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Michael Hess, Claudia Neubauer

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Interlaboratory comparison of ability to detect nucleic acid of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by PCR

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Figure 1.

Standard curves derived from the amplification plot of tenfold dilutions of plasmids containing the 16S rRNA genes of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, tested in triplicate. Dots represent the C_T value for each dilution within a range of $\geq 99\%$ efficiency.

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M. Hess*, C. Neubauer & R. Hackl

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*Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna,
Veterinärplatz 1, 1210 Vienna, Austria*

Short title: Interlaboratory study on *Mycoplasma* PCR

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*To whom correspondence should be addressed:

Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna,
Veterinärplatz 1, 1210 Vienna, Austria, e-mail: michael.hess@vu-wien.ac.at

tel: +43-1250775150; fax: +43-250775192

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Interlaboratory comparison of ability to detect nucleic acid of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by PCR

M. Hess *, C. Neubauer & R. Hackl

Summary

In recent years polymerase chain reaction (PCR) assays have become widely used as methods to confirm the presence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in poultry flocks, but there has been limited standardization of the protocols used. Thirteen laboratories from 5 different countries participated in an interlaboratory comparison of detection of *M. gallisepticum* and *M. synoviae* DNA by PCR in samples that contained ten fold dilutions of these bacteria. The concentration of bacteria ranged from 10^5 to 10^2 genome copies / 100 μ l sample, as quantified by real time PCR, and the samples were supplied on dry cotton swabs. Each laboratory was asked to use its standard method for PCR testing of these pathogens. A questionnaire was supplied with the samples to obtain details of the methods that were used in testing. Half of the laboratories used a commercially available test kit, while the others used an in-house protocol. The protocols used for DNA extraction varied greatly, even among those using commercially available test kits. Two laboratories had developed the primers for nucleic acid amplification themselves, and one of these used real time PCR for amplification. While the majority of the laboratories detected *M. synoviae* down to the 100 copy limit of the comparison, the detection limit for *M. gallisepticum* was somewhat higher. Furthermore, different results were obtained from laboratories that used the same commercial test kit. To the best of our knowledge this is the first investigation of its kind in the field of avian diseases.

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Introduction

Infections in poultry with *Mycoplasma gallisepticum* or *Mycoplasma synoviae* are a major concern for the poultry industry (Kleven, 2003; Ley, 2003). Horizontal transmission occurs within a flock and the progeny of breeder birds can be infected by vertical transmission (Bradbury, 2005). Prevention of infection and precise diagnosis of breeder flocks is therefore an important element in control strategies.

Various diagnostic methods have been described for confirmation of the presence of a mycoplasma infection (Kleven, 1998). Serological monitoring systems for detection of antibody against *M. gallisepticum* and *M. synoviae* are widely used in routine diagnosis, even though there are problems with sensitivity and specificity (Levisohn & Kleven, 2000). Isolation of the organism is laborious and time-consuming and therefore far from a routine procedure (Zain & Bradbury, 1996). Furthermore, culture takes several days and sometimes is confounded by overgrowth by other bacteria or suppression of growth by antimicrobial treatments administered to the birds. As a consequence, PCR has been regarded as a valuable tool under practical diagnostic conditions and may offer similar, or even higher, sensitivity than isolation (Kempf *et al.*, 1993; Ewing *et al.*, 1996; Salisch *et al.* 1998; Mekkes & Feberwee, 2005). Consequently, nucleic acid detection methods are preferred as an adjunct to serological investigations (Kempf, 1997; Feberwee *et al.*, 2005). In general, PCR tests for diagnostic purposes in the poultry industry are being increasingly used because of the limitations of conventional methods.

Most veterinarians, producers and even laboratory scientists have little experience using molecular tests, especially in the diagnostic laboratory, which is very different from the research environment (Apfalter *et al.*, 2005), but PCR tests to detect *Mycoplasma* species are on the list of assays of nearly every laboratory working in the field of avian diseases. In recent

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years several different commercial test kits have been introduced to supply the market with appropriate tools for routine testing.

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This investigation was set up to compare PCR assays of different laboratories for detection of *M. gallisepticum* and *M. synoviae*. Protocols that were used in the individual laboratories for DNA extraction and PCR were compared, as were the results obtained when testing identical samples in the different laboratories. In order to supply identical samples to all participating laboratories a DNA standard was developed to determine the number of copies of DNA extracted from cultured bacteria.

