

# *Stenotrophomonas maltophilia* isolated from the airways of animals with chronic respiratory disease

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## Abstract

*Stenotrophomonas maltophilia* (*S. maltophilia*) is a nonfermentative bacterium, which is naturally resistant against a panel of commonly-used antibiotics. It is frequently isolated from humans with chronic respiratory disease, e.g. cystic fibrosis or chronic obstructive pulmonary disease. In veterinary medicine *S. maltophilia* is perceived to be a mere coloniser. We herewith report 7 strains of *S. maltophilia* isolated from animals, of which 5 strains were harvested from 3 horses, a dog and a cat with chronic respiratory disease. The dog isolate showed resistance to trimethoprim / sulphamethoxazole, which was confirmed by detection of the *sul 1* gene. Analysis with pulsed field gel electrophoresis revealed that 2 horses, which were boarded in the same clinic but two years apart, harboured the same strain of *S. maltophilia*. This is indicative of a hospital acquired colonisation / infection, which contradicts involvement in the pre-existing chronic disease.

Keywords: *Stenotrophomonas maltophilia*, chronic respiratory disease, *sul 1* gene, pulsed field gel electrophoresis

## Isolierung von *Stenotrophomonas maltophilia* aus dem Respirationstrakt von Tieren mit chronischen Atemwegserkrankungen.

*Stenotrophomonas maltophilia* (*S. maltophilia*) ist ein nichtfermentatives Bakterium, welches natürlich resistent ist gegen viele routinemässig eingesetzte Antibiotika. Es wird häufig bei Menschen mit chronischen Atemwegserkrankungen wie Cystischer Fibrose oder Chronisch Obstruktiver Lungenerkrankung isoliert. In der Veterinärmedizin wird *S. maltophilia* als simpler Besiedler betrachtet. Wir berichten von 7 aus Tieren isolierten *S. maltophilia* Stämmen, von welchen 5 von 3 Pferden, einem Hund und einer Katze mit chronischen Atemwegserkrankungen isoliert werden konnten. Das Isolat des Hundes war resistent gegen Trimethoprim / Sulfamethoxazol, was durch Detektion des *sul 1* Gens bestätigt wurde. Die Analyse mit Pulsed Field Gel Elektrophorese zeigte, dass derselbe Stamm bei zwei Pferden vorhanden war, welche im Abstand von zwei Jahren an derselben Klinik standen. Dies lässt auf eine im Spital erworbene Besiedlung / Infektion schliessen, was einer Beteiligung an der vorbestehenden chronischen Krankheit widerspricht.

Schlüsselwörter: *Stenotrophomonas maltophilia*, chronische Atemwegserkrankung, *sul 1* Gen, Pulsed Field Gel Electrophoresis

## Introduction

*Stenotrophomonas maltophilia* (*S. maltophilia*) is a gram-negative, oxidase-negative rod, which was previously known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*. The bacterium is ubiquitous in nature, prevailing in soil, plant material, water and sewage (Denton and Kerr, 1998a). In animals it was isolated, e.g. from squirrel faeces (Cloud-Hansen et al., 2007), porcine semen (Althouse et al., 2005), oral flora of snakes (Hejnar et al., 2007) and fleece rot in sheep (MacDiarmid and Burrell, 1986). Despite earlier doubts about its pathogenicity, it is now established as an important emerging nosocomial pathogen in human medicine and is no lon-

ger regarded as a harmless coloniser. In patients that are immunocompromised or suffering from chronic disease, e.g. cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD), infection with *S. maltophilia* contributes to higher fatalities (Denton and Kerr, 1998a). Unlike other nosocomial pathogens, outbreaks with *S. maltophilia* are rarely due to single emerging strains. Rather, different strains from sources like water taps, water dispensers, inhalation therapy equipment, blood-sampling tubes or hands of medical staff may be isolated in parallel from different patients (Denton and Kerr, 1998a; Denton et al., 1998b; Abbassi et al., 2008). *S. maltophilia* is mainly considered as a nosocomial bacterium on the basis that it is naturally resistant to many commonly-used antibiotics.

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The drug of choice is trimethoprim / sulphamethoxazole, but resistance mediated by *sul* genes is emerging (Tolman et al., 2007). Little is known about the occurrence of *S. maltophilia* in animals with respiratory disease. We herein describe isolates from the routine diagnostic laboratory, which were harvested with routine culture methods. We were interested, whether the finding of *S. maltophilia* in three horses, a dog and a cat with chronic respiratory disease is suggestive of a similar importance as described in human CF or COPD patients and how the finding of this bacterium should be interpreted in diagnostic samples.

