1 **Quantitative real-time PCR assay for the rapid identification of the multidrug-resistant**

2 **bacterial pathogen *Stenotrophomonas maltophilia***

3 Tamieka A. Fraser[a,b], Mikaela G. Bell[a,b], Patrick N.A. Harris[c,d], Scott C. Bell[e,f], Haakon

4 Bergh[c], Thuy-Khanh Nguyen[e], Timothy J. Kidd[e,g], Graeme R. Nimmo[c], Derek S. Sarovich[a,b],

5 and Erin P. Price[a,b#]

6 [a] GeneCology Research Centre, University of the Sunshine Coast, Sippy Downs, Queensland,

7 Australia

8 [b] Sunshine Coast Health Institute, Birtinya, Queensland, Australia

9 [c] Microbiology Department, Central Laboratory, Pathology Queensland, Royal Brisbane &

10 Women's Hospital, Herston, Queensland, Australia

11 [d] University of Queensland Centre for Clinical Research, Royal Brisbane & Women's

12 Hospital, Herston, Queensland, Australia

13 [e] QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia

14 [f] Adult Cystic Fibrosis Centre, The Prince Charles Hospital, Chermside, Queensland,

15 Australia

16 [g] School of Chemistry and Molecular Biosciences, Faculty of Science, The University of

17 Queensland, St Lucia, Queensland, Australia

18 # Corresponding author

19 University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, Qld, 4558, Australia

20 Email: eprice@usc.edu.au

21 Phone: +61 7 5456 5568

1

Abstract

Stenotrophomonas maltophilia is emerging as an important cause of disease in nosocomial and community-acquired settings, including bloodstream, wound and catheter-associated infections. Cystic fibrosis airways also provide optimal growth conditions for various opportunistic pathogens with high antibiotic tolerance, including *S. maltophilia*. Currently, there is no rapid, cost-effective, and accurate molecular method for detecting this potentially life-threatening pathogen, particularly in polymicrobial specimens, suggesting that its true prevalence may be underestimated. Here, we used large-scale comparative genomics to identify a specific genetic target for *S. maltophilia*, with subsequent development and validation of a real-time PCR assay for its detection. Analysis of 165 *Stenotrophomonas* spp. genomes identified a 4kb region specific to *S. maltophilia*, which was targeted for Black Hole Quencher assay design. Our assay yielded the positive detection of 89 of 89 (100%) clinical *S. maltophilia* strains, and no amplification of 23 non-*S. maltophilia* clinical isolates. *S. maltophilia* was detected in 10/16 CF sputa, demonstrating the utility for direct detection in respiratory specimens. The assay demonstrated good sensitivity, with limits of detection and quantitation on pure culture of ~10 and ~100 genome equivalents, respectively. Our assay provides a highly specific, sensitive, and cost-effective method for the accurate identification of *S. maltophilia*, and will improve the diagnosis and treatment of this under-recognized pathogen by enabling its accurate and rapid detection from polymicrobial clinical and environmental samples.

2

Introduction

42

43 *Stenotrophomonas maltophilia* is a Gram-negative, intrinsically multidrug-resistant bacterium

44 that is ubiquitous in aqueous environments such as soils, plant roots, water treatments and

45 distribution systems (1). Whilst conventionally overlooked as a laboratory contaminant, or as

46 a common commensal in hospitalized patients, *S. maltophilia* is increasingly being

47 recognized as an important nosocomial pathogen in its own right due to its ability to cause

48 life-threatening disease in immunocompromised individuals (2). This opportunistic pathogen

49 has been isolated from a variety of hospital settings including faucets, sinks, central venous

50 catheters, ice machines, tap water, and water fountains, reinforcing its nosocomial importance

51 (1, 3, 4). *S. maltophilia* most commonly infects people with meningitis, cancer, chronic

52 obstructive pulmonary disease (COPD), or cystic fibrosis (CF), with pneumonia, bacteraemia,

53 and wound and urinary infections being the most frequent clinical manifestations (5, 6). Risk

54 factors for *S. maltophilia* infection include prolonged hospitalization, neutropenia,

55 catheterization, and previous use of broad-spectrum antibiotics (7). The recommended

56 antibiotic treatment for *S. maltophilia* infections is co-trimoxazole; however, resistance

57 towards this antibiotic combination has been documented (2, 8, 9). Indeed, treatment options

58 are limited for *S. maltophilia*, with this pathogen also exhibiting resistance towards several

59 antibiotic classes including fluoroquinolones, macrolides, β-lactams, aminoglycosides,

60 carbapenems, tetracyclines, polymyxins, chloramphenicol and cephalosporins (1, 2). With a

61 mortality rate approaching 70%, the importance of timely identification and effective

62 treatment of *S. maltophilia* infections is paramount (10).

63 *S. maltophilia* is a common pathogen in CF airways due to its ability to evade many

64 antipseudomonal antibiotics, with chronic *S. maltophilia* infection associated with an

65 increased risk of respiratory disease and mortality (11, 12). CF is an autosomal recessive

66 genetic disorder effecting multiple organs; however, its pathogenesis is most prominent in

3

67 airways, with ~90% of CF deaths associated with respiratory failure (13). The excessive

68 production of mucus in CF airways provides optimal growth conditions for opportunistic

69 pathogens, which drives most CF morbidity and mortality. Molecular methods have

70 confirmed that CF lower airways harbour diverse microbial communities, with *Pseudomonas*

71 *aeruginosa* and *Burkholderia cepacia* complex species of greatest concern due to frequent

72 rapid respiratory decline in people infected with these pathogens (14, 15). However, other co-

73 infecting opportunistic pathogens such as *Achromobacter* spp., *S. maltophilia*,

74 *Staphylococcus aureus*, *Haemophilus influenzae* and certain fungal species (e.g. *Aspergillus*)

75 are also prominent in CF airways, and are known to contribute to pathogenesis (14). Indeed,

76 recent studies have shown a mutualistic relationship between *S. maltophilia* and *P.*

77 *aeruginosa* in CF airways, with compounds produced by *S. maltophilia* under exposure to

78 certain antibiotics, such as imipenem, supporting the survival of otherwise antibiotic-

79 susceptible *P. aeruginosa* strains (16). Furthermore, these *S. maltophilia* compounds can

80 enhance *P. aeruginosa* stress tolerance, increasing polymyxin tolerance (17). These studies

81 highlight the importance of *S. maltophilia* in CF airway pathogenesis, particularly during

82 antibiotic treatment, and emphasize the need for correct species identification in

83 polymicrobial infections.

