*PAO	*aph(3')-IIb, qacE, fosA, catB7, crpP, sul1*	446	

ST: Sequence Type identified by multi-locus sequence typing method; CT: Cluster Type identified by core genome multi-locus sequence typing method.

The three isolates were positive for beta-lactams resistance genes such as MBLs (VIM-2 or IMP-13), blaPAO and OXA-type beta-lactamases but belonged to different STs.

Pse-1 (ST621) harbored blaIMP-13. Instead, Pse-2 produced a VIM-2 carbapenemase and belonged to the ST233, a clonal strain, referred as an international high-risk clone, responsible for nosocomial infection identified worldwide [14]. Additional resistance profile of Pse-2 isolates, showed the presence of 3 aminoglycoside resistance genes [aac(6')-II, aph(3')-IIb, aac(3')-Id], 3 phenicol resistance genes [catB7 cmlA1 floR], a quaternary ammonium compound qacE, and tetracycline, fosfomycin, sulfonamide, trimethoprim and quinolone resistance genes tet(G), fosA, sul1, dfrB5 and crpP, respectively.

According to the cgMLST method, the three isolates did not cluster into a Cluster-Type, and were categorized as belonging to 3 STs showing high allelic distance (more than 3000 allele difference) (**Figure 1**).

 doi:10.20944/preprints202412.1056.v1

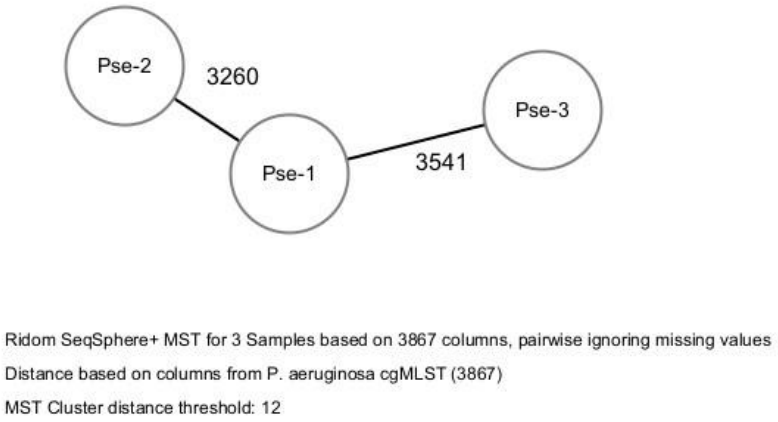

Figure 1. Bacterial epidemiological typing of the three isolates.

3.3. The Evaluation of Antibiotic Synergy by Fractional Inhibitory Concentration Index (FICI)

The evaluation of antibiotic synergy by the E-test method and the FICI showed the presence of synergy/additivity of cefiderocol in combination with imipenem-relebactam in the two *P. aeruginosa* isolates producing IMP-13. Pse-1 had a FICI of 0.34 (synergy), Pse-3 had a FICI of 0.84 (additivity). No synergy between cefiderocol and imipenem-relebactam was observed for the Pse-2 isolate producing VIM-2 (FICI of 2, defined as indifference) (**Figure 2**).

Bacterial epidemiological typing was performed by the whole genome sequencing-based core genome multi-locus sequence typing (cgMLST). A gene-by-gene approach with 3867 target genes was used to compare the genomes.

Pse-1 (IMP-13 producer)	**Agar diffusion (E-test) MIC**	**Fractional Inhibitory Concentration Index**
Cefiderocol + Imipenem	Cefiderocol alone: 0,064 Cefiderocol combination: 0,016 Imipenem alone: >32 Imipenem combination: 2	0,31
Cefiderocol + Imipenem/relebactam	Cefiderocol alone: 0,064 Cefiderocol combination: 0,016 Imipenem/relebactam alone: >32 Imipenem/relebactam combination: 3	0,34
Cefiderocol + Fosfomycin	Cefiderocol alone: 0,064 Cefiderocol combination: 0,016 Fosfomycin alone: 16 Fosfomycin combination: 6	0,62
Pse-2 (VIM-2 producer)	**Agar diffusione (E-test) MIC**	**Fractional Inhibitory Concentration Index**
Cefiderocol + Imipenem	Cefiderocol alone: 0,19 Cefiderocol combination: 0,19 Imipenem alone: >32 Imipenem combination: 32	2
Cefiderocol + Imipenem/relebactam	Cefiderocol alone: 0,19 Cefiderocol combination: 0,19 Imipenem/relebactam alone: >32 Imipenem/relebactam combination: 32	2
Cefiderocol + Fosfomycin	Cefiderocol alone: 0,19 Cefiderocol combination: 0,19 Fosfomycin alone: >256 Fosfomycin combination: 256	2

