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PII: S0378-1135(07)00492-0
DOI: doi:10.1016/j.vetmic.2007.10.007
Reference: VETMIC 3850

To appear in: *VETMIC*

Received date: 13-8-2007
Revised date: 8-10-2007
Accepted date: 10-10-2007

Please cite this article as: Boot, R., Van de Berg, L., Reubsaet, F.A.G., Vlemminx, M.J., Positive *Streptobacillus moniliformis* PCR in guinea pigs likely due to *Leptotrichia spp*, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.10.007

# Positive *Streptobacillus moniliformis* PCR in guinea pigs likely due to *Leptotrichia spp*

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key words: *Streptobacillus moniliformis* – PCR - *Leptotrichia*

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## Summary

*Streptobacillus moniliformis* is a zoonotic bacterium. We obtained positive *S. moniliformis* PCR results in oral swab samples from guinea pigs from an experimental colony and the breeding colony of origin. Comparison of the DNA sequence of an amplicon with deposited 16S rDNA sequences revealed that *Leptotrichia* sp can be the source of a false positive *S. moniliformis* PCR outcome.


## Introduction

*Streptobacillus moniliformis* is the causative agent of rat bite fever and Haverhill fever in man (Elliott, 2007). Streptobacillosis is a risk associated with work with laboratory rodents (Wullenweber, 1995) and *S. moniliformis* infection has been detected in various mouse, rat and guinea pig colonies in the last twenty years (references in Boot et al., 2002). The host spectrum of the bacterium might comprise also non rodent species (Elliott, 2007). The infection may be detected by serology (Boot et al., 1993, 2006), culture and the polymerase chain reaction (PCR) (Boot et al., 2002). *S. moniliformis* is listed in the European recommendations for the routine monitoring of laboratory rodents for unwanted infectious microorganisms (Nicklas et al., 2002).

We report a case in which guinea pigs used as experimental animals and showing antibodies to *S. moniliformis* were positive by *S. moniliformis* PCR used as a confirmatory test. DNA sequencing of an amplicon revealed that *Leptotrichia* sp can be the source of a false positive *S. moniliformis* PCR outcome.

## Material and Methods

### Animals and samples

Positive results of testing Dunkin Hartly guinea pigs used in vaccine control experiments by *S. moniliformis* enzyme linked immunosorbent assay (ELISA) and immunoblot (IB or Western blot) made us to sample four animals for testing by *S. moniliformis* PCR (Boot et al., 2002). Sampling was carried out in a sterilized class 2 safety cabinet and strict precautions were taken to avoid cross-contaminations between animals and samples.

The animals were sacrificed under anesthesia, inspected for gross abnormalities of the lungs, the larynx en the cervical lymphnodes (*lnn cervicalis profundus cranialis*). The oral

cavity was sampled using a moistened cotton swab and the cervical lymphnodes and a part of the anterior lobe of the lung was excised. Samples were stored at 4 °C until testing by PCR.

Our experimental colony contained guinea pigs originating from two breeding colonies. Positive PCR findings in the experimental guinea pigs made us to test additional oral samples from animals from our institutional (n=24) and a commercial breeding colony (n=12) by PCR.

Culture for *S. moniliformis* was not attempted as the bacterium is notoriously difficult to grow and in rats PCR has been found more sensitive than culture to detect the infection (Boot et al., 1995).

Animal experiments were approved by the institute's Ethical Committee on Animal Experiments and were conducted in compliance with national legislation which is based on European Community directive 86/609/EEC.

## PCR

Extraction of DNA from samples and amplification of DNA were carried out as described (Boot et al., 2002). We used a primer set designed around 1990 on the basis of the DNA base sequence of the 16S rRNA gene of *S. moniliformis* comprising the forward primer 5'GCT TAA CAC ATG CAA ATC TAT 3' and the reverse primer 5'AGT AAG GGC CGT ATC TCA 3'. Length of the *S. moniliformis* amplicon is expected to be 296 bp.

Lysate of *S. moniliformis* control strain KUN-3 was included as a positive control and MQ as a negative control in all runs.

Identification of the source of the amplicons was attempted by *Bfa*1 restriction enzyme analysis (Boot et al., 2002).

Measures to prevent DNA contamination

Preparation of PCR mixtures, DNA extraction from tissue specimens, addition of template DNA, and detection and analysis of PCR products were done in separate rooms to prevent intercurrent contamination of reaction mixtures by *S. moniliformis* DNA.

