Clonal transmission and new mechanism of resistance to trimethoprim-sulfamethoxazole in *Stenotrophomonas maltophilia* strains isolated in a neonatology unit at Antananarivo, Madagascar, deciphered by whole genome sequence analysis

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ABSTRACT

Stenotrophomonas maltophilia has been recognized as an emerging multidrug resistant organism in hospital settings due to its resistance to a broad range of antimicrobial agents. These include β-lactams and aminoglycosides, afforded by the existence of intrinsic and

acquired resistance mechanisms. Trimethoprim/sulfamethoxazole (SXT) is recommended as one of the best treatment choices against *S. maltophilia* infections; however increasing resistance to SXT has complicated the treatment. From July 2014 to March 2015, individuals and surfaces from a neonatology ward in Antananarivo, Madagascar, were longitudinally followed to assess the transmission of bacteria resistant to antibiotics between neonates, individuals (parents and nurses) and ward environments. Four *S. maltophilia* strains were successively isolated from a water-tap (N=1), from feces obtained from a newborn (N=1), and nursing staff (N=2). Antimicrobial susceptibility testing and whole genome sequencing were performed on each isolate. Based on coregenome alignment, all strains were identical and belonged to the new sequence type ST-288. They were resistant to trimethoprim-sulfamethoxazole, carbapenems and intermediate to levofloxacin. Each isolate carried the *aadB*, *strA*, *strB* and *sul1* genes located in a class I integron but variants of the *dfrA* gene were absent. We assessed by PROVEAN analysis the single nucleotide mutations found in *folA*, *folC* and *folM* genes and only the mutation in *folA* (A114T:GCC→ACC) has an effect on the activity of trimethoprim. Our findings demonstrated the prolonged presence of SXT-resistant *S. maltophilia* in a clinical setting with consecutive transfers from the environment to a newborn and staff based on the isolation dates. We also hypothesized that single nucleotide mutations in *folA* could be responsible for trimethoprim resistance.

INTRODUCTION

Stenotrophomonas maltophilia is a non-fermentative Gram-negative bacterium generally found throughout the environment (soil, sewage, plants). It is also occasionally isolated in hospitals where this bacterium is currently regarded as an important opportunistic pathogen. It causes a large range of clinical syndromes such as bacteraemia, sepsis, pneumonia, meningitis, endocarditis, septic arthritis, urinary infections, and endophthalmitis (1, 2), especially in hospitalized immunocompromized patients or patients with underlying disease. *S. maltophilia* has been recognized as one of the leading nosocomial multidrug resistant

organisms due to its resistance to a broad range of antimicrobial agents, including β-lactams and aminoglycosides, afforded by the existence of intrinsic and acquired resistance mechanisms (3). However it remained susceptible to fluoroquinolones, polymyxins and trimethoprim/sulfamethoxazole (SXT) (4).

SXT in association with ticarcillin and clavulanic acid is traditionally recommended as one of the first choices against *S. maltophilia* infections but fluoroquinolones (also in association with other antibiotics) are an attractive option due to their *in vitro* activity (5). However, increasing resistance to SXT has complicated the treatment and resistance determinants such as *sul* and *dfrA* genes, class 1 integrons and mobile genetic elements have been reported to contribute to SXT resistance (6–8). The aim of this study was to establish the link between 4 SXT resistant *S. maltophilia* isolates collected in a neonatalogy unit and to decipher the genetic basis of resistance to SXT.

MATERIALS AND METHODS

Study design

The longitudinal study was conducted in the neonatal intensive care unit in CENHOSOA hospital in Antananarivo, Madagascar (08/27/2014–03/06/2015) and was already described in Bonneault *et al*. 2019 (9). Briefly, 22 newborns (NBs) were included in the cohort and were followed until discharge or death. Average unit stays lasted 18 days. All health-care workers (HCWs) and NBs' accompanying family members (FMs; usually the mother, involved in the basic infant care, except for one child who had four distinct accompanying FMs) were also followed. In total 22 NBs, 21 HCWs and 24 FMs were included in the study. At enrollment, a rectal swab was obtained from the NB and a stool sample from the FM to detect E-ESBL colonization. Rectal swabs were systematically obtained from the NB on a weekly basis. For stays <7 days in the unit, a stool sample was obtained the day of discharge. Stools were also collected from the FMs and from the HCWs every week. Environmental swabbing was performed at the beginning, middle and the end of the investigation. The study was approved

by the Madagascar Public Health Ministry Ethics Committee (Reference number: 040–MSANP/CE).

