From the Institute of Parasitology and Zoology[1] and the Institute of Bacteriology, Mycology and Hygiene[2] (Department of Pathobiology), the Institute of Clinical Virology[3] (Clinical Department of Diagnostic Imaging, Infectious Diseases and Clinical Pathology) and the Institute of Medical Physics[4] (Department of Natural Sciences), University of Veterinary Medicine, Vienna

Detection of *Mycoplasma (Eperythrozoon) suis* by real-time PCR

B. GATTINGER[1], J. SPERGSER[2], W. WILLE-PIAZZAI[1], J. KOLODZIEJEK[3], A.G. TICHY[4] and A. JOACHIM[1]

received May 27, 2007
accepted for publication December 5, 2007

Keywords: *Mycoplasma suis, Eperythrozoon suis*, real-time PCR, quantitative PCR, minor groove binder probe, diagnosis, blood, spleen, pig.

Summary

Mycoplasma (syn. *Eperythrozoon*) *suis* is a haemotrophic facultative pathogen of swine which can cause acute or chronic infections. Its accurate diagnosis is often hampered by the lack of sensitive and specific methods, therefore a real-time PCR based assay was developed using PCR amplification of a 101 bp fragment and a minor groove binder probe which could detect <2 copies of the 16S ribosomal RNA gene of *M. suis*. In comparison to a previously developed nested PCR technique the real-time was more specific and more sensitive. It detected several strains of *M. suis* but no other *Mycoplasma* species of pigs. Probe-based PCR was more specific than SYBR® Green using only the primers (without the probe) and detected *M. suis* in 76 of 372 blood samples and 18 of 224 spleen samples. Samples positive with nested PCR or SYBR® Green but negative with the probe (n=4) turned out to be false positive results. Field samples could be quantified using a standard curve of a cloned PCR amplicon. Real-time PCR can be used to quantitatively detect *M. suis* DNA in blood and spleen samples from infected pigs, supporting the diagnosis of eperythrozoonosis in the field.

Abbreviations: BLAST = basic local alignment search tool; Ct = threshold cycle; dRn = baseline-subtracted fluorescence reading normalized to the reference dye; dRnLast = final dRn minus first dRn; MGB = minor groove binder; PCR = polymerase chain reaction; qPCR = quantitative PCR; Rn = the fluorescence reading normalized to the reference dye in a real-time experiment; RSq = fit to all data to the standard curve of the fluorescence reading; tRNA = transfer RNA

Schlüsselwörter: *Mycoplasma suis, Eperythrozoon suis*, real-time PCR, quantitative PCR, minor-groove-binder-Sonde, Diagnose, Blut, Milz, Schwein.

Zusammenfassung

Nachweis von *Mycoplasma (Eperythrozoon) suis* mittels real-time PCR

Mycoplasma (syn. *Eperythrozoon*) *suis* ist ein haemotropher fakultativer Krankheitserreger beim Schwein, der akute oder chronische Infektionen verursachen kann. Die genaue Diagnose wird häufig dadurch erschwert, dass kaum spezifische und sensitive Methoden zur Verfügung stehen. Daher wurde eine real-time PCR entwickelt, bei der mittels Amplifikation eines 101-bp-Fragments und einer Minor-groove-binder-Sonde schon geringe Mengen (< 2 Kopien) des 16S-ribosomalen RNA-Gens von *M. suis* detektiert werden konnten. Im Vergleich zur bereits entwickelten nested-PCR-Technik ist die real-time PCR sensitiver und spezifischer. Sie detektierte verschiedene Stämme von *M. suis,* aber keine anderen Mykoplasmenarten vom Schwein. Die PCR mit der Sonde war spezifischer als die SYBR®-Green-PCR, bei der nur die PCR-Primer (ohne Sonde) verwendet wurden, und detektierte *M. suis* in 76 von 372 Blutproben und 18 von 224 Milzproben. Proben, die in der nested-PCR oder in der SYBR®-Green-PCR positiv und in der Sonden-PCR negativ waren (n=4) stellten sich als falsch positiv heraus. In Feldproben war eine Quantifizierung anhand einer Standardkurve mit einem klonierten PCR-Fragment möglich. Die real-time PCR kann eingesetzt werden, um *M. suis* DNA in Blut- und Milzproben infizierter Schweine quantitativ zu detektieren, und ergänzt somit die Diagnose der Eperythrozoonose im Feld.