## Sequencing

An amplicon was sequenced in both directions using the amplification primers as sequencing primers. The concensus (of both directions) DNA sequences was compared

with bacterial 16S rDNA sequences deposited in the NCBI database (Genbank) using BlastN 2.2.12 and AlignPlus 5.1 (SciEd software). Sequencing was performed on a Perkin Elmer 310 sequencer, with Big Dye Terminator Cycle Sequencing Ready reaction kit.

### Reevaluation of primer set

The primer set used for the detection of *S. moniliformis* (Boot et al., 2002) was reevaluated by comparison with bacterial 16S rDNA sequences deposited in Genbank.

## *Results*

Observations in guinea pigs

PCR testing of oral samples from four experimental guinea pigs yielded amplicons which by their size were suggestive of being produced from *S. moniliformis* but samples from the cervical lymph nodes and the lungs were negative. No gross abnormalities were found in the lungs, the larynx and the cervical lymphnodes.

Oral samples from the 24 guinea pigs from our institutional breeding colony were negative by PCR but an amplicon suggestive for *S. moniliformis* infection was obtained from all 12 samples from animals from the commercial breeding colony.

Amplicon identification

*Bfa*1 restriction enzyme analysis did not yield the expected fragments from *S. moniliformis* control strain KUN-3 and could therefore not be used for identification of the source of the amplicons. Sequencing of one amplicon yielded a sequence of 289 bp which by *in silico* evaluation showed significant homology with the 16S rDNA sequences of four uncultured bacterial clones partly deposited in Genbank as *Leptotrichia* sp (Table 1 and Fig. 1) and only limited homology with the 16S rDNA sequence of the *S. moniliformis* type strain. The remaining amplicons were not kept for sequencing.

Reevaluation of the primer set

Reevaluation of the *S. moniliformis* primer set revealed that in addition to *Sebaldella* (*Bacteroides*) *termitidis* and *Fusobacterium necrogenes* (Boot et al., 2002) unnamed human oesophageal bacteria and *Leptotrichia* species from the female reproductive tract (Shukla et al., 2002; Gundi et al., 2004; Hebb et al., 2004; Zhou et al., 2004), would lead to a positive *S. moniliformis* PCR result.

Differences in base sequences between *S. moniliformis* and these species (Fig. 2) suggest the possibility to develop one or two probes or primers that might be used in a *S. moniliformis* specific real time PCR and nested PCR respectivily. *Bfa*1 restriction enzyme treatment of the amplicons would yield similar sized fragments from *S. moniliformis* and its four closest relatives (Table 1 and Fig. 2).

### *Discussion*

Detection of *S. moniliformis* in laboratory animals by culture is notoriously difficult and in rats PCR has been found to be more sensitive than culture (Boot et al., 2002).
We recently obtained the expected 296 bp *S. moniliformis* amplicon from oral swab samples taken from seropositive pet rats kept by a human rat bite fever patient in which *S. moniliformis* infection was confirmed by culture (Van Nood and Peters, 2005).
The detection of similar sized amplicons in oral samples from guinea pigs from our experimental colony therefore seemed to confirm our serological observations.
The fact that the commercial breeding colony could be traced as the likely source of the infection of course raised concern given the consequences of the distribution of *S. moniliformis* infected animals to various research institutes.

When we developed our primer set we became aware that our *S. moniliformis* PCR would also generate a 296 bp amplicon from *Sebaldella* (*Bacteroides*) *termitidis* (from termite intestine so not likely occuring in laboratory animals) and from *Fusobacterium necrogenes* but then we were able to discriminate the amplicons through a different number of fragments obtained by *Bfa*1 restriction enzyme treatment (Boot et al., 2002).
As, for unknown reasons, we now failed to produce the three fragments to be expected from the *S. moniliformis* amplicon, we used amplicon sequencing for confirmation and found *Leptotrichia* sp or closely related bacterial species as the most likely source.
We believe that the new organism identified was present in all PCR positive guinea pigs. Unfortunately amplicons from other animals were not kept. Sequencing more amplicons would however not alter the obervation that our *S. moniliformis* PCR can yield a false positive outcome.
We informed the breeder about our findings as our primer set for the detection of *S. moniliformis* might be in use by other laboratory animal diagnostic laboratories which might then report streptobacillosis to the breeder.

Reevaluation of the *S. moniliformis* primer set with 16S rDNA sequence data accumulated in GenBank up to august 2006 indicated that apart from human *Leptotrichia* species also unnamed human oesophageal bacteria might lead to a false positive *S. moniliformis* PCR result. Human bacteria are easily transmitted to contemporary specified pathogen free laboratory rodents and rats have been found infected by *Leptotrichia* (McNamara et al., 1982) and *Fusobacterium* species (Isogai et al., 1985; Wang et al., 1996).