84 Although there are a variety of diagnostic methods available for *S. maltophilia* detection,

85 such as PCR amplicon sequencing, VITEK mass spectrometry identification, or key

86 morphological characteristics on growth media, these methods suffer from issues such as

87 limited access to equipment with a large capital expenditure (e.g. ~USD$200,000 for VITEK

88 MS instrumentation), high per-assay cost, the need for highly trained personnel,

89 laboriousness, slow turn-around time, the requirement for purified colonies, or

90 misidentification issues (16, 18, 19). For example, *S. maltophilia* and *P. aeruginosa* exhibit

91 colonial colour differences when grown on bromothymol blue-containing media, which

4

92   reflects their different metabolic processes (16). However, the use of media containing

93   bromothymol blue is not routine, and thus the retrieval of *S. maltophilia* from polymicrobial

94   specimens requires clinical expertise in identifying appropriate culture media for

95   differentiation of this bacterium from other pathogens. As a non-exhaustive list, *S.*

96   *maltophilia* has been misidentified as several other organisms, including *Bordetella*

97   *bronchiseptica*, *Alcaligenes faecalis*, *B. cepacia*, and numerous *Pseudomonas* species, which

98   are common in clinical settings, including CF sputa (1, 20). Diagnostic inconsistencies in *S.*

99   *maltophilia* detection from clinical specimens can lead to inappropriate or even detrimental

100   treatment (16), particularly for those patients requiring urgent care. There is therefore a need

101   to accurately identify this emerging pathogen to improve antibiotic treatment regimens,

102   stewardship, and patient outcomes. Here, we report the development and validation of a

103   Black Hole Quencher (BHQ) probe-based real-time PCR assay for the specific detection of *S.*

104   *maltophilia*. Our results indicate that our real-time PCR assay is more sensitive than routine

105   culture for detecting *S. maltophilia*, particularly in polymicrobial respiratory specimens.

106   **Materials and Methods**

107   **Ethics.** This study was approved by The Prince Charles Hospital Human Research Ethics

108   Committee (HREC/13/QPCH/127).

109   **Genomes of *Stenotrophomonas* spp. and closely related species.** All non-redundant

110   *Stenotrophomas* spp. genomes available on the NCBI GenBank database were downloaded as

111   of December 2018. Additional non-redundant *Stenotrophomonas* spp. genomes generated

112   using paired-end Illumina sequencing were downloaded from the NCBI Sequence Read

113   Archive database (SRA; https://www.ncbi.nlm.nih.gov/sra). In total, 165 publicly available

114   genomes were available for this study (Table S1), represented by *S. maltophilia* (including all

115   "*S. pavanii*"; *n*=132), *S. acidaminiphila* (*n*=4), *S. bentonitica* (*n*=3), *S. chelatiphaga* (*n*=1), *S.*

116   *daejeonensis* (*n*=1), *S. ginsengisoli* (*n*=1), *S. humi* (*n*=1), *S. indicatrix* (*n*=4), *S. koreensis*

117   (*n*=1), *S. lactitubi* (*n*=1), *S. nitritireducens* (*n*=2), *S. panacihumi* (*n*=1), *S. pictorum* (*n*=1), *S.*

118   *rhizophila* (*n*=2), *S. terrae* (*n*=1), and unassigned *Stenotrophomonas* spp. (*n*=9). Three strains

119   listed on the NCBI database as *Pseudomonas geniculata* (95, AM526, and N1) were included

120   in the phylogenomic analysis to confirm that they were in fact *S. maltophilia*. Sequence data

121   from assembled genomes were converted to simulated 100bp paired-end Illumina reads at

122   85x coverage using ART version MountRainier (21). SRA data were quality-filtered using

123   Trimmomatic v0.33 (22) using previously described filtering parameters (23) prior to

124   analysis.

125   **Bioinformatic analysis to identify a *S. maltophilia*-specific genetic locus.** The haploid

126   comparative genomics pipeline SPANDx v3.2.1 (24) was used to phylogenetically delineate

127   *S. maltophilia* from non-*S. maltophilia* species, and subsequently, to identify *S. maltophilia*-

128   specific loci. Illumina reads for the publicly available strains (Table S1) were mapped to the

129   closed 4.85Mbp *S. maltophilia* K279a genome (GenBank accession NC_010943.1) (25).

130   Phylogenetic reconstruction of the 165 taxa was performed using 31,246 core, biallelic

131   single-nucleotide polymorphisms (SNPs) using the maximum parsimony function of PAUP*

132   v4.0a.164 (26), rooted with *Stenotrophomonas daejeonensis* JCM 16244 (GenBank accession

133   LDJP01000001.1), and with bootstrapping carried out using 1,000 replicates. To identify

134   genetic loci present in all *S. maltophilia* but absent in all other organisms, including other

135   *Stenotrophomonas* spp., the BEDcov output (27) from SPANDx was examined. Candidate

136   regions were assessed further for specificity and PCR assay design.

137   *S. maltophilia* **probe-based real-time PCR assay design.** Upon identification of a putative

138   *S. maltophilia*-specific genetic target, sequence alignments were used to locate conserved

139   regions. Although some variation was allowed within the amplicon, primer- and probe-

140   binding regions required 100% sequence identity in all *S. maltophilia* strains to avoid false

141   negatives. Oligo self-dimers and heterodimers were assessed *in silico* using NetPrimer

142   (http://www.premierbiosoft.com/netprimer/) and Beacon Designer

143   (http://www.premierbiosoft.com/qOligo/Oligo.jsp/), with configurations resulting in ΔG

144   values of <-8.0 (NetPrimer) and <-4.0 (Beacon Designer) excluded. The following sequences

145   and probe label were chosen for the assay: Smalto-For: 5'-

146   AAGGACAAGGCGATGACCATC, Smalto-Rev: 5'-CCCCACCACGAYTTCATCA, and

147   Smalto-Probe: 5'-FAM-CAGAACGACATCTGGTTGGCG-BHQ1, resulting in an amplicon

148   length of 344 bp. NCBI Microbial Nucleotide discontiguous MegaBLAST

149   (http://blast.ncbi.nlm.nih.gov/) analysis of this amplicon was used to determine assay

150   specificity for only *S. maltophilia*. No mismatches in the probe-binding site were tolerated in

151   any *S. maltophilia* strain, whereas ≥2 mismatches in the probe-binding site were considered

152   sufficient for conferring *S. maltophilia* specificity.

