Rapid identification of *Stenotrophomonas maltophilia* by peptide nucleic acid fluorescence *in situ* hybridization

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Abstract

The objective of this study was to develop a novel peptide nucleic acid (PNA) probe for *Stenotrophomonas maltophilia* identification by fluorescence *in situ* hybridization (FISH). The probe was evaluated using 33 human and veterinary clinical *S. maltophilia* isolates and 45 reference strains representing common bacterial species in the respiratory tract. The probe displayed 100% sensitivity and 100% specificity on pure cultures and allowed detection in sputum from cystic fibrosis patients. The detection limit was 10^4 CFU/mL in spiked tracheal aspirate and bronchoalveolar lavage from healthy horses. Altogether the study shows that this species-specific PNA FISH probe facilitates rapid detection of *S. maltophilia* in biological specimens.

Keywords: Cystic fibrosis, identification, PNA FISH, *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia is a Gram-negative bacterium ubiquitous in nature [1]. It is capable of causing serious disease in immuno-compromised patients and has been reported in cystic fibrosis (CF) patients with a yearly prevalence of up to 25% [2, 3]. In veterinary medicine, an increasing number of studies indicate possible associations with respiratory disease [4, 5], bacterial cystitis and endocarditis [6], urinary tract infections [7] and lymphadenitis [8]. Within the genus, *S. maltophilia* is the only species that is known to colonize and cause respiratory tract infections in humans and animals.

The objective of this study was to design and evaluate a novel peptide nucleic acid (PNA) probe for *S. maltophilia* identification by fluorescence *in situ* hybridization (FISH). A PNA probe targeting 16S rRNA (Flu-OO-CGCCATG-GATGTTCC, 5′-3′) specific for *S. maltophilia* and conjugated with fluorescein isothiocyanate (FITC) was added a PNA blocker probe (Ac-CCACGGATGTTCC, 5′-3′) to prevent cross-reaction with closely related species such as *Xanthomonas campestris* (Panagene, Daejeon, Korea). Sensitivity and specificity of the probe were evaluated using 35 human and veterinary clinical *S. maltophilia* isolates, and 43 reference and clinical strains representing common bacterial species in the airways of humans and animals (Table 1). All strains were cultured on solid media (5% calf blood agar, chocolate agar or nutrient/yeast/glycerol agar as deemed appropriate) and sub-cultured in tryptic soy broth. Ten microlitres of broth were placed onto a microscopic slide prepared with one drop of fixation solution (phosphate-buffered saline with detergent) and fixed by heating (methanol fixation for sputum smears). After adding one drop of hybridization solution (AdvanDx, Woburn, MA, USA) containing the *S. maltophilia* PNA probe, a coverslip was applied and hybridization performed by incubating the slides at 55°C for 30 min (90 min for sputum smears). Limited to Gram-negative strains, slides were immersed in preheated deionized water (55°C) for 1 min. All slides were then placed in a wash jar with a preheated (55°C) wash solution (diluted Tris-buffered saline with detergent) in a water bath for 30 min, coverslips removed. After air drying, a drop of mounting medium (photobleaching inhibitor in glycerol) and a coverslip were applied. A positive control slide with *S. maltophilia* and a negative control slide with the relevant strain hybridized with a universal PNA FISH probe (BacUni PNA, AdvanDx) according to manufacturer's instructions were included in all runs. All slides were evaluated within 2 h under a fluorescence microscope (×60 objective, Olympus BX51, Ballerup, Denmark; Mercury U-LH100HG 100 W lamp) equipped with an FITC/Texas Red Dual Band Filter. Fluorescence images were obtained using an Olympus DP72 camera (1360 × 1024 pixels, 1 s exposure). Samples were considered positive when single cells had a strong fluorescence and clear

TABLE 1. Reference and clinical strains used for probe evaluation.

Proteus mirabilis	ATCC 12453	*Streptococcus dysgal.* subsp. *dysgalactiae*^{T}	CCUG 27301
Bacillus cereus[a]	S31A8[a]	*Streptococcus dysgal.* subsp. *equisimilis*	ATCC 12388
Escherichia coli	ATCC 35218	*Streptococcus equi* subsp. *zooepidemicus*	CCUG 23256
Enterococcus faecalis^T	ATCC 19433	*Streptococcus equi* subsp. *equi*	ATCC 9528
Enterobacter aerogenes^T	ATCC 13048	*Streptococcus suis*^{T}	CCUG 7984
Serratia marcescens	ATCC 14756	*Streptococcus bovis*^{T}	CCUG 17828
Citrobacter freundii	ATCC 43864	*Streptococcus pneumoniae*	ATCC 10015
Staphylococcus epidermidis	ATCC 12228	*Actinobacillus equuli* subsp. *equuli*[a]	S55G3[a]
Staphylococcus aureus^T	ATCC 12600	*Alcaligenes* spp.[a]	S61E5[a]
Hafnia alvei[a]	S24B4[a]	*Corynebacterium diphtheriae*	ATCC 27010
Pasteurella multocida	ATCC 12945	*Bordetella bronchiseptica*[a]	S31B7[a]
Neisseria meningitidis	ATCC 13102	*Klebsiella pneumoniae* subsp. *pneumoniae*^{T}	ATCC 13883
Acinetobacter baumannii^T	ATCC 19606	*Klebsiella pneumoniae* subsp. *pneumoniae*	ATCC 10031
Moraxella bovis[a]	S31B1[a]	*Xanthomonas campestris* pv. *campestris*	Strain 8004
Moraxella catarrhalis	ATCC 25240	*Pseudomonas fluorescens*^{T}	ATCC 13525
Moraxella nonliquefaciens^T	ATCC 19975	*Pseudomonas alcaligenes*^{T}	ATCC 14909
Haemophilus influenzae	ATCC 19418	*Pseudomonas putida*	ATCC 49128
Burkholderia cepacia	ATCC 25416	*Pseudomonas stutzeri*^{T}	ATCC 17588
Burkholderia multivorans	CF 54[b]	*Pseudomonas aeruginosa*^{T}	ATCC 10145
Stenotrophomonas acidaminiphila^T	ATCC 700916	*Pseudomonas aeruginosa*	PAO1[b], CF15[b], CF16[b]
Stenotrophomonas maltophilia^T	ATCC 13637		
Stenotrophomonas maltophilia	Veterinary clinical strains $n = 22$[a] and human clinical strains $n = 13$[b]		