## Animals, Material and Methods

### Bacterial strains and culture methods

Bacterial isolates used in this study were derived from routine diagnostic specimens from veterinary patients from 2005 to 2007: Tracheobronchial wash (TBW) from 120 horses, 5 dogs and 4 cats, bronchoalveolar lavage (BAL) from 7 horses, 59 dogs and 29 cats and nasal swabs from 117 horses, 53 dogs and 72 cats. From these, a total of 5 strains were obtained from 3 horses (isolates 1, 2 and 3), one dog (isolate 4) and one cat (isolate 6). Further, isolate 5 was obtained from a wound infection in a dog and isolate 7 from an oral swab of a ball python (*Python regius*). Isolates 1 (from 2005) and 3 (from 2007) derived from horses from the same clinic. Also, isolates 4 (from 2007) and 5 (from 2005) derived from one practice (Tab. 1). All animals, apart from the snake, had been pre-treated with antibiotics and were presented to the veterinarian because of persisting respiratory disease (case isolates 1, 2, 3, 4, 6) or post operative wound infection (isolate 5). Samples were routinely cultured on various media, but *S. maltophilia* was always isolated from tryptone soy agar with 5% sheep blood (TSA; Oxoid, Basel, Switzerland) incubated at 37 °C. *S. maltophilia* and concomitant flora was identified with the API ID system (BioMérieux, Geneva, Switzerland). The type strain *S. maltophilia* DSM 50170 (ATCC 13637<sup>T</sup>) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

### PCR analysis from TBW or BAL samples

For PCR analysis only, TBW or BAL from routine material deriving from 42 horses was additionally tested. Sediments from the samples obtained by centrifugation were lysed in 450 µl lysis buffer containing 0.1 M Tris-HCl [pH 8.5], 0.05% Tween 20, 0.24 mg of proteinase K (Roche, Reinach, Switzerland) per ml and were incubated for 60 min at 60 °C followed by inactivation of the proteinase K for 15 min at 97 °C and subjected to *S. maltophilia* PCR.

### Species-specific *S. maltophilia* PCR

For confirmation of biochemical identification, 5 colonies were lysed as described above. Lysates were subjected to a previously described *S. maltophilia* species-specific PCR amplifying a 531 bp product (Whitby et al., 2000), carried out with the following modifications: 3 µl of sample lysate was added to 22 µl of reaction mixture containing 0.2 µM of each Primer (SM1 5'-CAGCCTGCGAAAAG-TA-3'; SM4 5'-TTAAGCTTGCCACGAACAG-3'), 100 µM of each dNTP, 2.5 mM MgCl<sub>2</sub> and 1 U of Taq polymerase (FIREPol®, Solis Biodyne, Tartu, Estonia) in 1 × Solis PCR reaction buffer B (Solis Biodyne), with the thermal cycling conditions 5 min at 95 °C, followed by 10s at 95 °C, 10s at 58 °C and 60s at 72 °C for 30 cycles and final elongation for 2 min at 72 °C. The type strain *S. maltophilia* ATCC 13637<sup>T</sup> was used as a positive control and *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* (reference strain D 510/96) and *Brevundimonas diminuta* (field isolate Ue 2020/04) were used as negative controls.

### Antimicrobial susceptibility testing

All isolates were tested with the automated susceptibility test strips ATB®PSE 5 and ATB®VET strips (BioMérieux), a micro-dilution technique, performed according to the manufacturer's recommendations. Briefly, the bacteria are grown on TSA, a few colonies are suspended in sterile saline to a turbidity of 0.5 × McFarland, transferred to a semi-solid growth medium, and pipetted into each well of the strip, containing different antibiotics. The strip is then incubated at 37 °C for 18–24 h and hence read with the automated miniAPI system.

### Detection of *sul 1* gene by PCR and verification by sequencing

For one PCR reaction for the detection of *sul 1* gene the same PCR reaction mixture was used as described above with 20 pmol of each primer Sull-L GTGACGGTGTTCGGCATTCT and Sull-R TCCGAGAAGGTGATTGCGCT (Lanz et al., 2003). The product was sequenced with the same primers, as described by Kuhnert et al. (2002).