Introduction

Infections with *Mycoplasma* (syn. *Eperythrozoon*) *suis* were first described by KINSLEY (1932) as a "protozoan-like body in blood of swine", and by DOYLE (1932) as "rickettsia-like or anaplasmosis-like disease in swine". SPLITTER and WILLIAMSON (1950) named the causative agent of porcine icteroanaemia *Eperythrozoon* (*E.*) *suis*. Analysis of the 16S rRNA gene sequences placed *Eperythrozoon* close to *Mycoplasma* (RIKIHSA et al., 1997; MESSICK et al., 1999) and now *E. suis* is commonly called *Mycoplasma* (*M.*) *suis* (NEIMARK et al., 2001; HÖLZLE, 2007), although this nomenclature is not generally accepted (UILENBERG et al., 2004).

Acute eperythrozoonosis, characterised by anaemia, jaundice, pyrexia, lethargy, respiratory disorders and occasional cyanosis of the ears, tail and distal limbs (HEINRITZI, 1983) or reproductive disorders in infected sows (HENRY, 1979; SCHWEIGHARDT et al., 1986), is rare. The most common form is the latent infection which HEINRITZI (1989) describes as „multi-factorial disease". Transmission occurs orally (by ingestion of blood) or through

various vectors (e.g. haematophageous arthropods like lice or iatrogenic via injections etc.), but is unlikely through mating, placentophagy or in utero (HEINRITZI, 1992).

Since *M. suis* is regarded as unculturable (HÖLZLE, 2007; HÖLZLE et al., 2007a), accurate and sensitive tests applicable in the field must rely on the direct or indirect detection of infection in vivo. Bacteria can be found in blood smears stained with Giemsa or acridine orange (HEINRITZI, 1990) during the acute stage of infection with high levels of bacteriaemia (which can be provoked by splenectomy; HEINRITZI, 1984). Anti-*M. suis* antibodies can be detected by complement fixation reaction (SCHULLER et al., 1990), indirect haemagglutination assay (SMITH and RAHN, 1975) or enzyme-linked immunosorbent assay (SCHULLER et al., 1990). The first assay for the detection of *M. suis* DNA was based on a hybridisation probe (OBERST et al., 1990a, b) and GWALTNEY and coworkers described the first PCR test for *M. suis* (GWALTNEY et al., 1993).

Previous studies on the qualitative detection and prevalence of *M. suis* in blood and spleen samples from naturally infected pigs have employed a sensitive nested PCR (STRIMITZER et al., 2004); however, this technique is time-consuming and prone to contamination, therefore we attempted to evaluate and establish an assay based on real-time PCR technology for qualitative and quantitative evaluation for the accurate diagnosis of field samples.

Material and methods

Sources of DNA

Blood (n=372) and spleen (n=224) samples from a routine screening program for Austrian pig farms were used irrespective of age, management or health status of the animals. All samples had previously been tested for *M. suis* by nested PCR (STRIMITZER et al., 2004) and the respective DNA preparations (stored at -20 °C) were used.

Real-time PCR

Real-time assays (both based on SYBR® Green and fluorescent probes) were performed in a 96-well format (25 µl) in the MX 3000 P® (Stratagene, La Jolla, CA, USA) in duplicates for each sample. In each run known positive samples and no-template-controls were included. Pig-specific PCR was used as an amplification control for negative samples in separate runs, using primers P19 (5'-CGG CCA TGC ACC ACC-3'; SOGIN and GUNDERSON, 1986) and S2 (5'-ATG CGG CGG CGT TAT TCC CA-3'; RUTTKOWSKI, 2006) with a product length of 239 bp.