Bacteriological analyses

All samples were cultivated on CHROMagar™ ESBL (CHROMagar, Paris, France) and each colony morphotype was identified by mass spectrometry (MS) MALDI-TOF (Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibility testing was performed on each isolate according to the standard disc methods described in the 2018 CASFM guidelines. In this study, we only focused on *S. maltophilia* positive samples.

Whole genome sequencing (WGS) & Bioinformatic analysis

DNA extraction was performed on 5 mL of liquid cultures grown overnight at 37°C in a Luria Bertani infusion medium by using the Cador Pathogen Extraction Kit (Indical Bioscience) on the Qiacube HT (QIAGEN, France) device according to the manufacturer's protocol for Gram-negative bacteria. DNA quantity and purity was assessed by using Nanodrop 2000/200C (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was conducted by using the Nextera XT DNA Sample Kit (Illumina, San Diego, CA, USA). WGS was performed on a NextSeq 500 platform (Illumina) by using 2 × 150-bp runs. FqCleaner version 3.0 was used to eliminate adaptor sequences (10, 11), reduce redundant or overrepresented reads (12), correct sequencing errors (13), merge overlapping paired reads, and discard reads with Phred scores (measure of the quality of identification of nucleobases generated by automated DNA sequencing) <20. Illumina reads *de novo* assembly was performed using Spades (14). Acquired resistance genes were detected by the resfinder software (15). The genomes were annotated by using prokka and PATRIC web server (16, 17). The sequence types (ST) were determined *in silico* with the public database for *S. maltophilia* (https://pubmlst.org/smaltophilia/). Phylogenetic analysis based on whole genome

sequences was done using the Parsnp program from the harvest suite, gubbins and RaxML (18–20). Mutation detection in SXT resistant *S. maltophilia* strains was performed with the Breseq 0.31.1 software (*S. maltophilia* D457 was used as reference genome) (21). All non-synonymous mutations were analyzed with PROVEAN (Protein Variation Effect Analyzer) (13) to predict the functional deleteriousness caused by the missense mutation. Plasmid detection, typing and reconstruction were performed with the MOB-suite software (22).

Predicted structure and molecular docking

The secondary and tertiary structures of proteins were predicted by using the Raptor X server (23–26). The wild-type amino acid sequence from the D457 strain was chosen as template for homology modeling. We used autodock vina to calculate Gibbs free energy of binding (ΔG_{bind}) of ligands (trimethoprim) with targets. They were further converted to the predicted inhibition constants (Ki_{pred}) with this formula (27):

$$Ki_{pred} = \exp([\Delta G_{bind} * 1{,}000]/[R * T])$$

where R (gas constant) is 1.98 cal(mol*K)−1, and T (room temperature) is 298.15 Kelvin.

Quantification of biofilm formation

The biofilm assay was performed as previously described (28) but with slight modifications. Overnight cultures of *S. maltophilia* in 5 mL Luria Bertani infusion medium reaching an optical density at 620 nm equivalent to 1 (OD620) (approximately $1*10^9$ CFU/mL) were transferred to the wells of a sterile flat-bottomed 96-well polystyrene microtitre plate and incubated for 24h at 35°C +/- 2°C. Non adherent cells were subsequently removed by washing twice with 200 µl of sterile distilled water. The amount of biofilm biomass was assessed by crystal violet staining. Biofilms were stained with 125 µl of 1.0 % (w/v) crystal violet for 15 min. The dye solution was discarded, and the plate was washed three times with sterile distilled water and allowed to air-dry for 24 h at room temperature. Stained biofilms were exposed to 30.0 % (v/v) acetic acid for 15 min, and the OD 620 of the extracted dye was