 doi:10.20944/preprints202412.1056.v1

Pse-3 (IPM-13 producer)	Agar diffusione (E-test) MIC	Fractional Inhibitory Concentration Index
Cefiderocol + Imipenem	Cefiderocol alone: 0,047 Cefiderocol combination: 0,016 Imipenem alone: 2 Imipenem combination: 0,5	0,59
Cefiderocol + Imipenem/relebactam	Cefiderocol alone: 0,047 Cefiderocol combination: 0,016 Imipenem/relebactam alone: 1,5 Imipenem/relebactam combination: 0,75	0,84
Cefiderocol + Fosfomycin	Cefiderocol alone: 0,047 Cefiderocol combination: 0,023 Fosfomycin alone: 6 Fosfomycin combination: 2	0,81

Figure 2. The measure of antibiotic synergy by fractional inhibitory concentration index (FICI).

An inoculum equal to a 0.5 McFarland turbidity was prepared from each isolate. Determination of MICs by E-test was first performed for the two drugs separately, and the MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip. For synergy testing, the two E-test strips were placed on the same culture plate in a cross formation, so that they intersected each other at their respective MICs with a 90° angle or at the highest concentration present on the E-test strip, when the MIC exceeded this value (e.g., > 256 mg/L). The plates were then incubated at 37°C for 24 hr. The Fractional Inhibitory Concentration Index (FICI) was calculated on the basis of the resulting zone of inhibition as follows: FICI = FI A+FIC B, where FIC A is the MIC of the combination/MIC of drug A alone, FIC B is the MIC of the combination/MIC of drug B alone. interpretation of the FIC results, according to accepted criteria, were as follows: <= 0.5, synergy; 0.5 to 1.0, additivity; >1.0 to 4.0, indifference; and >4, antagonism.

4. Discussion

The global occurrence of carbapenem-resistant and MDR P. aeruginosa is alarming because infections by these bacteria often result in limited treatment options [15]. The antimicrobial cefiderocol might be considered a last resort drug for some MBLs, non-New Delhi metallo-β-lactamase producing P. aeruginosa strain. Alarmingly, the emergence of cefiderocol resistance has been reported in non-New Delhi metallo-β-lactamase producing P. aeruginosa strains [16,17]. The emergence of resistance to cefiderocol in P. aeruginosa has been demonstrated both in vitro and in vivo, and was linked to alterations of the iron uptake pathways [18–21]

Here, we present three cases of severe MDR P. aeruginosa infection sustained by MBL producing strains, successfully treated with an antibiotic combination of cefiderocol and imipenem/relebactam. It is important to remark that currently there is no clear evidence that association therapy is more effective than monotherapy for infection due to MDR P. aeruginosa [22], unless may be in severe infections like septic shock.

A recent multicentre, retrospective cohort study [23] compared outcomes of patients with septic shock due to P. aeruginosa BSI receiving adequate empirical combination therapy to those on adequate empirical monotherapy. Adequate empirical combination therapy was associated with a lower 30-day all-cause mortality (25%, six out of 24) compared to adequate empirical monotherapy (56.8%, 42 out of 74; P = 0.007). Multivariate Cox regression analysis indicated Adequate empirical combination therapy as the only factor significantly associated with improved survival (aHR 0.30; 95% CI 0.12–0.71; P: 0.006) [23].

The administration of cefiderocol in combination with other antibiotics, i.e. ceftazidime/avibactam, ampicillin/sulbactam or meropenem, has been recently proposed to treat MDR and pandrug-resistant Gram negative bacteria, including P. aeruginosa [24,25]. The rationale

 doi:10.20944/preprints202412.1056.v1

for administering cefiderocol in combination with other antimicrobials is to avoid resistance development and to exploit a potential synergistic effect [24].