Material and methods

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Participating laboratories¶

Participating laboratories. Thirteen laboratories from 5 different countries participated. Eleven of these laboratories used a single method to process the swabs - either a commercially available kit or an in-house system. After extraction of DNA, two laboratories used two completely different methods in parallel to process the samples. One of these laboratories obtained identical results for both methods, whereas the other one did not. As a consequence the two differing results obtained by the second laboratory were regarded as coming from two different laboratories (numbers 4 and 5). Consequently, 14 different results are listed in Tables 1 and 2.

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Mycoplasma gallisepticum and
Mycoplasma synoviae strains¶

***Mycoplasma gallisepticum* and *Mycoplasma synoviae* strains.** The reference strains *M. gallisepticum* ATCC 19610 and *M. synoviae* ATCC 25204 obtained from the American Type Culture Collection (ATCC). Each reference strain was suspended in 2 ml Mycoplasma liquid medium (Mycoplasma Experience, Reigate, UK). Following inoculation, the broth cultures

were incubated at 37°C, and were examined daily for acidity, as indicated by a colour change from red to orange or yellow.

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Calculation of rRNA gene copy numbers¶

Calculation of rRNA gene copy numbers. DNA was extracted from 200 µl of cultures of each reference strain using Chelex[®] 100 Resin (BioRad, Munich, Germany) according to the manufacturer's instructions. For amplification of the 16S rRNA genes from extracted DNA from *M. gallisepticum* the forward primer was MG14F (5' GAGCTAATCTGTAAAGTTGGTC 3') and the reverse primer was MG13R (5' GCTTCCTTGC GGTTAGCAAC 3') (Lauerman, 1998), while the rRNA genes of *M. synoviae* were amplified using the forward primer MS-1 (5' GAAGCAAAAATAGTGATATCA 3') and the reverse primer MS-2 (5' GTCGTCTCCGAAGTTAACAA 3') (Lauerman *et al.*, 1993).

Following amplification the PCR fragments were cloned using the TA Topocloning[®] Kit for Sequencing (Invitrogen, Lofer, Austria) according to the manufacturer's instructions. Plasmid concentrations were measured by spectrophotometry (Smartspec, BioRad, Munich, Germany) and number of copies calculated as described by Whelan *et al.* (2003).

Real time PCRs were performed with the Brilliant[®] Sybr[®] Green QPCR Master Mix (Stratagene, Amsterdam, The Netherlands) in 25 µl of PCR mixture containing a primer concentration of 1 pmol. For both species the PCRs were performed in a Stratagene MX 3000 with an initial denaturation step of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min. Standard curves were created using a 10 fold dilution series of each plasmid, with 2 µl of each dilution used in the real time PCRs.

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Preparation of samples¶

Preparation of samples. The number of copies of the rRNA genes in the cultures of each of the mycoplasma strains were quantified and each culture was diluted to contain concentrations of 10⁵ to 10² copies of rRNA gene / 100 µl. The swabs of the four dilutions of

M. gallisepticum ATCC 19610 were assigned sample numbers 1, 8, 12 and 4, respectively, and the swabs of the four dilutions of *M. synoviae* ATCC 25204 were assigned the numbers 11, 13, 6 and 3, respectively. In addition, a series of 4 swabs (samples 5, 9, 10 and 7) were supplied that contained a 50 µl volume of the dilutions of both reference strains. The total volume of 100 µl of each of the dilutions of *M. gallisepticum* ATCC 19610 or *M. synoviae* ATCC 25204, as well as the mixtures of both (2 x 50 µl) were absorbed into sterile cotton swabs (Sarstedt, Nürmbrecht, Germany), which then dried at room temperature. One negative swab (sample 2) that contained 100 µl of the broth media was also included. Each swab was supplied as an individual sample in a separate tube.

Results

Quantification of samples by real time PCR. Standard curves derived from the 10-fold dilution series of *M. gallisepticum* ATCC 19610 and *M. synoviae* ATCC 25204 are shown in Figure 1. Each reaction was performed in triplicate and the manufacturer's software was used to calculate the threshold cycle. Only reactions with an efficiency close to 100% were included in the calculation.