### Pulsed Field Gel Electrophoresis (PFGE)

*S. maltophilia* strains were incubated overnight on TSA at 37 °C. Organisms were analysed according to the „*Pseudomonas aeruginosa* PFGE protocol“ (www.hpa.org.uk). Bacteria were suspended in 1 ml SE Buffer (75 mM NaCl, 25 mM EDTA Na<sub>2</sub>, sterile filtered) to a turbidity of 2.5 McFarland. 350 µl bacterial suspension was carefully mixed with 350 µl 2% pulsed field certified agarose (Bio-Rad, Reinach, Switzerland) in a waterbath at 56 °C and immediately transferred to the plug moulds. Moulds were allowed to set at 4 °C. Plugs were transferred to sterile glass vials and incubated in 3 ml gram-positive lysis buffer (pH

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7.5; consisting of 6 mM Tris-HCl, 100 mM EDTA Na<sub>2</sub>, 1 M NaCl, 0.5% (w/v) Brij 58, 0.2% (w/v) sodium deoxycholate, 0.5% lauroyl sarcosine) containing 500 µg/ml lysozyme (Roche) at 37°C overnight. The gram-positive lysis buffer was hence drawn off and the plugs resuspended in gram-negative lysis buffer (pH: 9.5; consisting of 1% N-lauroyl sarcosine sodium salt, 500 mM EDTA Na<sub>2</sub>, sterile filtered) containing 500 µg/ml Proteinase K (Roche) and incubated overnight at 56°C. The gram-negative buffer was removed and the plugs washed thrice in 3 ml TE buffer at 4°C for 30 min. A 2 mm portion of the plugs was incubated in 100 µl 1x reaction buffer at 4°C for 30 min. The reaction buffer was discharged and restriction digestion with 30 U of either *SpeI* or *XbaI* (Roche)

was done in 100 µl 1x enzyme buffer at 37°C overnight. The separation of the DNA fragments was performed in a Chef-Dr® III System (BioRad). The running buffer 0.5 × TBE contained 9 mg/l thiourea (Merck, Darmstadt, Germany). With this 0.5 × TBE buffer, a 1.2% pulsed field certified agarose gel was made, the digested plugs were loaded and run with Lambda 1 marker (New England BioLabs, Ipswich, MA, USA) under the following conditions: 12°C, initial start time 1 s, final start time 50 s, gradient 6V, angle 120°, run time 30 h. The gel was then stained for 40 min in 300 ml TBE with 30 µl ethidiumbromide, destained in 300 ml aqua bidest for 1 h and analysed in a GelDoc 2000 (BioRad).

Table 1: Species and clinical samples whereof *Stenotrophomonas maltophilia* and concomitant flora was isolated.

Strain / number	Clinic / Practice	species	clinical specimen	anamnesis	numbers <sup>1</sup> of <i>S. maltophilia</i> isolated	other bacteria than <i>S. maltophilia</i> cultured from the sample <sup>1</sup>
1	1	horse	tracheobronchial wash	chronic cough	+/-	+/- <i>Pseudomonas</i> sp.
2	2	horse	tracheobronchial wash	chronic cough	+/-	+/- α-haemolytic streptococci
3	1	horse	tracheobronchial wash	dyspnoea	+	none
4	3	dog	bronchoalveolar lavage	chronic cough	++	++ various unspecified
5	3	dog	wound abdomen	suture dehiscence after surgery	+++	+++ none
6	4	cat	nasal swab	mucopurulent nasal discharge	++	+++ <i>Pasteurella</i> sp.
7	5	ball python	oral cavity	not specified	++	+++ <i>Pasteurella</i> sp. non-haemolytic staphylococci

<sup>1</sup>Semiquantitative scheme: less than ten bacterial colonies (+/-), ten to 30 colonies (+), 30 to 100 colonies (++) and more than 100 colonies (+++) per plate

Table 2: Antibiotic susceptibilities<sup>1</sup> of *Stenotrophomonas maltophilia* determined with ATB® strips.

strain number	species	Ticarcillin	Piperacillin	Imipenem	Ceftazidime	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin / Enrofloxacin	Colistin / Polymyxin	Trimethoprim / Sulphamethoxazole	Chloramphenicol	Tetracycline
1	horse	R	R	R	R	R	R	R	S	S	S	S	R
2	horse	R	R	R	R	S	R	R	S	S	S	S	R
3	horse	R	R	R	R	R	R	R	S	S	S	S	R
4	dog	R	S	R	R	S	R	R	S	S	R <sup>2</sup>	S	R
5	dog	R	R	R	R	I	R	I	S	S	S	R	R
6	cat	R	R	R	R	R	I	S	S	S	S	S	R
7	python	R	R	R	R	S	S	S	I	S	S	R	R

<sup>1</sup>S: sensitive, I: intermediate, R: resistant, <sup>2</sup> *sul1* gene detected by PCR

## Results

### Identification of *S. maltophilia* with biochemical reactions and PCR

From 244 equine, 117 canine and 105 feline respiratory tract samples five isolates of *S. maltophilia* were harvested with routine culture methods. All strains biochemically identified as *S. maltophilia* were subsequently confirmed with the *S. maltophilia* species-specific PCR, whilst negative controls did not produce a signal. From 42 equine TBW or BAL samples crude lysates of two TBW samples were found to be positive in a *S. maltophilia* specific PCR, where *S. maltophilia* had not been found in the culture. The two samples derived from horses that were boarded at clinic 1 (Tab. 1).