The antibiotic combination may provide a synergistic effect, determining a greater and faster bactericidal effect, with improved patient outcome [26]. In addition, a potential benefit of the combination therapy is the reduction in the emergence of resistance to cefiderocol [27,28]. To consider, combination therapy may also have disadvantages, such as potentially increasing toxicity, or increased C. difficile infections [29]. Moreover, there is currently little data available to guide the choice of ancillary drug to be given with cefiderocol in the treatment of MBL-producing P. aeruginosa infections, i.e. fosfomycin or carbapenems.

In literature, there is scant data on the evaluation of synergy between cefiderocol and other antimicrobials against P. aeruginosa [30,31].

Therefore, an assay to evaluate the synergy between cefiderocol and imipenem, imipenem/relebactam, fosfomycin as well as other antibiotics, may have potential therapeutic utility in the management of MBL-producing P. aeruginosa infections to assist in the selection or discontinuation of the companion drug in combination with cefiderocol. Of course, this approach may only be useful if supported by prior microbiological testing of the isolate susceptibility profile and molecular characterisation. To our knowledge, this is the first report using the E-test technique and FICI to evaluate the synergy of cefiderocol plus imipenem/relebactam against MBL-producing P. aeruginosa strains.

By E-test and FICI evaluation, our two P. aeruginosa producing IMP-13 MBL showed synergy or additivity of cefidercol plus imipenem/relebactam, whilst the isolate producing VIM-2 showed no synergy effect. The mechanisms behind the synergy between cefiderocol and imipenem against P. aeruginosa are still unclear. One possible explanation could be the inhibition of multiple PBP targets by the two drugs (i.e., PBP 3, which is preferentially bound by cefiderocol, and other PBPs including PBP2, which are bound by imipenem at concentrations that might partially evade degradation by MBLs [31–33].

Further studies on the potential synergistic effects of cefiderocol combinations with carbapenems and other antimicrobials would be useful.

Understanding of the specific MBL type may also be of potential benefit. In our study, the difference in the phenotypic profiles of the three P. aeruginosa isolates could be a result of the difference in the resistome. All isolates were positive for β-lactam resistance genes such as MBLs (VIM or IMP), blaPAO and an OXA-type beta-lactamase and belonged to three different STs. Pse-1 and Pse-3, respectively ST621 and ST446, harboured blaIMP-13 and an identical additional resistance profile. Interestingly, they showed a completely different phenotypic profile. This could be due to a difference in membrane permeability and efflux pumps, as both OXA-50 and OXA-395 are oxacillinases with weak carbapenemase activity (unique difference in the molecular profile of isolates Pse-1 and Pse-3) [34,35].

5. Conclusions

Until further data are available, clinicians may consider combination therapy with cefiderocol and another antimicrobial for the treatment of severe infections caused by MBL-producing *P. aeruginosa* in order to avoid the development of cefiderocol resistance and to take advantage of a potential synergistic effect.

The E-test and FICI evaluation may prove a valuable tool in the treatment of MBL-producing *P. aeruginosa* infections, assisting in the selection or discontinuation of the companion drug in combination with cefiderocol.

Author Contributions: Conceptualization, G.G., C.V., C.F. and S.C.; methodology, C.R., V.D., S.D., A.G.; software, G.G., C.V.; validation, G.G, C.F., A.C. and S.C.; formal analysis, G.P., A.C., C.V., C.F.; investigation, G.P., A.C., C.V., C.F.; resources, G.P., S.C., C.F.; data curation, C.R., V.D., S.D., A.G.; writing—original draft preparation, G.G., C.V.; writing—review and editing, G.G., A.C., S.C.; visualization, G.G., C.V., C.F., S.C.; supervision, S.C., C.F.; project administration, S.C., C.F.; funding acquisition, S.C., C.F. All authors have read and agreed to the published version of the manuscript.".

 doi:10.20944/preprints202412.1056.v1

Funding: This work was supported by the Italian Ministry of health, through Ricerca Corrente Line n. 1, Project n. 2.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Acknowledgments: We gratefully acknowledge the staff of our BioBank: Gianluca Prota, Alberto Rossi, Melissa Baroni, Valentina Antonelli for their helpful activity in the storage of samples and microorganisms.

Conflicts of Interest: The authors declare no conflicts of interest.

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