subsequently measured. The average OD values was calculated for all tested strains and negative controls, since all tests are performed in triplicate and repeated three times. Second, the cut-off value (ODc) was established. It is defined as three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). OD values of a tested strain are expressed as average OD value of the strain reduced by ODc value (OD=average OD of a strain -ODc). ODc value is calculated for each microtiter plate separately. Based upon the previously calculated OD values: OD ≤ODc = no biofilm producer; ODc ≤OD ≤2 × ODc = weak biofilm producer; 2 × ODc≤OD≤4×ODc = moderate biofilm producer; 4ODc<OD = strong biofilm producer. All tests were performed in triplicate and repeated three times.

RESULTS

Epidemiological data

During the cohort conducted in the neonatal unit - (see Materials and Methods), 4 *S. maltophilia* were isolated. A first *S. maltophilia* strain (MS MALDI-TOF score of 2.736) was isolated in January, 07th 2015 from an environmental swab (476SM) realized on a water tap out of the three sinks present in the neonatalogy unit. Fourteen days later a second *S. maltophilia* strain (MS MALDI-TOF score of 2.120) was isolated from a rectal swab of a premature newborn (517SM) admitted to the neonatal ICU. Subsequently, two strains of *S. maltophilia* were isolated from the fingerprints of 2 nurses at day 47 (629SM) and 56 (646SM) after the first isolation from the water tap (MS MALDI-TOF scores of respectively 2.102 and 2.052) (Table 1).

Antimicrobial susceptibility testing

All 4 isolates were resistant to cotrimoxazole (SXT), ertapenem (ETP), meropenem (MEM) and imipenem (IPM), intermediate to levofloxacin (LVX) and sensitive to minocycline

(MNO), ceftazidime (CAZ) and ticarcillin-clavulanate (TCC). The antimicrobial susceptibility testing results of the 4 strains are showed in table1.

Table1: Antimicrobial susceptibility testing of the four *S. maltophilia* strains. Abbreviations: SXT: trimethoprim-sulfamethoxazole, TCC: Ticarcillin-clavulanate, CAZ: ceftazidim, LVX: levofloxacin, MNO: minocycline, MEM: meropenem, ETP: ertapenem and IPM: imipenem.

Strains	Origin	Isolation date	Sample	inhibition zone diameters (mm)							
				SXT	TCC	CAZ	LVX	MNO	MEM	ETP	IPM
476SM	Watertap	January 07, 2015	Environemental swab	6 (R)	35 (S)	22 (S)	21 (I)	25 (S)	6 (R)	6 (R)	6 (R)
517SM	Baby	January 21, 2015	Rectal swab	6 (R)	37 (S)	23 (S)	21 (I)	27 (S)	6 (R)	6 (R)	6 (R)
646SM	Nursing staff1	February 23, 2015	Fingerprint	6 (R)	36 (S)	23 (S)	21 (I)	24 (S)	6 (R)	6 (R)	6 (R)
629SM	Nursing staff2	March 04, 2015	Fingerprint	6 (R)	34 (S)	21 (S)	21 (I)	26 (S)	6 (R)	6 (R)	6 (R)

Phylogenetic analysis and multilocus sequence-types

A phylogenetic analysis based on whole genome sequences was performed using the Parsnp program. The 4 isolates are identical and clustered with a *S. maltophilia* (LQQS01000001) originating from Malaysia (figure 1). The sequence types (ST) of our four isolates were determined with MLST *in silico* with the public database (https://pubmlst.org/smaltophilia/). They all belonged to a new ST: ST-288.