### Antimicrobial susceptibility and detection of *sul* genes

Antimicrobial susceptibilities are shown in Table 2. Isolate 4 that originated from BAL from a dog with chronic cough, showed resistance to trimethoprim / sulphamethoxazole. Therefore a specific PCR was carried out for the detection of *sul* 1, which was consequently detected in isolate 4, bearing a sequence identity of 100%.

### Pulsed Field Gel Electrophoresis (PFGE)

Analysis with *Spe*I showed an identical pattern in strains 1 and 3, while all other strains were unique (Fig. 1). The identical strains had been collected two years apart, but

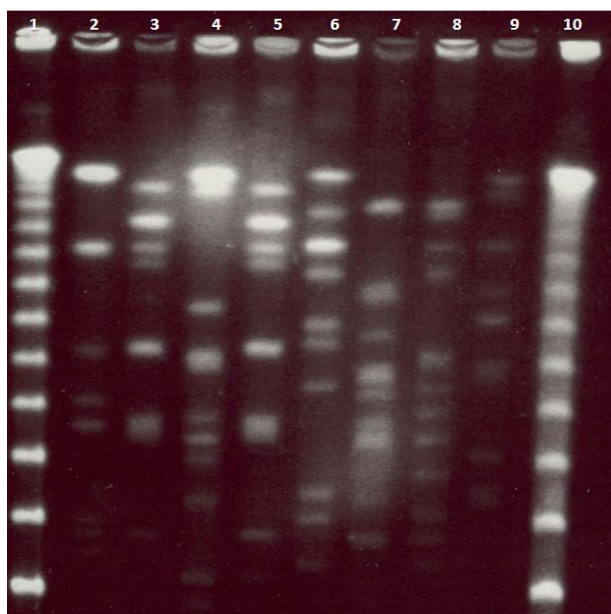


Figure 1: Pulsed Field Gel Electrophoresis of *Stenotrophomonas maltophilia* strains isolated from animals with respiratory disease, digested with *Spe*I. Lane 1, 10: marker, lane 2: *S. maltophilia* type strain, lane 3–9: study isolates 1–7.

were derived from the same horse clinic. For confirmation new plugs were made and the procedure repeated for all strains with *Xba*I, yielding the same result (data not shown).

## Discussion

Isolation of *S. maltophilia* is probably underdiagnosed in the routine veterinary diagnostic laboratory, as no selective media that increase the isolation success of *S. maltophilia* (Kerr et al., 1996a) are used. In highly contaminated samples *S. maltophilia* may not be retrieved. In most samples of this study from where *S. maltophilia* was isolated a variety of other bacteria were growing as well (Tab. 1). Isolation of *S. maltophilia* in mixed culture is not a rare event, and was long seen as evidence that this bacterium may only be a coloniser without contribution to disease, unless the host is immunocompromised. However, it is now widely established that isolation of *S. maltophilia* from normally sterile sites is significant (Denton and Kerr, 1998). Nonetheless, the significance of *S. maltophilia* in veterinary routine specimens is not easy to pinpoint. First, it is difficult to distinguish infection from colonisation. *S. maltophilia* was isolated from the upper respiratory tract in horses from oral-tracheal swabs (Mancini et al., 2005) and guttural pouch (DeBiaio, 2003) without association with respiratory disease. Nevertheless, samples from normally sterile sites in the lower respiratory tract should not yield this bacterium. Secondly, *S. maltophilia* is known to inhibit potentially significant fungi (Kerr, 1996b), therefore an underlying disease may be missed. Furthermore, it must be noticed that *S. maltophilia* was shown in vitro to activate influenza virus by cleavage of haemagglutinin, a fact that could be of significance in unvaccinated horses (Mancini et al., 2005).