153   **Microbiological cultures and CF sputum DNA extractions.** A total of 89 *S. maltophilia*

154   isolates were obtained for PCR testing: *S. maltophilia* control strain LMG 957, 16 sputum-

7

155   derived isolates cultured from individuals with CF identified either by amplified rRNA gene

156   restriction analysis (*n*=8 (28)) or *in silico* multilocus sequence typing (*n*=8 (29)), and 72

157   isolates from various clinical presentations that had previously been identified as *S.*

158   *maltophilia* by Pathology Queensland Central laboratory, Brisbane, Australia, according to

159   the VITEK2 GNI card (98% of isolates) or VITEK MS (2% of isolates). Strains were grown

160   on Luria-Bertani (LB) agar for 24h at 37 °C. DNA was extracted from a small (toothpick

161   head) swatch of the primary culture by heat soaking in 80 µL of a 5% chelex solution at 95

162   °C for 10 min, with the resultant DNA diluted 1:10 with molecular grade H_2O prior to real-

163   time PCR testing.

164   Sixteen sputa collected from nine Australian adult CF patients underwent DNA extractions

165   using the Zymo Quick-DNA Miniprep Plus Kit (ZYMO Research) protocol, with the addition

166   of an enzymatic lysis solution (20 mg/mL lysozyme, 22U/mL lysostaphin, and 250 U/mL

167   mutanolysin) at 37 °C for 1-3 hours prior to the addition of Proteinase K. Three patients had

168   sputa collected over three time points (range: 13-46 days), and one patient had two sputa

169   collected 320 days apart. "Day 1" samples represent sputa collected on the day of intravenous
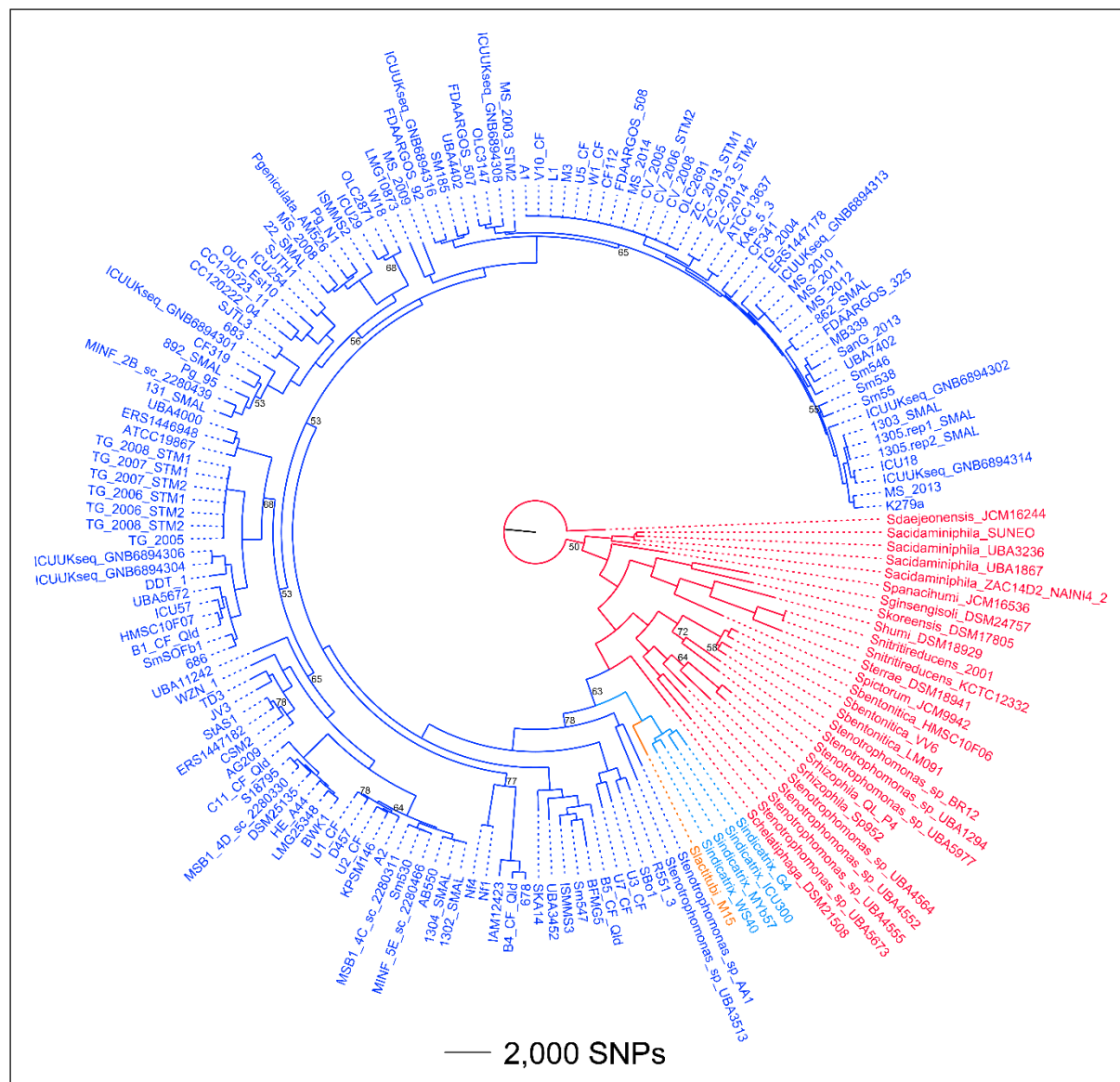
170   antibiotic commencement.