For optimum results primers and probe were titrated for the respective SYBR® Green or probe-based PCR in order to obtain low threshold cycle values (Ct) and high dRnLast values *(*dRNLast= the normalized final fluorescence reading minus the normalized first fluorescence reading in a real-time experiment).

For the SYBR® Green-based assays the Brilliant® SYBR®Green QPCR Master Mix and for the probe-based assays the Brilliant®QPCR Core Reagent Kit (both from Stratagene, La Jolla, CA, USA) were used.

PCR profiles were as follows:

- *M. suis* PCR (both SYBR®-Green and probe-based): initial heating step: 95 °C 10 min, followed by 40 cycles: 95 °C 30 s, 65 °C 1 min, 72 °C 1 min.
- pig-specific PCR: initial heating step 95 °C 10 min, followed by 28 cycles: 95 °C 30 s, 55 °C 1 min, 72 °C 1 min.

SYBR® Green profiles were finished by the dissociation determination (95 °C 1 min, 58 °C 30 s, 95 °C 30 s).

Primer and probe design

Based on the 16S ribosomal DNA sequence of the *M. suis* strains available in Genbank ("Illinois", U88565; "Guangdong", AY492086 and "Zachary", AF029394) primers were designed using the Program Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). M.s.713_for (5'-AAC ACC AGA GGC TAA GGC GA-3') and M.s.713_rev (5'-TTA CGG CGT GGA CTA CTG GG-3') yield a product size of 101 bp (annealing temp.: 65 °C). Additionally, the minor groove binder (MGB) probe 713 (5'-FAM-TAA TTG ACG CTG AGG CTT-3' MGB; annealing temp.: 65 °C; Applied Biosystems, Warrington, UK) was designed with the Beacon Designer 2.1 software program (Premier Biosoft International, Palo Alto, CA, USA).

The primers were evaluated in a SYBR® Green assay with a cohort of 3 *M. suis* nested PCR positive samples using 25-100 pmol of each primer using the Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA, USA).

Sequence determination

Selected samples (mainly those positive in conventional PCR but negative in the probe-based real-time PCR) were PCR-amplified using primers M.s.287_for (5'-TAG CTG GAC TGA GAG GTT GA-3') and M.s.1287_rev (5'-GTT TGA CGG GCG GTG TGT AC-3) for a product of 1,100 bp (conventional PCR with 36 cycles, annealing temperature: 63 °C) and subjected to sequencing employing the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the appropriate oligonucleotides in a final concentration of 0.2 µM. Reaction products were sequenced in both directions by the ABI Prism 310 genetic analyzer (Perkin Elmer, Waltham, MA, USA) automated sequencing system. The compiled nucleotide sequences were submitted to BLAST (Basic Local Aligment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/) for comparison with all corresponding sequences deposited in gene bank databases.

Other *Mycoplasma* species from swine

Mycoplasma type strains were purchased from the National Collection of Type Cultures (NCTC; Colindale, UK) (*M. flocculare* Ms42^T NCTC 10143, *M. hyorhinis* BTS-7/PG42^T NCTC 10130, *M. hyopneumoniae* J^T NCTC 10110, *M. hyosynoviae* S16^T NCTC 10167) or the American Type Culture Collection - ATCC (LGC Promochem, Wesel, Germany) (*M. hyopharyngis* H3-6B-F^T ATCC 51909). Standard cultures were used to test the specificity of the PCR assays.

Production of a PCR-standard for quantitative real-time PCR

To obtain a standard for quantitation of the PCR, a product obtained from primers M.s.287_for and M.s.1287_rev (sequencing primers) was cloned into a plasmid vector (TOPO TA Cloning® Kit for Sequencing; Invitrogen, La Jolla, CA, USA) and amplified in *E. coli*. Clones were grown in liquid medium and plasmid DNA was prepared (E.Z.N.A.® Plasmid

Tab. 1: Comparison of PCR results with 3 different methods: nested PCR, SYBR® Green real-time PCR and MGB (minor groove binder) probe-based PCR; n=372 blood samples and 224 spleen samples from pigs were compared for nested PCR vs. SYBR® Green, 104 and 123 samples were compared for nested PCR vs. probe-based PCR, and 113 and 123 samples were included in the comparison of SYBR® Green with probe-based PCR.