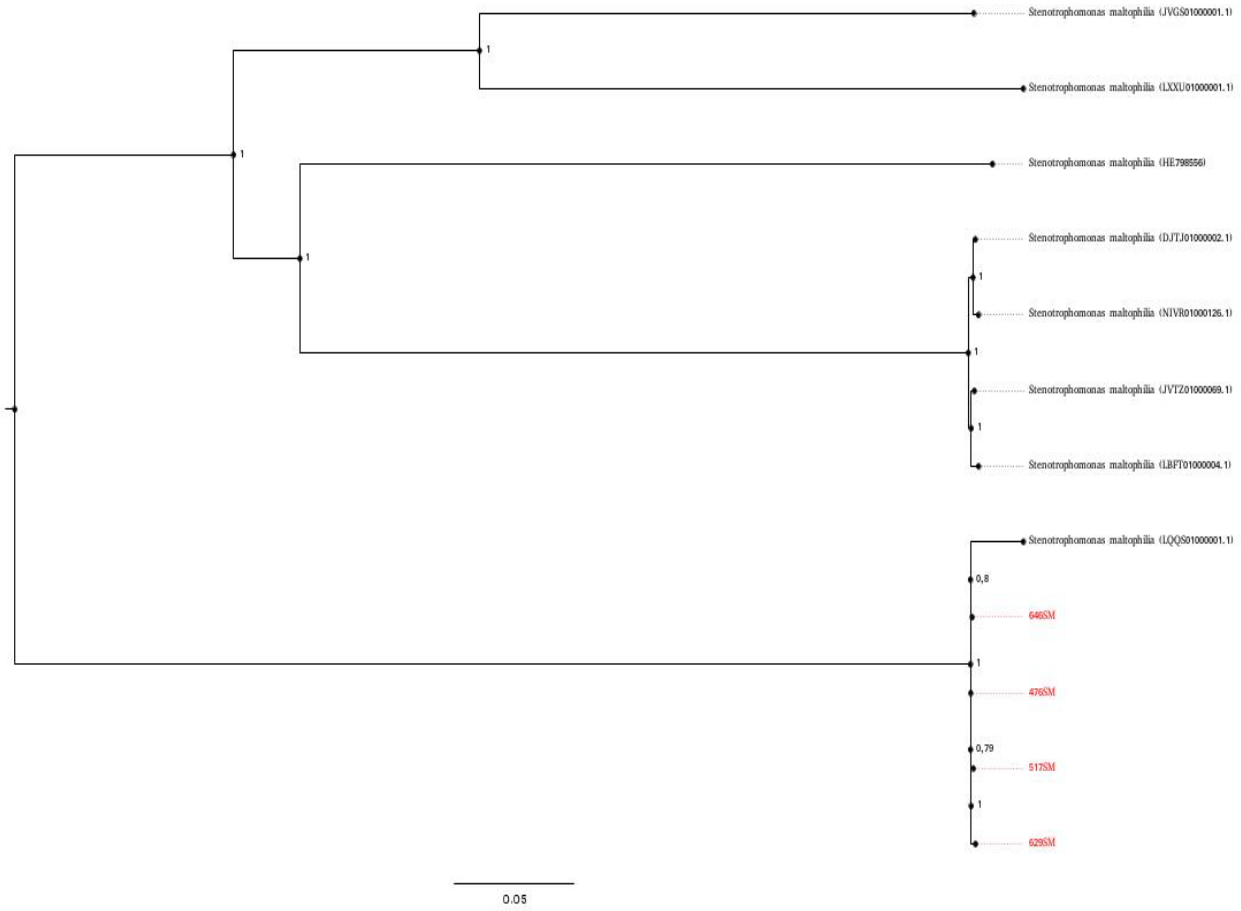

Figure 1: Phylogenetic tree based on coregenome alignment of four *S. maltophilia* including representative genomes from NCBI genbank. Sequences were aligned using ParSNP, genetic recombinaison were removed using Gubbins and phylogenetic inferences were obtained using the maximum likelihood method within raxML software. Bootstrap values are expressed by decimal of 1 000 replicates with a parameter test and shown at the branching points. The branches of the tree are indicated by the genus and species name of the type strains followed by the NCBI gene accession numbers. The four *S. maltophilia* isolates from this study are represented in red color.