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Quantification of samples by real time PCR ¶

DNA extraction protocols. A summary of the extraction protocols used by the laboratories is given in Table 1. There was considerable variation between the buffers used in the different protocols. Only 6 laboratories (1, 2, 7, 8, 12 and 13) processed all the initial sample for DNA extraction, the others processing only a portion. For example, laboratory 14 used only 2.5% of the material retrieved from the swabs. The extracted DNA was resuspended in different volumes of buffer, ranging from 25 µl to 250 µl. With the exception of laboratory 10, which

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¶ DNA extraction protocols ¶

used 15 µl of the extracted material, all other laboratories used 2 – 5 µl as template DNA in the PCR assay (Table 1).

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PCR variables¶

PCR variables. Half of the laboratories (1-7) used a commercial kit while the other 7 labs used an in-house system (Table 1). Laboratories 1 and 2 used the same commercial test kit. A different commercial kit was used by laboratories 3 and 4 and a third detection kit was used by three laboratories (5, 6 and 7). There were some variations in the protocol between laboratories using the same commercial test kit.

PCRs were performed in volumes of between 20 and 50 µl. With the exception of laboratory 12, all used primers that hybridized within the 16S rRNA genes of *M. gallisepticum* or *M. synoviae*. Information on the target sequence was not supplied for the commercial kits as this information is not publicly available for some of the kits. In all laboratories the detection of *M. gallisepticum* and *M. synoviae* was done by separate PCR tests. Laboratory 14 used real time PCR for detection. Some diversity was noticed with regard to the volume taken from the PCRs to confirm successful amplification of the nucleic acid. The processed volume ranged from 6 to 100%. Half of the laboratories used at least 40 - 50% of the volume of the PCR to perform the final step of the protocol, the detection of amplified PCR products.

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PCR results¶

PCR Results. The results of the PCR testing are given in Table 2. All laboratories supplied the correct result for the negative control (sample 2). A false positive result was only reported once. Laboratory 12 detected both *M. gallisepticum* and *M. synoviae* in sample 1, which contained only 10⁵ copy numbers of the *M. gallisepticum* rRNA gene. Laboratory 8 provided a graded result for positive samples, ranging from +++ to +, which was in accordance with the concentrations on the swabs. Some laboratories (2, 3, 6 and 14) described samples that were closest to the detection limit as “weak positive”.

Nearly all of the PCR results corresponded with the declining concentrations of target organisms supplied on the swabs. The exception was laboratory 4, which reported a negative result for sample 6, which contained 10^3 copies of the *M. synoviae* rRNA gene, but a positive result for sample 3, which contained fewer bacteria. This also occurred at laboratory 10 with the samples containing both *M. gallisepticum* and *M. synoviae*. Sample 9, with 10^4 copies, was reported as negative, while samples containing fewer bacteria were found to be positive.

The majority of laboratories detected the lowest concentrations of *M. synoviae* supplied (10^2 rRNA gene copies), whereas the detection limit for *M. gallisepticum* was no lower than 10^3 copies. Three laboratories (5, 7 and 9) detected *M. gallisepticum* only in samples with the highest concentrations supplied (10^5 copies).

Discussion

This investigation was set up to compare the results and methods of several laboratories doing PCR tests to diagnose the presence of *M. gallisepticum* and *M. synoviae*. Dried swabs were supplied to standardise the samples, even though wet swabs are sometimes used in field diagnostic investigations in order to allow isolation by culture (Zain & Bradbury, 1996).

A questionnaire enabled identification of variations in the DNA extraction methods. At two different stages of the procedure the dilution factor was calculated in order to investigate the possible influence of this on results. However the extraction procedure had very little influence on the outcome of the PCR. Laboratories 12 and 13, and laboratories 3 and 4, obtained different results and had different detection limits even though they transferred identical quantities of the samples through the steps of the extraction procedure. The extraction procedure also had little influence on the results obtained by the laboratory that analysed the samples by real time PCR and achieved very high analytical sensitivities, even

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though a relatively small proportion of the extracted DNA was used in the assay. This might be expected given the high sensitivity reported for the detection of *M. gallisepticum* by real time PCR (Carli & Eyigor, 2003) and confirmed by comparing this PCR technique with isolation (Mekkes & Feberwee, 2005).