171   ***S. maltophilia* real-time PCR assay validation and specificity testing.** Each PCR consisted

172   of 1X Sso Advanced Universal Probes Supermix (Bio-Rad Laboratories, Gladesville, NSW,

173   Australia), optimised primer and probe concentrations of 0.30 and 0.35 µM, respectively

174   (Macrogen Inc., Geumcheon-gu, Seoul, Rep. of Korea), 1 µL of DNA template, and

175   RNase/DNase-free PCR grade water (Thermo Fisher Scientific), to a final per-reaction

176   volume of 5 µL. Thermocycling parameters included an initial hot start activation at 95 °C

177   for 2 minutes, followed by 45 cycles of 95 °C for 3 seconds and 60 °C for 10 seconds, using

178   the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Results were analysed

179   using CFX Maestro v4.1.2433.1219 software.

8

180   The 89 *S. maltophilia* isolates and 16 CF sputa were tested using the optimised PCR

181   conditions to ensure accurate detection of *S. maltophilia* DNA in known positive isolates, and

182   in sputum samples from which *S. maltophilia* was variably detected using selective culture

183   (Horse Blood Agar, McConkey Agar, *Burkholderia cepacia* Agar Base and Bacitracin Agar

184   Base). Relative *S. maltophilia* abundance in sputa was determined by performing a previously

185   described 16S rDNA real-time PCR (30) and calculating the cycles-to-threshold difference

186   (ΔC_T) between 16S and the *S. maltophilia* assays. Further specificity testing was performed

187   on 23 non-*S. maltophilia* DNA: *Burkholderia thailandensis* (*n*=1), *Enterobacter aerogenes*

188   (*n*=1), *Enterobacter cloacae* (*n*=4), *Klebsiella oxytoca* (*n*=1), *Klebsiella pneumoniae* (*n*=2),

189   *P. aeruginosa* (*n*=11), *S. aureus* (*n*=1), and *Staphylococcus epidermidis* (*n*=2).

190   Limit of detection (LoD) and limit of quantification (LoQ) were determined for our newly

191   designed *S. maltophilia* real-time PCR assay using serial dilutions of 50 ng/µL to 0.05 fg/µL

192   *S. maltophilia* DNA across eight replicates/dilution. Twenty-four no-template controls were

193   also included. The upper and lower LoD/LoQ limits were determined as described elsewhere

194   (31).

Results

Comparative genomics of *Stenotrophomonas* spp. identifies a *S. maltophilia*-specific gene target. Phylogenomic reconstruction of 165 *Stenotrophomonas* spp. genomes using 31,306 core-genome, biallelic, orthologous SNPs demonstrated a close relationship between *S. maltophilia* and two recently described *Stenotrophomonas* species, *S. indicatrix* and *S. lactitubi* (32), and a clear distinction of these taxa from other *Stenotrophomonas* spp. (Fig 1). This phylogenomic analysis was used to delineate *S. maltophilia* from other species, and to modify incorrect species designations for 43 taxa, including reclassification of all four "*S. pavanii*", three *P. geniculata*, and 17 *Stenotrophomonas* sp. as *S. maltophilia*, and nine *S. maltophilia* as *Stenotrophomonas* spp. (Table S1).

Figure 1: Phylogenomic analysis of *Stenotrophomonas* spp. Dark blue, *S. maltophilia* (target species); red, distantly related *Stenotrophomonas* spp.; light blue, *S. indicatrix*; orange, *S. lactitubi*. The tree was rooted with *S. daejeonensis* JCM16244. Consistency index=0.20. Branches with bootstrap values with <80% support are labelled.

Following identification of the species boundary for *S. maltophilia*, we next identified loci specific for *S. maltophilia*. A single 4kb region was found to be *S. maltophilia*-specific, being present in the 134 *S. maltophilia* genomes but absent or highly divergent in the other *Stenotrophomonas* spp., including *S. indicatrix* and *S. lactitubi*. The genetic coordinates for

11

214   these loci are 3,935,000-3,939,000 in *S. maltophilia* K279a, spanning gene-coding regions for

215   formate dehydrogenase (α, β, and γ-subunits; encoded by *fdnG, fdnH,* and *fdnI*, respectively).

216   Upon identification of a suitable 344 bp *S. maltophilia*-specific amplicon, a microbial

217   discontiguous MegaBLAST analysis was conducted to confirm specificity (performed

218   15Mar19). For all available *S. maltophilia* genomes, 100% sequence identity for both primers

219   and probe was attained. Unexpectedly, the genomes of two recently published *S. indicatrix*

220   strains, which were obtained from soil in Lebanon (RS1 and RS7; GenBank accessions

221   NZ_RKSQ00000000 and NZ_RKSR00000000, respectively), provided close BLAST hits to

222   this amplicon, despite this locus being absent or highly divergent in the four *S. indicatrix*

223   genomes used in the phylogenomic analysis. Fortunately, RS1 and RS7 contained two SNPs

224   in the probe-binding region, including a mismatch at the very 3' end, which would be

225   expected to inhibit or substantially reduce fluorophore detection in these strains due to poor

226   binding kinetics.

227   **BLAST analysis identifies further *S. maltophilia* misclassification.** In addition to RS1 and

228   RS7, BLAST analysis revealed several non-*S. maltophilia* matches to this amplicon,

229   including three *P. geniculata* strains, several misclassified *Stenotrophomonas* spp. (Table

230   S1), and even misclassifications assigned to distant bacteria e.g. *Acinetobacter baumannii*

231   (4300STDY7045681; GenBank ref. UFGS00000000.1), *Pseudomonas aeruginosa*

232   (E15_London_28_01_14; GenBank ref. CVWF00000000.1) and *Pseudomonas* sp.

233   (UBA10046; GenBank ref. DPXK00000000.1). In all cases, these isolates were confirmed to

234   be *S. maltophilia* according to Microbes BLAST analysis of the whole genome, thereby

235   representing species assignment errors in the NCBI database, and demonstrating the

236   specificity of our *S. maltophilia* target.

237 ***S. maltophilia* real-time PCR assay specificity.** To further assess assay specificity, DNA

238 from 89 *S. maltophilia* isolates derived from both CF and non-CF acute infections was tested.