Resistome analysis

Multiple antimicrobial, heavy-metal and arsenic resistance markers were identified in the chromosome of each isolate (Table 2). The resistome of these 4 strains revealed the presence of 27 antibacterial-resistant genes using the resfinder software and PATRIC webservers. The

192 isolates possess two different types of β-lactamases, i.e., Amber class A genes (L2 family),
193 Amber class B metallo-β-lactamase (MBL) genes (L1 family), and five aminoglycoside
194 inactivation enzymes: *aph(2'')-ia, aph(3'')-i, aph(3')-ii/aph(3')-xv, aph(6)-ic/aph(6)-id*. We
195 also identified 9 efflux pumps conferring antibiotic resistance and two regulators modulating
196 expression of antibiotic resistance genes
197
198 **Plasmid analysis**
199 Mobsuite software enabled us to identify and reconstruct two plasmids: an IncP conjugative
200 plasmid harboring the aminoglycoside 6-phosphotransferase gene (*sph* gene) and which
201 presented 86% nucleotidic identities with the plasmid p35734 found in *E. cloacae*. Moreover
202 we founda non-transferable plasmid with a RND efflux transporter conferring resistances to
203 cobalt, zinc, cadmium and which presented 99% nucleotidic identities with the plasmid
204 pLMG930 found in *Xanthomonas euvesicatoria*. As we used the short-reads sequencing
205 technique, we were unable to circularize the plasmids.
206
207 **Genetic environment of resistance genes**
208 All isolates carried class I integrons that contains *sul*1, *aad*B, *aph*3, *aph*6 genes and the entire
209 heat shock protein gene *groEL* upstream the *sul1* gene (Figure 2).

210
211 Figure2: Structure of class I integrons in the *S. maltophilia* isolated in the neonatology unit.

212 Table 2: Resistome of *S. maltophilia* isolated in the neonatology unit

Function	Enzymes commission numbers	Classification
Subclass B3 beta-lactamase (L1 family)	EC 3.5.2.6	antibiotic inactivation enzyme
Putative aminoglycoside 6-phosphotransferase		
Aminoglycoside 3'-phosphotransferase (*aph(3')-ii/aph(3')-xv*)	EC 2.7.1.95	
Aminoglycoside 2"-nucleotidyltransferase (*aph(2")-ia (aadb* family))	EC 2.7.7.46	
Aminoglycoside 6-phosphotransferase (*aph(6)-ic/aph(6)-id*)	EC 2.7.1.72	
Class A beta-lactamase	EC 3.5.2.6	
Aminoglycoside 3"-phosphotransferase (*aph(3")-i*)	EC 2.7.1.87	
Outer membrane channel TolC (*OpmH*)		efflux pump conferring antibiotic resistance
Multidrug efflux system EmrAB-OMF, membrane fusion component EmrA		
Macrolide-specific efflux protein MacA		
Multidrug efflux system MdtABC-TolC, inner-membrane proton/drug antiporter MdtB (RND type)		
Macrolide export ATP-binding/permease protein MacB		

213 Table 2: Continued

Function	Enzymes commission numbers	Classification
Multidrug efflux system EmrAB-OMF, inner-membrane proton/drug antiporter EmrB (MFS type)		efflux pump conferring antibiotic resistance
Multidrug efflux system MdtABC-TolC, inner-membrane proton/drug antiporter MdtC (RND type)		
Multidrug efflux system MdtABC-TolC, membrane fusion component MdtA		
Outer membrane factor (OMF) lipoprotein associated wth EmrAB-OMF efflux system		
Outer membrane low permeability porin, OprB family		gene modulating permeability to antibiotic
Hydrogen peroxide-inducible genes activator (*OxyR*)		regulator modulating expression of antibiotic resistance genes