We might develop a new primer set for the specific detection of *S. moniliformis*. The number of bacterial species on which 16S rDNA sequence data is added to Genbank increases however exponentially and we presume that also a new primer set will soon become outdated. It seems more practical to sequence the amplicon to identify its source (*S. moniliformis* or another bacterial species). Sequencing of amplicons has been used in the diagnosis of human *S. moniliformis* infection (Berger et al., 2001; Wallet et al., 2003). A nested or real time PCR with a specific probe (Heid et al., 1996) would be an improvement of the assay used in this study. However since PCR oligonucleotide primers and probes comprise a limited number of base pairs only they provide less information than the sequence of the amplicon (Fig. 2).

### *Conclusion*

Our observations show that PCR testing for detection of *S. moniliformis* may yield false positive results. Therefore PCR results should be further evaluated by amplicon sequencing in order to prevent unnecessary eradication measures in laboratory animal colonies.

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240 Table 1. Homology of a *S. moniliformis* PCR amplicon obtained from guinea pig oral samples with 16S rDNA sequences of *Leptotrichia* sp (%)

242

| | Accession number | % homology with an amplicon obtained by *S. moniliformis* PCR |
|---|---|---|
| Uncultured bacterium clone-nE22-31 | DQ634071 | 98 |
| Uncultured *Leptotrichia* species oral clone-IS019B26 | AY806831 | 98 |
| Uncultured bacterium clone-nE12-14 | DQ633193 | 98 |
| Uncultured *Leptotrichia* species oral clone-FP036 | AF432138 | 98 |
| *Leptotrichia goodfellowii*-LB57$^T$ | AY029807 | 84 |
| *Leptotrichia buccalis*-ATCC 14201$^T$ | L37788 | 82 |
| *Streptobacillus moniliformis*-ATCC 14647$^T$ | Z35305 | 80 |

$^T$ = type strain

244

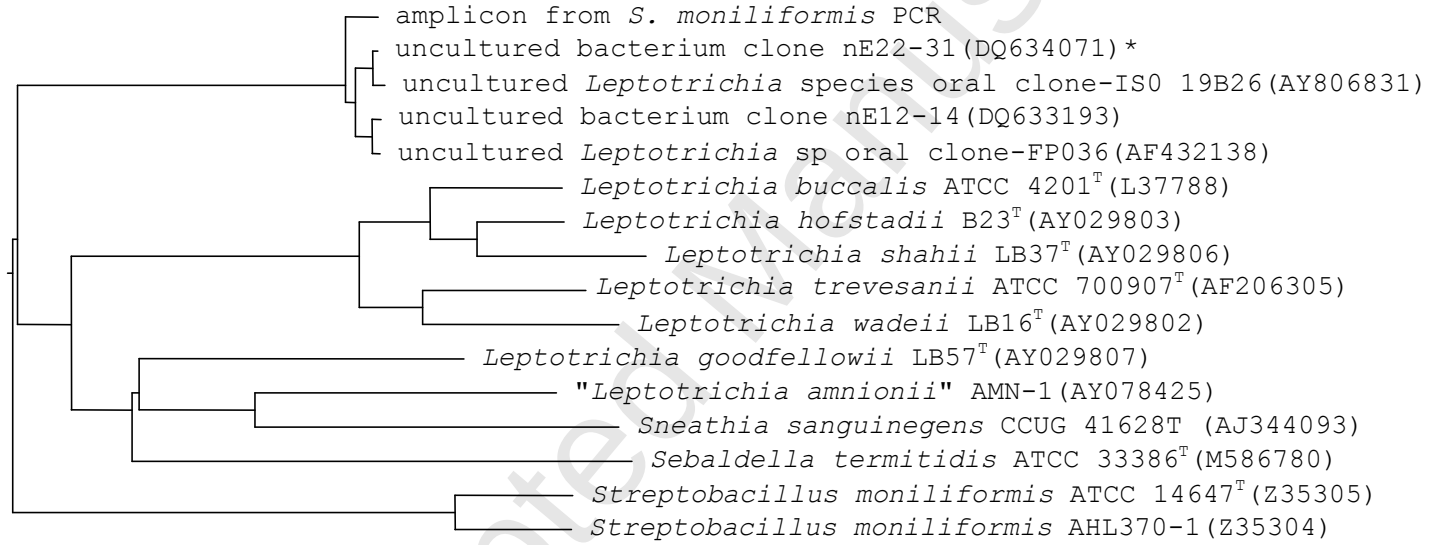


Fig. 1. the 16S rRNA gene sequence of an amplicon generated by *S. moniliformis* PCR is closely related to *Leptotrichia* species. The dendrogram was created with AlgnPlus5.0; multiway alignment using neighbor-joining phylogeny.