239 The real-time PCR assay accurately detected 100% of the *S. maltophilia* isolates but did not

240 amplify across any of the tested non-*S. maltophilia* species (*B. thailandensis* [*n*= 1], *P.*

241 *aeruginosa* (*n*=11), *K. pneumoniae* (*n*=2), *K. oxytoca* (*n*=1), *E. cloacae* (*n*=4), *E. aerogenes*

242 (*n*=1), *S. epidermidis* (*n*=2) and *S. aureus* (*n*=1)). As *S. maltophilia* is known to be a common

243 bacterium in CF sputa, we next tested the assay across 16 CF sputum samples obtained from

244 nine patients. Of these, 10 sputa demonstrated *S. maltophilia* presence at various abundances,

245 with 16S rDNA: *S. maltophilia* ΔC_T values ranging from 2.5 (SCHI0019 Day 11; same as

246 pure *S. maltophilia* DNA control) to 17.0 (Table 1). In two longitudinal samples (SCHI0020

247 and SCHI0021), *S. maltophilia* was only detected at very low levels at the Day 1 time point,

248 with the two subsequent sputa being PCR-negative for this organism. In contrast, *S.*

249 *maltophilia* persisted in the other two longitudinal samples (SCHI0002 and SCHI0019).

250 There was an 84.6% congruence between the two methods, with two PCR-positive sputa

251 (SCHI0020 Day 1 and SCHI0021 Day 1) being negative by culture (Table 1). However, these

252 two specimens had the lowest *S. maltophilia* load (ΔC_T values of 13.8 and 17.0), indicating

253 higher sensitivity with the real-time PCR.

Table 1. Real-time PCR quantification of *Stenotrophomonas maltophilia* on 16 sputa

obtained from cystic fibrosis airways, with concurrent culture diagnosis.

Sample	ΔC_T[#]	Culture Result
S. maltophilia isolate control	2.5	Positive
SCHI0002 Day 1	7.9	Positive
SCHI0002 Day 320	6.0	Positive
SCHI0008	NA	ND
SCHI0010	NA	Negative
SCHI0011	12.4	ND
SCHI0013	9.7	ND
SCHI0014	8.0	Positive
SCHI0019 Day 1	6.7	Positive
SCHI0019 Day 11	2.5	Positive
SCHI0019 Day 46	6.5	Positive
SCHI0020 Day 1	17.0	Negative
SCHI0020 Day 6	NA	Negative
SCHI0020 Day 31	NA	Negative
SCHI0021 Day 1	13.8	Negative
SCHI0021 Day 5	NA	Negative
SCHI0021 Day 13	NA	Negative

[#]Cycles-to-threshold difference; NA, no amplification; ND, not determined.

***S. maltophilia* real-time PCR assay sensitivity.** To assess assay sensitivity, LoD and LoQ

values were determined (Figure 2). Using a 10-fold DNA dilution series (50 ng/µL to 0.05

fg/µL), the LoD for this assay is ~5 fg/µL, or ~9 genome equivalents (GEs), and the LoQ is
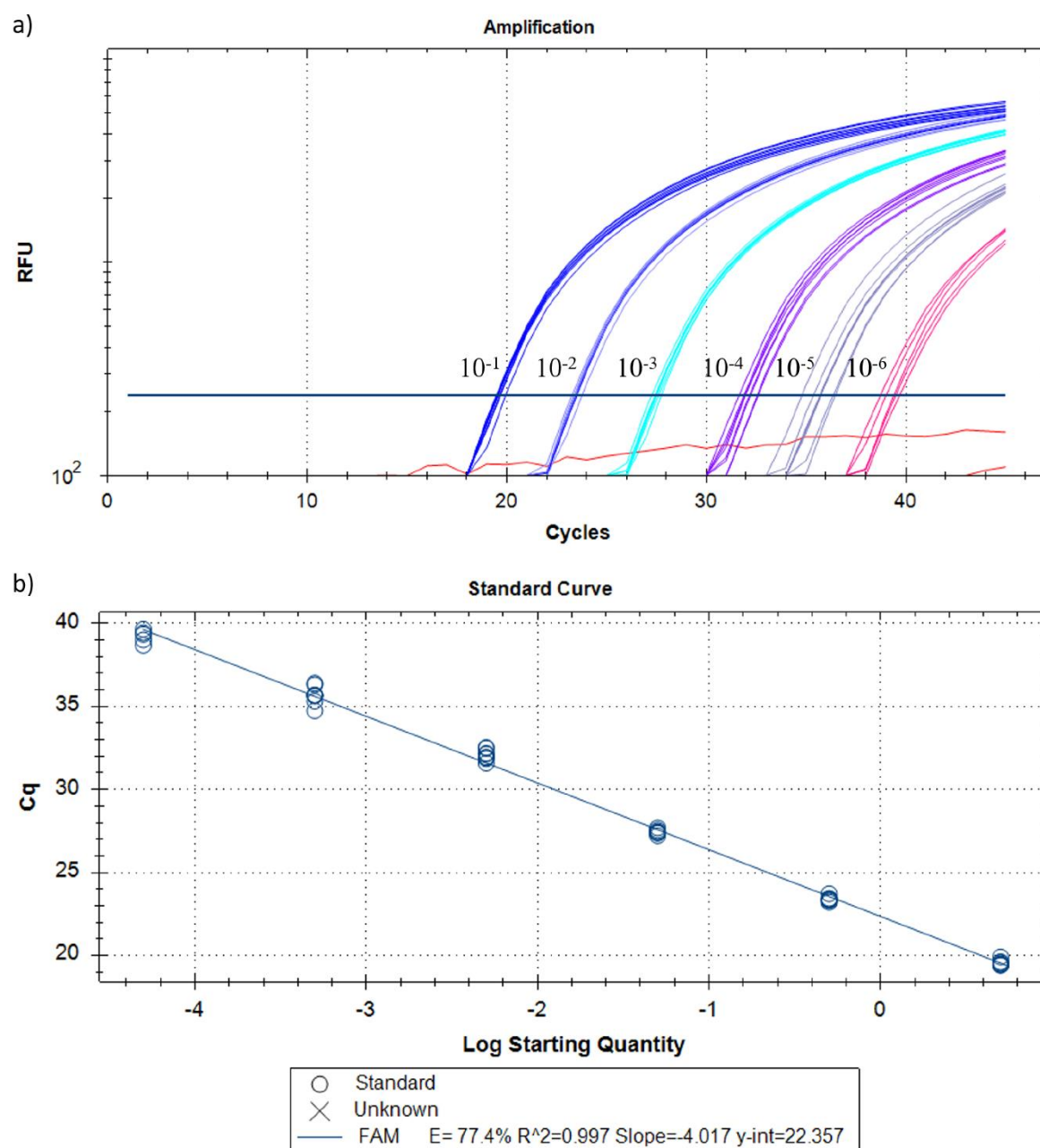
0.5 pg/µL, or ~94 GEs.

a)

b)

262

Figure 2. Limits of detection (LoD) and quantitation (LoQ) for the *Stenotrophomonas maltophilia*-specific real-time PCR assay. To determine the LoD and LoQ, serial dilutions of a *S. maltophilia*-positive control DNA sample were performed across eight replicates, ranging from 50 ng/uL to 0.05 fg/µL (a). To assess the correlation coefficient, a standard curve was also included for these dilutions, resulting in an R^2 of 0.997 (b). LoD and LoQ was identified as ~5 fg/µL and ~0.5 pg/µL, respectively. All 24 negative controls (red) were negative.