214

215 **Mutations and resistance to SXT**

216 When using *S. maltophilia* D457 as reference genome a total of 327 deletions, 286 insertions
217 and 2133 substitutions were detected with the Breseq 0.31.1 software. In all isolates, single
218 nucleotide mutations in genes encoding enzymes involved in folic acid metabolism which can
219 be inactivated by trimethoprim were detected in *folA* (A114T:GCC→ACC), *folC*
220 (L339V:TTG→GTG) and *folM* (A72P:GCC→CCC) which encode respectively the
221 dihydrofolate reductase, dihydrofolate synthase and FolM alternative dihydrofolate reductase
222 1. Only the mutation in *folA* has an effect on the activity of the protein as assessed by
223 PROVEAN analysis

224

225 **Predicted structure and molecular docking**

226 The Gibbs free energy of binding (ΔG_{bind}) between the FolA protein (mutated and wild-type)
227 and trimethoprim was calculated and values of - 6.9 kcal/mol and -7.1 kcal/mol were found
228 for the mutated and the wild-type proteins, respectively. The predicted inhibition constants
229 (Ki_{pred}) between trimethoprim and the FolA wild-type protein was $3.59x10^{-6}$ and $8.39X10^{-6}$
230 between trimethoprim and the mutated FolA indicating a reduced susceptibility to
231 trimethoprim.

232

233 **Quantification of biofilm formation**

234 The amount of biofilm biomass was assessed by crystal violet staining as explained in table 4.
235 All 4 isolates had an average OD_{650nm} value of 0.145 (ODc= 0.086), meaning they are weak
236 biofilm producers. Additionally, we detected in the four isolates modifications in genes
237 involved in biofilm production: 43 substitutions in the rmlA gene, 32 substitutions in the
238 spgM gene, two insertions and three substitutions in the intergenic region between the manA
239 and spgM genes, two substitutions in the intergenic region between the rfbB and rmlA genes
240 and one substitutions in the intergenic region between the rpfC and rpfF genes. These genetic

241 modifications are probably the cause of weak biofilm production. The list of all mutations
242 detected is showed in supplementary material S1.

243 Table 4: Optical density (650nm) of biofilm formation of four strains

244

ID strain	517SM	476SM	629SM	646SM	Standard deviation	$OD_{negative}$
test 1	0,212125	0,11725	0,174125	0,1245	0,00698851	0,08659053
test2	0,174125	0,19625	0,125375	0,100625	0,01431034	0,08368101
test3	0,13575	0,135625	0,124	0,12375	0,00484031	0,08952092
Average	0,174	0,14970833	0,14116667	0,11629167	0,00871305	0,08659749

245

246 **Discussion**

247 The prevalence of *S. maltophilia* has increased in hospitals worldwide simultaneously
248 with the emergence of a myriad of other antibiotic resistant bacteria (29). Here, we
249 demonstrated, based on WGS phylogeny, the clonal transmission of *S. matophilia* resistant to
250 SXT in a neonatalogy unit, which might be explained by its ecology and fitness in hospitals
251 wards and/or by poor hygiene management. The determination of a new ST is in accordance
252 with the high plasticity and capacity of this bacterium to adapt to specific niches and develop
253 new characteristics. We showed in our study that the same strain of *S. maltophilia* was
254 isolated four times on a period of two months in a neonatal ward of a hospital in
255 Antananarivo, confirming its ability to persist and spread in the medical environment.
256 Selective pressure imposed by specific conditions in a hospital environment could promote
257 the survival of certain STs with an adaptive advantage for this specific setting and lead
258 afterwards to their clonal spread.

259 The reduced susceptibility of *S. maltophilia* to most antibiotics can be attributed to
260 both intrinsic and acquired resistances. The proteins mediating intrinsic resistance in *S.*
261 *maltophilia* include chromosomally encoded multidrug efflux pumps such as SmeABC,
262 SmeDEF, SmeYZ, SmeOP-TolCSm, antibiotic-inactivating enzymes (L1/L2 β-lactamases

and aminoglycoside inactivating enzymes), and the chromosomally encoded Qnr pentapeptide repeat proteins (30) which are present in most if not all strains of *S. maltophilia*, as in our Madagascan strains, suggesting that they did not arise during the recent evolution of resistance caused by antibiotic therapy. In addition, *S. maltophilia* can acquire mechanisms to increase its resistance pattern through horizontal gene transfer via plasmids, and subsequently by recombination processes with integrons, transposons and genomic islands (GIs).