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All of the laboratories that commented about the intensity of the PCR signal obtained detected the weakest signal from the samples with the lowest concentrations of bacteria, whether the swabs contained one or both species. This confirmed the validity of the test and the samples that were supplied, despite the differing detection limits of the assays performed by different laboratories. As every laboratory processed each sample for *M. gallisepticum* and *M. synoviae*, a total of 364 (14 x 26) PCRs were performed by the laboratories and only one false positive result (laboratory 12) was obtained. One possible reason for this may be that the primers used by this laboratory may have been cross-reactive when high concentrations of DNA are present in the samples, as none of the other laboratories used the same primers to detect *M. gallisepticum*. To some extent this demonstrates the high standard of the participating laboratories, keeping in mind that processing a large number of samples at the same time increases the risk of cross-contamination of samples, potentially creating false positives (Kwok & Higuchi, 1989). This has been reported in a previous ring test in which field samples were used for investigation (Hess *et al.*, 2004).

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Only two laboratories obtained false negative results. However, to address this issue more fully a second testing method would need to be included, preferably isolation by culture, but this would not reflect the usual diagnostic situation. The influence of the medium on the results could be excluded as all samples were set up in an identical way using the same volume of medium. Two of the PCR kits used include an internal positive control, which is co-amplified in every sample and demonstrates the absence of inhibitors of the PCR. The PCR products obtained from the internal positive control can be easily distinguished from those obtained from the target sequences as they have different amplicon lengths. Therefore,

false negative results due to inhibition of the PCR can be easily identified when using PCR in diagnostic investigations (Moalic *et al.*, 1998; Carli & Eyigor, 2003). However the presence of an internal positive control may interfere with amplification of the target DNA, resulting in false negative results (Sachadyn & Kur, 1998). In our investigation there was no evidence of an influence of internal positive controls on the likelihood of obtaining false negative results. Furthermore, the presence of both *M. gallisepticum* and *M. synoviae* in a sample had no influence on the likelihood of obtaining false negative results. In fact some laboratories achieved a tenfold higher sensitivity with mixed samples than with those containing a single species. This is difficult to explain, but it was noticed in the majority of laboratories. In swabs that contained both bacteria the samples used to inoculate the swabs were concentrated twofold in order to ensure that the volume of medium on the swab (100 µl) was identical to the volume on swabs containing only single species and the negative control.

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The preparation of reference samples was based upon the determination of the number of copies of bacterial DNA using a standard curve established with a purified plasmid in which the gene for the 16S rRNA had been cloned (Whelan *et al.*, 2003). This was considered more appropriate than enumeration of bacteria by culture for a comparison of PCR tests, as PCR is able to detect both viable and non-viable, or non-culturable, organisms. In addition, damaged and degraded DNA may serve as a template for PCR. This explains the numerous reports in which positive PCR results could not be confirmed by culture, as demonstrated recently for environmental samples and samples taken from experimentally infected birds (Marois *et al.*, 2002). In our study none of the laboratories detected 100 copies of the *M. gallisepticum* rRNA gene. The decreased sensitivity of detection of *M. gallisepticum* could be a limitation of certain PCR protocols under practical conditions. In this context it needs to be considered that the calculation of the numbers of copies was performed with primers that hybridize to the 16S rRNA gene, and that there are two copies of this gene on the *M. gallisepticum* and *M. synoviae* genomes (Papazisi *et al.* 2003; Vasconcelos *et al.*, 2005). This

indicates that the detection limit is actually half that given in Table 2. However, Garcia *et al.* (2005) found no differences in sensitivity between PCRs that targeted a single copy gene (*mgc2*) and those that targeted the 16S rRNA gene.

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Test specificity was only assessed by examining the potential for cross-recognition of *M. gallisepticum* and *M. synoviae* and other influences on specificity should be further examined in future investigations.

Most PCR diagnostic assays for mycoplasmas rely on detection of highly conserved regions of the 16S rRNA and the low levels of genetic variation in this region reduce the likelihood that some strains will not be detected (Weisburg *et al.*, 1989). In our investigation only one of the laboratories using an in-house system used oligonucleotides that did not hybridize with the 16S rRNA gene. It may well be that the relatively low sensitivity of the assays performed by this laboratory is compensated for by a somewhat increased specificity. Different levels of sensitivity between PCRs targeting 4 different genes of *M. gallisepticum* have been reported recently (Garcia *et al.*, 2005). In this investigation the *M. gallisepticum* 16S rRNA gene PCR developed by Lauerman (1998) had a higher analytical sensitivity than the other PCR methods tested. However, the 16S rRNA PCR method amplified DNA not only from *M. gallisepticum* but also from *M. imitans*. These two phylogenetically related avian mycoplasmas have very similar 16S rRNA genes, and PCR primers that are targeted to this gene amplify both species (Boyle, 1993; Harasawa *et al.*, 2004). *M. imitans* has been isolated from ducks, geese and partridges and therefore is considered of limited significance in diagnoses on samples from chickens (Abdul-Wahab *et al.*, 1996).