15

270 **Discussion**

271 *S. maltophilia* is emerging as an important multidrug-resistant nosocomial pathogen, being

272 amongst the top three most common non-fermentative Gram-negative bacilli identified in

273 hospitalized patients (5, 33). Despite *S. maltophilia* being well-adapted to many

274 environments, most infections occur in immunocompromised individuals in the nosocomial

275 setting (10), although community-acquired infections are also on the rise (1). Here, we

276 describe the first real-time PCR assay to detect *S. maltophilia* with 100% accuracy in purified

277 colonies, and demonstrate that this assay is superior to microbiological culture for detecting

278 this multidrug-resistant bacterium in polymicrobial respiratory specimens collected from CF

279 patients.

280 There is currently a lack of a rapid, cost-effective, accessible, and accurate diagnostic method

281 for *S. maltophilia* detection, particularly from polymicrobial clinical specimens such as CF

282 sputa. As *S. maltophilia* is thought to be the only *Stenotrophomonas* species to cause human

283 disease, mass spectrometry-based systems such as VITEK 2 and VITEK MS are a common

284 diagnostic method in large, centralized pathology laboratories. However, the accuracy of

285 species determination using mass spectrometry is heavily dependent on the quality of the

286 associated databases, and it is currently unknown whether other *Stenotrophomonas* spp. can

287 be accurately differentiated from *S. maltophilia* on these systems. In addition, access to this

288 instrument is limited to well-resourced laboratories owing to a large barrier-to-entry cost

289 (~USD$200,000) (34-36). From a genotyping standpoint, 16S rDNA PCR has been used to

290 identify *S. maltophilia* in blood samples for patients undergoing chemotherapy for leukemia

291 (37), and a multiplex PCR to detect *P. aeruginosa*, *S. maltophilia* and *B. cepacia* successfully

292 identified *S. maltophilia* in 85% of cases (38). However, these assays have either not been

293 optimized to avoid non-specific amplification in other *Stenotrophomonas* spp. and members

294 of the closely related *Xanthomonas* genus, or they require downstream processing (e.g. gel

16

295   electrophoresis, Sanger sequencing) to confirm results, which is laborious, time-consuming,

296   and raises potential laboratory contamination issues.

297   Therefore, the purpose of this study was to use large-scale comparative genomics to identify

298   a *S. maltophilia*-specific genetic target, and to subsequently design a highly-specific and

299   accurate real-time PCR-based assay for identifying *S. maltophilia*. Using this approach, we

300   identified a genetic region specific to *S. maltophilia*, which was subsequently targeted for

301   assay development. We found that our newly developed assay correctly identified 89 *S.*

302   *maltophilia* isolates with 100% accuracy. The accuracy and specificity of this assay is both

303   highly sensitive and selective for *S. maltophilia*, with an LoQ and LoD of ~94 and ~9 GEs,

304   respectively. We chose the Black Hole Quencher probe real-time PCR format due to its

305   relatively inexpensive up-front cost (~USD$25,000 for real-time instrumentation), low per-

306   reaction cost (~USD$0.80 per sample when performed in duplicate), high-throughput

307   capacity, closed-tube format (which eliminates post-PCR contamination concerns), simple

308   set-up, and rapid turn-around-time (~1 h). This format also enables robust identification of

309   target species in polymicrobial specimens. Although not examined in this study, the multi-

310   fluorophore capacity of many real-time PCR instruments also enables multiplexing of probe-

311   based assays for the simultaneous identification of multiple organisms in a single specimen,

312   leading to further cost reductions.

313   Our *in silico* and laboratory results indicate that all non-*S. maltophilia* microorganisms failed

314   to amplify, with the possible exception of two *S. indicatrix* strains, RS1 and RS7. *S.*

315   *indicatrix* is a newly identified *Stenotrophomonas* species (32) that has so far been isolated

316   from dirty dishes in Germany (strain WS40; (32)), sewage in China (strain G4; unpublished),

317   a rotting apple in Germany (Myb57; unpublished), soil in Lebanon (strains RS1 and RS7;

318   unpublished), and an unknown source in Germany (ICU300; unpublished). The RS1 and RS7

319   *S. indicatrix* genomes became available subsequent to assay design; however, BLASTn

17

320   analysis showed that there were two SNPs in the probe-binding region, including a SNP at

321   the 5' ultimate base of the BHQ probe, which would likely result in poor or no amplification.

322   Taken together, we show that our assay is highly specific for *S. maltophilia*, particularly in

323   clinical samples, but it also has applicability for testing environmental samples, such as

324   hospital water supplies.

325   Although the quality of life and life expectancy for people with CF has markedly increased in

326   recent decades due to improvements in antibiotic treatments and clinical management,

327   persistent polymicrobial infections in CF airways remain the primary cause of morbidity and

328   mortality (39). A recent longitudinal study of a single CF patient's airways using a cutting-

329   edge metatranscriptomic approach, which measures only the 'active' microbial population

330   through messenger RNA characterisation, revealed that *S. maltophilia* was the second most

331   prevalent bacterium behind *P. aeruginosa* in the six months prior to death (39). Our results

332   also revealed a high prevalence of *S. maltophilia* in adult CF sputa, with 10/16 samples

333   positive for this bacterium according to our assay. As these sputa samples had concurrent

334   culture results, we demonstrated 84.6% congruence to real-time PCR results, with two culture

335   negative samples returning as positive by real-time PCR (SCHI0020 Day 1 and SCHI0021

336   Day 1). This finding demonstrates that our assay has a higher sensitivity for detecting *S.*

337   *maltophilia* in CF clinical specimens than culture methods. Of four longitudinally collected