[a]From the Department of Veterinary Disease Biology, University of Copenhagen, Denmark.
[b]From the Department for Clinical Microbiology, University Hospital of Copenhagen, Denmark.

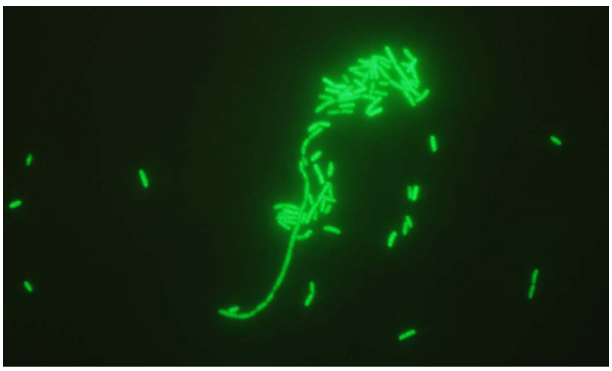

FIG. 1. PNA FISH (peptide nucleic acid fluorescence *in situ* hybridization) microscopy of *Stenotrophomonas maltophilia*.

morphology (Fig. 1). The probe yielded positive results with all target organisms and negative results with all non-target organisms (100% sensitivity and 100% specificity).

The probe was tested on spiked respiratory samples of equine origin to determine the detection limit. Tracheal aspirate (TA) and bronchoalveolar lavage (BAL) samples from three healthy horses were spiked with *S. maltophilia* ATCC 13637. Ten-fold dilution series were prepared from the spiked TA/BAL samples and PNA FISH smears were prepared for each dilution as described above but without fixation solution. The detection limit was determined by parallel plating of the dilutions on blood agar for colony counts. Fluorescence microscopy of the smears showed at least one to ten *S. maltophilia* cells in most fields when the concentration in the sample was 10^4 CFU/mL. The probe specificity was further tested on sputum samples from seven patients with CF. The samples underwent routine culture at the University Hospital of Copenhagen, Denmark. The probe produced positive results in all four samples from *S. maltophilia*-infected patients and negative results in all control samples (two patients infected with *Pseudomonas aeruginosa*, one patient infected with *Burkholderia multivorans*). No background fluorescence was observed.

Isolation and identification of *S. maltophilia* can be problematic [9–13]. Selective differential media have been recommended for improved detection of *S. maltophilia* from non-sterile sites such as respiratory secretions [14–16]. Problems related to misidentification of *S. maltophilia* by phenotypic methods can be overcome by the use of molecular methods. Pinot *et al.* [17] used vancomycin, imipenem, amphotericin B medium agar for isolation and multiplex PCR for identification of *S. maltophilia*. Hogardt *et al.* [18] designed a species-specific DNA probe for *S. maltophilia* identification and demonstrated that the probe could be used successfully on sputum and throat samples from CF patients. However, the limit for microscopic detection of bacteria within sputum was 4×10^5 CFU/mL and the sensitivity of the DNA FISH method (90%) was lower than that of the PNA FISH approach described in this study. PNA probes are small in size with a non-charged polyamide backbone that renders them easy to hybridize and increases the binding strength compared to DNA probes [19]. PNA probes have better sensitivity and specificity, show improved penetration into cells and through biofilm matrices and are not susceptible to bacterial endonucleases which may be present in clinical samples [20]. PNA FISH is a very fast (less than 90 min) and reliable molecular identification method which is easy to perform with very little hands-on time. Furthermore, FISH is useful for *in situ* detection of this microorganism directly in clinical samples and mixed bacterial populations without prior cultivation. Thus, the *S. maltophilia* PNA FISH probe described in this study has

important applications for studies of biofilm infections and *S. maltophilia* colonization in patients with CF, where colonization and chronic infection with *S. maltophilia* is commonly reported.

In conclusion, the *S. maltophilia* PNA FISH probe demonstrated excellent sensitivity and specificity when tested against clinically relevant bacteria occurring in the respiratory tract of humans and animals. The PNA FISH assay can be implemented in diagnostic laboratories for rapid, simple and reliable *in situ* identification of *S. maltophilia* in clinical specimens. It can only be a valuable tool for research aimed at understanding the role played by this organism in CF and in equine respiratory tract infections. For this purpose, further studies are warranted to evaluate the use of the probe for studying spatial distribution of *S. maltophilia* in polymicrobial biofilms.

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Conflict of Interest

None declared.

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