* (..) Genbank accession number.

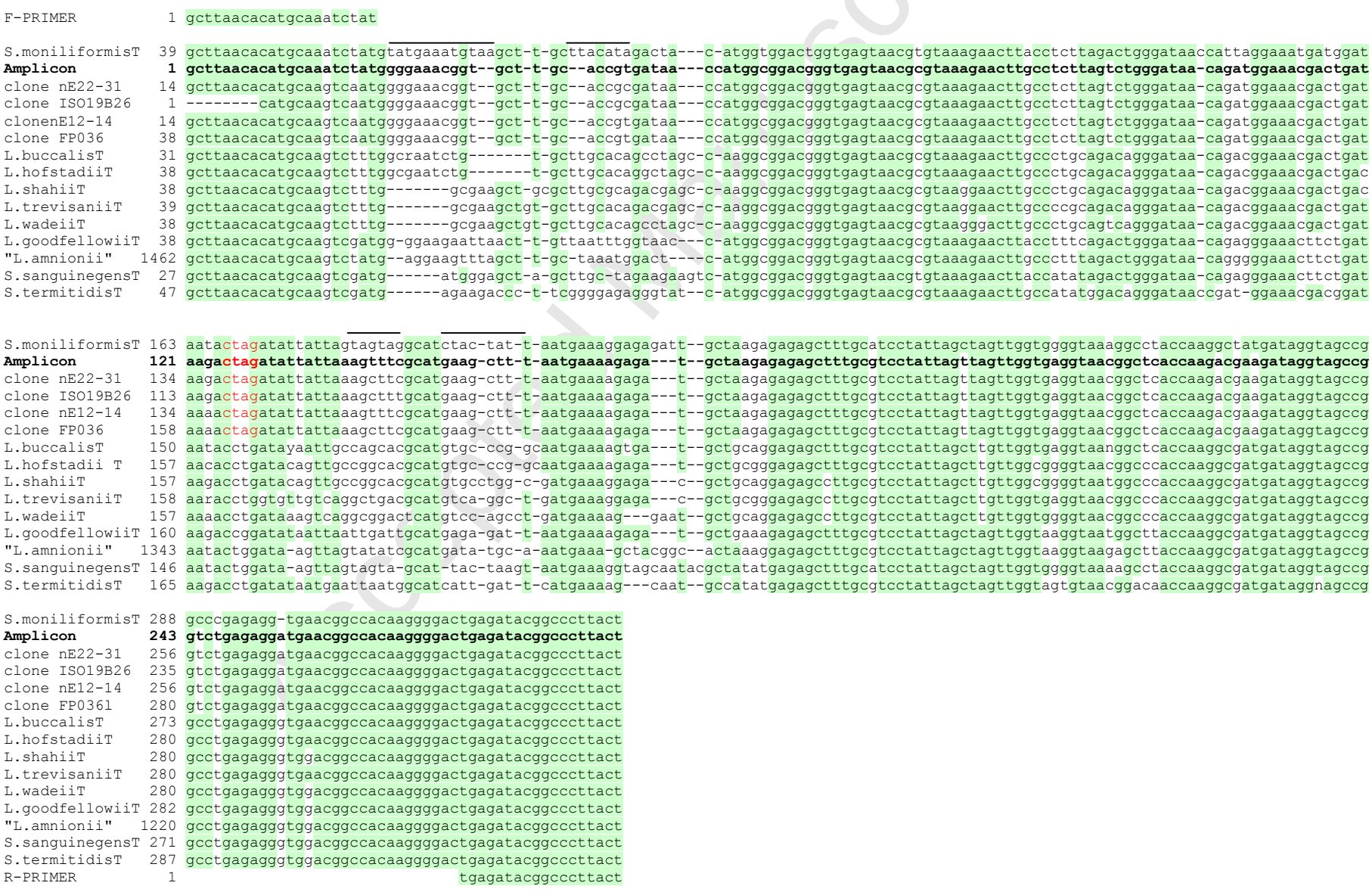

Fig. 2. Alignment of the 16S rDNA base sequences of *S. moniliformis*, its closest relatives and the amplicon obtained by *S. moniliformis* PCR. ____ : sequence sites specific for *S. moniliformis* and thus likely suitable for development of specific probe(s).; consensus sequences in green; Bfa1: restriction enzym sequence CTAG in red.