The yield of a pure culture of *S. maltophilia* from a suture dehiscence in a dog (isolate 5) can be easily interpreted as a secondary wound infection, while the finding in the ball python (isolate 7) denotes a part of the normal oral flora of snakes (Hejnar et al., 2007). Contrary, the finding of *S. maltophilia* in horses with chronic coughing (isolates 1, 2 and 3) and in a dog (isolate 4) and a cat (isolate 6) with chronic respiratory disease was intriguing at first sight, and bears similarities to the occurrence of *S. maltophilia* in human patients with COPD or CF. Risk factors for *S. maltophilia* colonisation and infections are likely to be similar in veterinary medicine as reported for human cases. *S. maltophilia* infections benefit from preceded antibiotic therapy, which in human medicine is often due to imipenem or vancomycin treatment (antibiotics that are not registered for veterinary use in Switzerland), but other antibiotics like cephalosporins, gentamicin or fluoroquinolones, also select for *S. maltophilia* (Suilen et al., 1999). The bacterium is also known to adhere to plastic parts like intravenous catheters, in-

halation equipment or bronchoscopes (Denton and Kerr, 1998a; Suilen et al., 1999). The finding of *S. maltophilia* should therefore always alert the veterinarian concerning the contamination of any equipment used. But unlike other hospitalism-agents where clonal occurrence and the establishment of one strain is the rule (Boerlin et al., 2001), *S. maltophilia* from different patients even in hospital environments are mostly different strains (Sader et al., 1994). Outbreaks with clonal occurrence do occur, but rarely (Abbassi et al., 2008). Analysis of isolates with PFGE may be useful to uncover such an establishment of a particular strain. When we did this, our study strains 1 and 3 that derived from horses that had been boarded in the same clinic, but two years apart, surprisingly yielded the same strain of *S. maltophilia* (Fig. 1). The two dog strains (4 and 5) also came from one practice, but were shown to be different (Fig. 1). Unfortunately, it was not possible to analyse environmental samples from clinic 1 and the precise source remains unknown. Nevertheless it can be deduced that this strain is present in the clinic environment, was acquired in the clinic and was not present in the horses beforehand. This is further supported by the positive PCR testing of horses only from clinic 1. Thus, at second sight, involvement of *S. maltophilia* in the observed clinical disease is therefore unlikely.

In conclusion, the significance of *S. maltophilia* is not always clear. Notwithstanding, the isolation, identification and susceptibility testing of this multi-resistant bacterium may be of particular importance in animals with chronic

coughing that are companion animals for humans with known chronic respiratory diseases. The finding of an isolate with *sul* 1-mediated resistance to trimethoprim / sulphamethoxazole in a dog may be regarded with caution, although transmission from animals to humans have not been reported to date.

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### Isolation de *Stenotrophomas maltophilia* dans le système respiratoire d'animaux souffrant de problèmes respiratoires chroniques.

*Stenotrophomas maltophilia* est une bactérie non fermentative qui est naturellement résistante face à de nombreux antibiotiques employés de façon routinière. Il est souvent isolé chez des êtres humains souffrant de maladies respiratoires chroniques telle la fibrose kystique ou les maladies obstructives chroniques. En médecine vétérinaire, *Stenotrophomas maltophilia* est considéré comme un simple opportuniste. Nous rapportons l'isolation de souches de *S. maltophilia* chez 7 animaux donc 5 provenant de 3 chevaux, 1 d'un chien et 1 d'un chat souffrant tous de maladies respiratoires chroniques. L'isolat du chien était résistant au Triméthoprim / Sulfaméthoxazole ce qui a été confirmé par la détection du gène *sul* 1. Les analyses par électrophorèses ont montré que le même germe était présent chez 2 chevaux présentés à 2 ans d'intervalles à la même clinique. Ceci laisse conclure à une infection contractée en milieu hospitalier, ce qui contredit son implication dans la maladie chronique pré-existante.

### Isolamento della *Stenotrophomonas maltophilia* dal tratto respiratorio di animali affetti da malattie croniche delle vie respiratorie

*Stenotrophomonas maltophilia* (*S. maltophilia*) è un battere non fermentativo che resiste naturalmente a molti antibiotici utilizzati normalmente. Viene spesso isolato negli uomini affetti da malattie croniche delle vie respiratorie come la fibrosi cistica o la malattia polmonare ostruttiva cronica. In medicina veterinaria, l'*S. maltophilia*, viene considerato come semplice colonizzatore. Qui di seguito facciamo riferimento a 7 ceppi di *S. maltophilia* isolati da animali di cui 5 sono stati isolati da 3 cavalli, un cane e un gatto affetti da malattie croniche delle vie respiratorie. L'isolato del cane si è dimostrato resistente alla trimethoprim-sulfametossazolo cosa confermata dal ritrovamento del gene *sul* 1. Le analisi con elettroforesi a campi pulsanti (Pulsed Field Gel) hanno mostrato che lo stesso ceppo era presente in due cavalli che si trovavano a distanza di due anni nella medesima clinica. Questi dimostra una colonizzazione/infezione in ospedale, escludendo una partecipazione dell'agente in questione alla malattia cronica preesistente.

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