# A specific polymerase chain reaction method to identify *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia *is a multidrug-resistant nosocomial pathogen that is difficult to identify unequivocally using current methods. Accordingly, because the presence of this microorganism in a patient may directly determine the antimicrobial treatment, conventional polymerase chain reaction (PCR) and real-time PCR assays targeting 23S rRNA were developed for the specific identification of* S. maltophilia. *The PCR protocol showed high specificity when tested against other species of* Stenotrophomonas, *non-fermentative Gram-negative bacilli and 100 clinical isolates of* S. maltophilia *previously identified using the Vitek system.*

Key words: *Stenotrophomonas maltophilia* - identification - PCR

*Stenotrophomonas maltophilia* is an important emerging opportunistic nosocomial pathogen that is found in different environmental sources and it is commonly described as an infecting agent in immunocompromised, oncologic and cystic fibrosis patients (Davies & Rubin 2007, Looney et al. 2009). The treatment of *S. maltophilia* infection is difficult due to its intrinsic resistance to important antimicrobial agents and mobile genetic elements, such as transposons and plasmids, which are frequently present and carry determinants of resistance inserted in integrons (Chang et al. 2004, Nicodemo & Paez 2007, Liaw et al. 2010).

*S. maltophilia* is usually isolated using a selective medium with the addition of imipenem and other antimicrobial agents and identified by biochemical methods (Foster et al. 2008a, Adjidé et al. 2010). However, the biochemical tests used to identify *S. maltophilia*, including such commercial systems as Vitek-2, API-20NE and Biolog, occasionally misidentify this species as other non-fermentative Gram-negative bacilli (Zbinden et al. 2007, Pinot et al. 2011). Moreover, the current polymerase chain reaction (PCR)-based protocols for the identification of *S. maltophilia* have also presented limitations related to low specificity due to the significant genetic similarity between *Stenotrophomonas* species and other non-fermentative Gram-negative bacilli (Berg et al. 1999, Foster et al. 2008b).

To overcome this problem, we developed a specific PCR protocol for the identification of *S. maltophilia* based on the design of a primer pair that targets a 278 bp fragment of the 23S rRNA gene (F, 5'GCTGGATTGGTTCTAGGAAAACGC3', and R, 5'ACGCAGTCACTCCTTGCG3'). The 23S rRNA gene was chosen due to the higher variability in this region among species of the *Stenotrophomonas* genus compared to the 16S rRNA gene, as determined using CLUSTALW software (data not shown). The primers targeting the 23S rRNA gene were designed based on the alignment of the following sequences: *S. maltophilia* (GenBank accession AF273255), *Stenotrophomonas acidaminiphila* ATCC 700916T (EU878276), *Stenotrophomonas nitritireducens* ATCC BAA-12T (EU878278), *Stenotrophomonas rhizophila* ATCC BAA-473T (EU878279), *Xanthomonas axonopodis* pv. *citri* DAR 65864 (EU878280), *X. axonopodis* pv. *phaseoli* IAC13755 (FJ828875), *Xanthomonas campestris* pv. *begoniae* HT1-14 (JQ337960), *X. axonopodis* pv. *poinsettiicola* HN-1 (GU144282), *Xanthomonas arboricola* pv. *poinsettiicola* LMG 8676 (GU144280), *X. axonopodis* pv. *manihotis* IBSBF1994 (FJ828873), *X. axonopodis* pv. *allii* IBSBF1770 (FJ828871) and *X. campestris* pv. *campestris* DAR 65808 (EU878277). The PCR mixture was composed of 100 ng target DNA, which was extracted according to Rademaker and de Bruijn (1997), 0.2 mM each deoxynucleoside triphosphate (Ludwig Biotecnologia, Alvorada, RS, Brazil), 2.5 µL 10X PCR buffer (Invitrogen, São Paulo, SP, Brazil), 1.0 mM $MgCl_2$, 0.2 U *Taq* DNA polymerase (Invitrogen) and 20 µM each primer in a total volume of 25 µL. The amplifications were performed using a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) with an initial denaturation step at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 68ºC for 45 sec and extension at 72ºC for 45 sec, with a final extension at 72ºC for 10 min. The amplicons were analysed by electrophoresis through a 2% agarose gel. In addition, the same primer pair was evaluated in a real-time PCR (RT-PCR) assay using a Platinum SYBR Green quantitative PCR (qPCR) SuperMix-UDG (Invitrogen). Each 20 µL reaction contained 100 ng of DNA sample, 10 µL of SYBR

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Green qPCR SuperMix reagent (Invitrogen) and 0.2 μL of each primer. The amplifications were performed using a StepOne RT-PCR system (Applied Biosystems) with the same annealing temperature and cycle number used in the conventional PCR described above.

The following reference strains of the *Stenotrophomonas* species and other non-fermentative Gram-negative bacilli were used to standardise the protocol: *S. maltophilia* ATCC 13637, *S. maltophilia* LMG 958T, *S. maltophilia*, formerly *Stenotrophomonas africana* LMG 22072 (Coenye et al. 2004), *S. acidaminiphila* LMG 22073, *S. nitritireducens* LMG 22074, five *Acinetobacter baumannii* isolates, three *Acinetobacter calcoaceticus-baumannii* complex isolates, one *Burkholderia cepacia*, five *Pseudomonas aeruginosa* isolates, two *Pseudomonas* spp and *X. axonopodis*. Specific amplification was detected using both PCR methods only for the *S. maltophilia* strains, including the sample previously classified as *S. africana*, whereas no amplification was observed for all other bacterial strains tested. The detection limits of RT-PCR were determined using the threshold cycle (Ct) values obtained from tests performed with *S. maltophilia* ATCC 13637 and *S. maltophilia*, formerly *S. africana* LMG 22072; the Ct values were 19.58 and 15.9, respectively. All tests were performed in duplicate. The standard deviation was 0.08 for both analyses. To test the applicability of the protocol, 100 different clinical isolates of *S. maltophilia*, which were previously identified using the Vitek system (bioMérieux, Hazelwood, MO, USA), were evaluated and produced specific amplicons.

One amplicon obtained from *S. maltophilia* ATCC 13637 was randomly chosen and sequenced using an ABI 3130 XL Genetic Analyzer (Applied Biosystems) automated DNA sequencer. The sequence was aligned with the *S. maltophilia* 23S rRNA sequences deposited in GenBank (AM743169 and AF273255) using MEGA 5.10 Beta software, presenting 100% identity.

Foster et al. (2008b) and Pinot et al. (2011) reported the use of a different region of the 23S rRNA gene as a target, but their results showed cross-reaction between *Stenotrophomonas* and *Xanthomonas* species, demonstrating low PCR specificity. Additionally, Pinot et al. (2011) developed a multiplex PCR targeting *sme*D and *ggp*S to differentiate *S. maltophilia* and *S. rhizophila*; although this technique was successful, it did not exclude the cross-reaction with *Xanthomonas* species. In contrast, no cross-reaction was observed with *X. axonopodis* using the protocol described in this study. It should be noted that the primer pair in this study was not evaluated against other species of *Xanthomonas* and/or *S. rhizophila*. Other studies have described protocols to detect *S. maltophilia* using RT-PCR, but the cross-reaction with *Xanthomonas* and/or other *Stenotrophomonas* species was not evaluated (Wellinghausen et al. 2004, Dark et al. 2011).

Therefore, the primers designed in this study are an important alternative to specifically detect *S. maltophilia* using rapid molecular methods, which can enable the timely determination of the appropriate antimicrobial protocol for the successful treatment of infections caused by this microorganism.

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