The four isolates, which are genotypically identical, were resistant to SXT, one of the therapeutic choices. The resistance of Gram-negative bacteria to sulfonamides is mainly conferred by the acquisition of either *sul1* or *sul2*, encoding dihydropteroate synthases (31). The *sul1* gene carried by class 1 integrons and sul2, which is linked to insertion sequence common region (ISCR) elements, was identified in SXT-resistant *S. maltophilia* isolates (5, 32, 33). The resistance to trimethoprim in *S. maltophilia* is mainly conferred by the dihydrofolate reductase *dfr* genes such as *dfrA1, dfrA5, dfrA12, dfrA17,* and *dfrA27* which are usually located within class 1 integrons as part of various resistance gene cassettes. Both types of *sul* and *dfr* genes can occur together in high-level SXT-resistant isolates (6, 34). Moreover, the efflux pumps SmeDEF, TolCsm, and SmeYZ are associated with SXT resistance (35–37). We found that the *sul1* gene was present but no *dfr* genes were detected, pointing out the presence of another mechanism of resistance to trimethoprim. We were able to show that a point mutation (A114T:GCC→ACC) in the dihydrofolate reductase gene (*folA*) decreased the affinity of trimethoprim to the FolA protein ensuring therefore resistance to this antibiotic.

We have also shown the presence of two plasmids, one of which harbored heavy metal resistance genes and which was almost identical to the pLMG930 plasmid found in *Xanthomonas euvesicatoria,* a bacterial spot-causing xanthomonads. This indicates a high probability of dissemination of the strain in different ecological niches, notably those contaminated with heavy metals. It is well known that the presence of both metals and antibiotic resistance genes play a major role in the persistence, selection and spread of

antibiotic- and metal-resistant bacteria in anthropogenic environments heavily contaminated with detergents, heavy metals and other antimicrobials [29, 30]. In developing countries, rivers, lakes and lagoons are often contaminated with untreated hospital and industrial effluents and also by urban storm-water containing anthropogenic pollutants due to intensive uncontrolled urbanization. These are optimal conditions for bacterial development and the spread of antibiotic-resistant bacteria.

Biofilm formation in bacteria is a multifactorial event that depends on surface characteristics, motility of strains, genes involved in biofilm formation, and other factors, and is usually correlated with a higher level of resistance to antibiotics and disinfectants (38). Different factors influence the physiology of biofilm formation in *S. maltophilia*, namely the SmeYZ efflux pump that confers resistance to antimicrobials (37), the iron level in the media (39), and histidine kinase and BfmAK system (40). Interestingly, our study revealed a negative correlation between the simultaneous presence of genes involved in biofilm formation such as *spgM, rmlA* and *rpfF* genes and the biofilm production. This correlation could be due to the mutations identified in the three previous genes. However, a clone was detected along a period of two months in the neonatlogy ward pointing out a persistence of the strain in this environment.

In conclusion, this work represents the first characterization at the genomic level of SXT resistant *S. maltophilia* strains circulating in a neonatology ward of a hospital in Antananarivo, Madagascar. We also pointed out the possible role of a point mutation in the *folA* gene conferring resistance to trimethoprim. Clonal relatedness between strains indicated the transmission and the persistence of *S. maltophlia* in the hospital setting and the threat it could represent for newborns, especially for preterms.

317 **Data availability:**

318 These whole genome shotgun projects have been deposited at DDBJ/ENA/GenBank. under
319 the accession VFJF00000000, VFJG00000000, VFJH00000000 and VFEX00000000
320 corresponding respectively for the strains.517SM, 629SM, 646SM and 476SM.

321

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328

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