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Laboratory 7 achieved a much greater sensitivity of detection of *M. synoviae* than the other two laboratories (5 and 6) that used the same commercial kit. Comparison of the different test systems themselves is not appropriate for several reasons. For example, one test is only licensed for research purposes and one of the test kits used recommends the transport of the swabs in specific transport medium. This limits the comparison, as samples were not

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handled and the tests not used as the manufacturers recommend. However, the present investigation better reflects the situations where swabs are collected for PCR testing on several pathogens at the same time. In addition, the number of laboratories using the same test kit was too low to enable a full comparison. If more laboratories participate in future investigations a scoring system could be established to enable the comparison of the outcomes of individual tests.

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However, as more test kits become available on the market and are used by an increasing number of laboratories, the tendency will be to improve these test systems due to the increasing competition. This will further increase the standardisation and quality of the PCR diagnosis of mycoplasma infections in poultry. In this context interlaboratory investigations are of some benefit for all the partners involved in order to establish a quality control system, not only for in-house protocols, which are usually less well evaluated and therefore more prone to error under uncontrolled conditions (Apfalter *et al.*, 2005).

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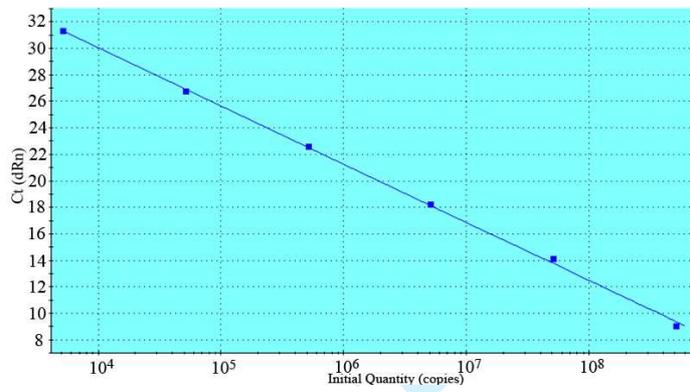
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Figure 1:

a)
Mycoplasma gallisepticum



b)
Mycoplasma synoviae

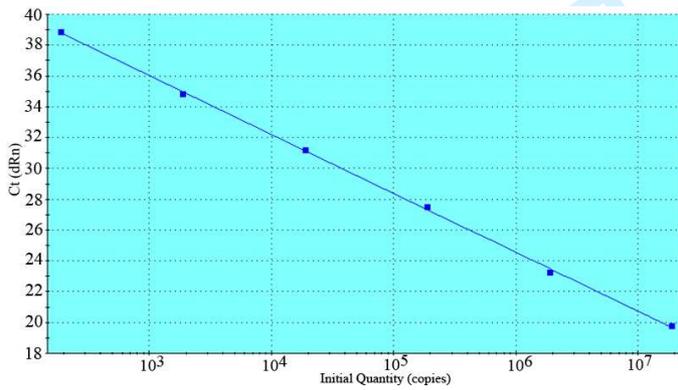


Table 1: Methods used for DNA extraction and PCR according to the information supplied by each laboratory 1-14.