338   sputa, one patient (SCHI0019) had *S. maltophilia* at all time points (Days 1, 11, and 46; Table

339   1) despite intravenous antibiotic therapy during this time, and another patient, SCHI0002,

340   was positive for *S. maltophilia* in samples that were collected nearly 12 months apart (Days 1

341   and 320), indicating either long-term airway persistence or reinfection with this organism. In

342   one sputum sample from SCHI0019 (Day 11), the ΔC_T value between the 16S rDNA and *S.*

343   *maltophilia* PCRs was identical to that of pure *S. maltophilia* culture (Table 1), indicating that

344   *S. maltophilia* had become the dominant, and potentially sole bacterial species, in this

18

345   specimen. Although outside the scope of this study, this finding demonstrates the potential

346   for *S. maltophilia* to persist and dominate in CF airways following antibiotic-driven

347   microbiome perturbations, which may have implications for rapid re-infection with more

348   formidable pathogens such as *P. aeruginosa*. A further two patients, SCHI0020 and

349   SCHI0021, had *S. maltophilia* at Day 1, but subsequent sampling (up to Day 31 and 13,

350   respectively) were PCR-negative during the intravenous antibiotic treatment phase, indicating

351   successful eradication of *S. maltophilia* in these cases. Future work will entail testing across

352   larger CF sputum panels, including longitudinal samples, to further examine potential

353   mutualistic relationships between *S. maltophilia* and other pathogens such as *P. aeruginosa*,

354   and on assessing assay performance directly on clinical specimens to further reduce sample

355   processing timeframes.

356   In conclusion, the ability to accurately, rapidly, and cheaply detect *S. maltophilia* is critical

357   for understanding the prevalence of this underappreciated opportunistic pathogen and for

358   reducing its burden of disease. The implementation of this assay in the clinical setting will

359   enable researchers, clinicians and pathologists to more accurately identify this multidrug-

360   resistant bacterium, particularly in isolates that have been ruled out as other multidrug-

361   resistant Gram-negative pathogens, such as *P. aeruginosa* or *Burkholderia* spp. Finally, the

362   correct and rapid identification of *S. maltophilia* will improve antibiotic stewardship

363   measures by enabling more targeted eradication of this pathogen, and in polymicrobial

364   infections such as those commonly found in CF airways, *S. maltophilia* eradication may

365   reduce the prevalence and persistence of more serious pathogens such as *P. aeruginosa*,

366   leading to improved quality of life and lifespans for people with CF.

Acknowledgements

367

References

1. Brooke JS. 2012. *Stenotrophomonas maltophilia*: an Emerging Global Opportunistic Pathogen. Clin Microbiol Rev 25:2-41.

2. Adegoke AA, Stenström TA, Okoh AI. 2017. *Stenotrophomonas maltophilia* as an emerging ubiquitous pathogen: looking beyond contemporary antibiotic therapy. Front Microbiol 8:2276.

3. Trifonova A, Strateva T. 2018. *Stenotrophomonas maltophilia*–a low-grade pathogen with numerous virulence factors. Infect Dis :1-11.

4. Waters V, Yau Y, Prasad S, Lu A, Atenafu E, Crandall I, Tom S, Tullis E, Ratjen F. 2011. *Stenotrophomonas maltophilia* in cystic fibrosis: serologic response and effect on lung disease. Am J Respir Crit Care Med 183:635-640.

5. Looney WJ, Narita M, Mühlemann K. 2009. *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. Lancet Infect Dis 9:312-323.

6. Jeon YD, Jeong WY, Kim MH, Jung IY, Ahn MY, Ann HW, Ahn JY, Han SH, Choi JY, Song YG, Kim JM, Ku NS. 2016. Risk factors for mortality in patients with *Stenotrophomonas maltophilia* bacteremia. Medicine (Baltimore) 95:e4375.

7. Senol E, DesJardin J, Stark PC, Barefoot L, Snydman DR. 2002. Attributable Mortality of *Stenotrophomonas maltophilia* Bacteremia. Clin Infect Dis 34:1653-1656.

8. Gales AC, Jones RN, Forward KR, Liñares J, Sader HS, Verhoef J. 2001. Emerging Importance of Multidrug-Resistant *Acinetobacter* Species and *Stenotrophomonas maltophilia* as Pathogens in Seriously Ill Patients: Geographic Patterns, Epidemiological Features, and Trends in the SENTRY Antimicrobial Surveillance Program (1997–1999). Clin Infect Dis 32:S104-S113.

397   9.   Al-Jasser AM. 2006. *Stenotrophomonas maltophilia* resistant to trimethoprim-

398   sulfamethoxazole: an increasing problem. nn Clin Microbiol Antimicrob 5:23-23.

399   10.   Falagas M, Kastoris A, Vouloumanou E, Dimopoulos G. 2009. Community-acquired

400   *Stenotrophomonas maltophilia* infections: a systematic review. Eur J Clin Microbiol

401   Infect Dis 28:719.

402   11.   Parkins MD, Floto RA. 2015. Emerging bacterial pathogens and changing concepts of

403   bacterial pathogenesis in cystic fibrosis. J Cyst Fibros 14:293-304.

404   12.   Waters V, Atenafu EG, Lu A, Yau Y, Tullis E, Ratjen F. 2013. Chronic

405   *Stenotrophomonas maltophilia* infection and mortality or lung transplantation in

406   cystic fibrosis patients. J Cyst Fibros 12:482-486.

407   13.   Klimova B, Kuca K, Novotny M, Maresova P. 2017. Cystic Fibrosis Revisited–a

408   Review Study. J Med Chem 13:102-109.

409   14.   Sherrard LJ, Tunney MM, Elborn JS. 2014. Antimicrobial resistance in the respiratory

410   microbiota of people with cystic fibrosis. Lancet 384:703-713.

411   15.   Muhlebach MS, Hatch JE, Einarsson GG, McGrath SJ, Gilipin DF, Lavelle G,

412   Mirkovic B, Murray MA, McNally P, Gotman N. 2018. Anaerobic bacteria cultured

413   from cystic fibrosis airways correlate to milder disease: a multisite study. Eur Respir J

414   52:1800242.

415   16.   Kataoka D, Fujiwara H, Kawakami T, Tanaka Y, Tanimoto A, Ikawa S, Tanaka Y.