Procedure	Laboratory													
	1 ^a	2 ^a	3 ^b	4 ^b	5 ^c	6 ^c	7 ^c	8	9	10	11	12	13	14
Buffer/fluid for DNA extr.	205 ^d	1200	1500	1500	1500	970	600	2000	500	500	300	180	200	1000
Volume processed	205	1200	1000	1000	150	200	600	2000	200	200	25	180	200	25
% ^e	<i>100</i>	<i>100</i>	<i>66.6</i>	<i>66.6</i>	<i>10</i>	<i>20.6</i>	<i>100</i>	<i>100</i>	<i>40</i>	<i>40</i>	<i>8.3</i>	<i>100</i>	<i>100</i>	<i>2.5</i>
Volume after extraction (µl)	100	150	25	25	100	100	100	50	200	100	25	250	200	25
Volume used in PCR (µl)	2.5	2.5	3	3	2	2	2	2	2	15	4.5	5	3	2
Proportion of sample in PCR (%)	<i>2.5</i>	<i>1.6</i>	<i>12</i>	<i>12</i>	<i>2</i>	<i>2</i>	<i>2</i>	<i>4</i>	<i>1</i>	<i>15</i>	<i>18</i>	<i>1.5</i>	<i>1.5</i>	<i>8</i>
Volume of PCR reaction (µl)	25	25	50	50	50	50	25	20	25	25	44.5	50	25	20
Primer	No detailed information supplied on instruction sheets or available in published literature							Publish. ^f	Publish.	MG: self designed Ms: publish.	Publish.	Publish.	Publish.	MG / MS: Self designed
Target gene								16S rRNA	16S rRNA	16S rRNA	16S rRNA	MG: pvpA MS: 16S rRNA	16S rRNA	16S rRNA
Volume used for detection (µl)	10	12	3	3	10	10	10	8	10	10	20	8	25	N.a.
Proportion of sample tested (%)	<i>40</i>	<i>48</i>	<i>6</i>	<i>6</i>	<i>20</i>	<i>20</i>	<i>40</i>	<i>40</i>	<i>40</i>	<i>40</i>	<i>45</i>	<i>16</i>	<i>100</i>	<i>100</i>

^{a, b, c} laboratories with identical letters used the same commercial kit for PCR

^f PCR primers were in a published protocol

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Table 2. Results from the different laboratories

Sample no.	MG conc.	MS conc.	Laboratory																											
			1 ^a		2 ^a		3 ^b		4 ^b		5 ^c		6 ^c		7 ^c		8		9		10		11		12		13		14	
			MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS	MG	MS
1	10 ^{5d}	0	+	—	Pos	—	pos	—	+	—	+	—	pos	—	+	—	+	—	+	—	pos	—	+	—	pos	pos	+	—	pos	—
8	10 ⁴	0	+	—	Pos	—	pos	—	+	—	—	—	—	—	—	—	+	—	—	—	pos	—	+	—	—	—	+	—	pos	—
12	10 ³	0	—	—	wp	—	wp	—	—	—	—	—	—	—	—	—	—	—	—	—	pos	—	+	—	—	—	—	—	pos	—
4	10 ²	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	10 ⁵	10 ⁵	+	+	Pos	pos	pos	pos	+	+	+	+	pos	pos	+	+	++	+++	+	+	pos	pos	+	+	pos	pos	+	+	pos	pos
9	10 ⁴	10 ⁴	+	+	pos	pos	pos	pos	+	+	+	+	pos	pos	+	+	+	++	+	+	—	—	+	+	pos	pos	+	+	pos	pos
10	10 ³	10 ³	+	+	wp	pos	pos	pos	—	+	—	—	wp	pos	—	+	+	+	—	+	pos	pos	+	+	—	pos	+	—	pos	pos
7	10 ²	10 ²	—	+	—	—	—	wp	—	—	—	—	—	—	—	+	—	+	—	+	—	pos	—	+	—	pos	—	—	—	wp
11	0	10 ⁵	—	+	—	pos	—	pos	—	+	—	+	—	pos	—	+	—	++	—	+	—	pos	—	+	—	pos	—	+	—	pos
13	0	10 ⁴	—	+	—	pos	—	pos	—	+	—	+	—	pos	—	+	—	++	—	+	—	pos	—	+	—	pos	—	+	—	pos
6	0	10 ³	—	+	—	pos	—	pos	—	—	—	—	—	—	—	+	—	++	—	+	—	pos	—	+	—	pos	—	—	—	pos
3	0	10 ²	—	—	—	wp	—	wp	—	+	—	—	—	—	—	+	—	+	—	—	—	—	—	+	—	pos	—	—	—	wp
2	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

^{a, b, c} identical letters indicate laboratories that used the same commercial kit

^d the number of copies of DNA were determined by real time PCR, without allowance for the presence of two copies of the 16S rRNA gene per genome

pos = positive

wp = weak positive