416   2003. The indirect pathogenicity of *Stenotrophomonas maltophilia*. Int J Antimicrob

417   Agents 22:601-606.

418   17.   Ryan RP, Fouhy Y, Garcia BF, Watt SA, Niehaus K, Yang L, Tolker-Nielsen T, Dow

419   JM. 2008. Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible

420   signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas*

421   *aeruginosa*. Mol Microbiol 68:75-86.

422   18.   Gallo SW, Ramos PL, Ferreira CAS, Oliveira SDd. 2013. A specific polymerase

423   chain reaction method to identify *Stenotrophomonas maltophilia*. Mem Inst Oswaldo

424   Cruz 108:390-391.

425   19.   Pinot C, Deredjian A, Nazaret S, Brothier E, Cournoyer B, Segonds C, Favre-Bonté S.

426   2011. Identification of *Stenotrophomonas maltophilia* strains isolated from

427   environmental and clinical samples: a rapid and efficient procedure. J Appl Microbiol

428   111:1185-1193.

429   20.   Denton M, Kerr KG. 1998. Microbiological and clinical aspects of infection

430   associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev 11:57-80.

431   21.   Huang W, Li L, Myers JR, Marth GT. 2011. ART: a next-generation sequencing read

432   simulator. Bioinformatics 28:593-594.

433   22.   Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina

434   sequence data. Bioinformatics 30:2114-2120.

435   23.   Price EP, Viberg LT, Kidd TJ, Bell SC, Currie BJ, Sarovich DS. 2018.

436   Transcriptomic analysis of longitudinal *Burkholderia pseudomallei* infecting the

437   cystic fibrosis lung. Microb Genom 4.

438   24.   Sarovich DS, Price EP. 2014. SPANDx: a genomics pipeline for comparative analysis

439   of large haploid whole genome re-sequencing datasets. BMC Res Notes 7:618.

440   25.   Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders

441   D, Arrowsmith C, Carver T, Peters N. 2008. The complete genome, comparative and

442   functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily

443   shielded by drug resistance determinants. Genome Biol 9:R74.

444   26.   Swofford DL, Sullivan J. 2003. Phylogeny inference based on parsimony and other

445   methods using PAUP*, p 160-206, vol 7. Cambridge University Press.

446 27. Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing
447   genomic features. Bioinformatics 26:841-842.

448 28. Kidd TJ, Ramsay KA, Hu H, Bye PT, Elkins MR, Grimwood K, Harbour C, Marks
449   GB, Nissen MD, Robinson PJ. 2009. Low rates of *Pseudomonas aeruginosa*
450   misidentification in isolates from cystic fibrosis patients. J Clin Microbiol 47:1503-
451   1509.

452 29. Wood ME, Stockwell RE, Johnson GR, Ramsay KA, Sherrard LJ, Kidd TJ, Cheney J,
453   Ballard EL, O'rourke P, Jabbour N. 2019. Cystic fibrosis pathogens survive for
454   extended periods within cough-generated droplet nuclei. Thorax 74:87-90.

455 30. Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE. 2002.
456   Quantitative Multiprobe PCR Assay for Simultaneous Detection and Identification to
457   Species Level of Bacterial Pathogens. J Clin Microbiol 40:3449-3454.

458 31. Price EP, Dale JL, Cook JM, Sarovich DS, Seymour ML, Ginther JL, Kaufman EL,
459   Beckstrom-Sternberg SM, Mayo M, Kaestli M. 2012. Development and validation of
460   *Burkholderia pseudomallei*-specific real-time PCR assays for clinical, environmental
461   or forensic detection applications. PloS One 7:e37723.

462 32. Weber M, Schünemann W, Fuß J, Kämpfer P, Lipski A. 2018. *Stenotrophomonas*
463   *lactitubi* sp. nov. and *Stenotrophomonas indicatrix* sp. nov., isolated from surfaces
464   with food contact. Int J Syst Evol Microbiol 68(6):1830-1838.

465 33. Zhao J, Xing Y, Liu W, Ni W, Wei C, Wang R, Liu Y, Liu Y. 2016. Surveillance of
466   Dihydropteroate Synthase Genes in *Stenotrophomonas maltophilia* by LAMP:
467   Implications for Infection Control and Initial Therapy. Front Microbiol 7.

468 34. AbdulWahab A, Taj-Aldeen SJ, Ibrahim EB, Talaq E, Abu-Madi M, Fotedar R. 2015.
469   Discrepancy in MALDI-TOF MS identification of uncommon Gram-negative bacteria

470   from lower respiratory secretions in patients with cystic fibrosis. Infect Drug Resist

471   8:83.

472   35. Gautam V, Sharma M, Singhal L, Kumar S, Kaur P, Tiwari R, Ray P. 2017. MALDI-

473   TOF mass spectrometry: An emerging tool for unequivocal identification of non-

474   fermenting Gram-negative bacilli. Indian J Med Res 145:665.

475   36. Nashid N, Yau Y. Evaluation of the Matrix-Assisted Laser Desorption Ionization

476   Time-of-Flight Mass Spectrometry for the Identification of Cystic Fibrosis Pathogens,

477   p. *In* (ed), Oxford University Press,

478   37. Nakamura A, Sugimoto Y, Ohishi K, Sugawara Y, Fujieda A, Monma F, Suzuki K,

479   Masuya M, Nakase K, Matsushima Y. 2010. Diagnostic value of PCR analysis of

480   bacteria and fungi from blood in empiric-therapy-resistant febrile neutropenia. J Clin

481   Microbiol 48:2030-2036.

482   38. da Silva Filho LV, Tateno AF, Velloso LdF, Levi JE, Fernandes S, Bento CN,

483   Rodrigues JC, Ramos SR. 2004. Identification of *Pseudomonas aeruginosa*,

484   *Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia* in respiratory

485   samples from cystic fibrosis patients using multiplex PCR. Pediatr Pulmonol 37:537-

486   547.

487   39. Güemes AGC, Lim YW, Quinn RA, Conrad DJ, Benler S, Maughan H, Edwards R,

488   Brettin T, Cantú VA, Cuevas D. 2019. Cystic Fibrosis Rapid Response: Translating

489   Multi-omics Data into Clinically Relevant Information. mBio 10:e00431-19.