	Nested + (%)	Nested - (%)	SYBR® Green + (%)	SYBR® Green - (%)
Blood samples				
SYBR® Green +	57 (15.3)	33 (8.9)		
SYBR® Green -	44 (11.8)	238 (64.0)		
Probe +	45 (43.3)	31 (29.8)	75 (66.4)	8 (7.1)
Probe -	19 (18.3)	9 (8.7)	19 (16.8)	11 (9.7)
Spleen samples				
SYBR® Green +	11 (4.9)	0 (0)		
SYBR® Green -	117 (52.2)	96 (42.9)		
Probe +	18 (14.6)	0 (0)	11 (8.9)	7 (5.7)
Probe -	105 (85.4)	0 (0)	0 (0)	105 (85.4)
Total				
SYBR® Green +	68 (11.4)	33 (5.5)		
SYBR® Green -	161 (27.0)	334 (56.0)		
Probe +	63 (27.8)	31 (13.7)	86 (36.4)	15 (6.4)
Probe -	124 (54.6)	9 (3.9)	19 (8.1)	116 (49.1)

Tab 2: Comparison of different plasmid templates for quantitative PCR

	circular + nuclease-free distilled water	circular + tRNA (5ng/ml)	linearised + tRNA (5ng/ml)
no. of runs	8	5	3
RSq (average)	0.901-0.998 (0.963)	0.992-0.998 (0.994)	0.991-0.999 (0.995)
% efficacy (average)	91-113.3 (105.3)	90.6-99.5 (95.2)	88-100.2 (95.3)

circular = circular DNA; tRNA = transfer-RNA

Miniprep Kit I, Peqlab, Erlangen, Germany) according to the manufacturers' instructions. Standard curves were prepared from purified plasmid DNA for 10^0 to 10^8 copies/PCR reaction. Dilutions were obtained with nuclease-free distilled water containing 5 ng/ml of tRNA. Circular plasmid was compared to linearised plasmid digested with restriction endonuclease EcoR I (Promega, Madison, WI, USA).

Quantitative PCR (qPCR)

Randomly chosen samples were compared to a standard curve of the cloned plasmid (10^1-10^8 copies) and the number of copies contained in the reaction was calculated by extrapolation using the MX 3000 P® software (Stratagene, La Jolla, CA, USA).

Results

PCR optimisation

The real-time PCR system was evaluated using the primers M.s.713_for and M.s.713_rev with an optimum primer concentration of 200 nM for the SYBR® Green assay and 300 nM for the probe-based assay, using a concentration of 200 nM for the probe. Positive samples were compared with dilutions over a range of 10^0-10^4. The undiluted samples were frequently inhibited as judged by the Ct values. The standard curves for pig DNA had RSq values (fit of the standard curve) of 0.996-1.000, slopes of -3.279 to -2.991 and efficiencies of 101.8-115.9 %. The SYBR® Green runs for *M. suis* detection had RSq values of 0.989-0.995, slopes of -3.451 to -3.189 and efficiencies of 94.9-105.9 % and the probe-based assays had RSq values of 0.991-0.997, slopes of -3.370 to -3.171 and efficiencies of 98.0-106.7 %.

Samples that reached the threshold value (i.e. the number of cycles with a significant fluorescence signal) until cycle 40 were considered positive. When the concentration of bacterial DNA was low, positive Ct values were close to the detection limit and differences in concentration were not detectable. High DNA concentrations on the other hand occasionally led to variable or false negative results (not shown). In quantitative assays with positive samples it was also noticeable that the first dilution step (1:10) produced lower Ct values than the undiluted sample, indicating inhibition of the PCR in the undiluted samples as described above. The effect of real-time PCR inhibition was noticed especially in the SYBR® Green PCR, less so in the probe-based PCR.

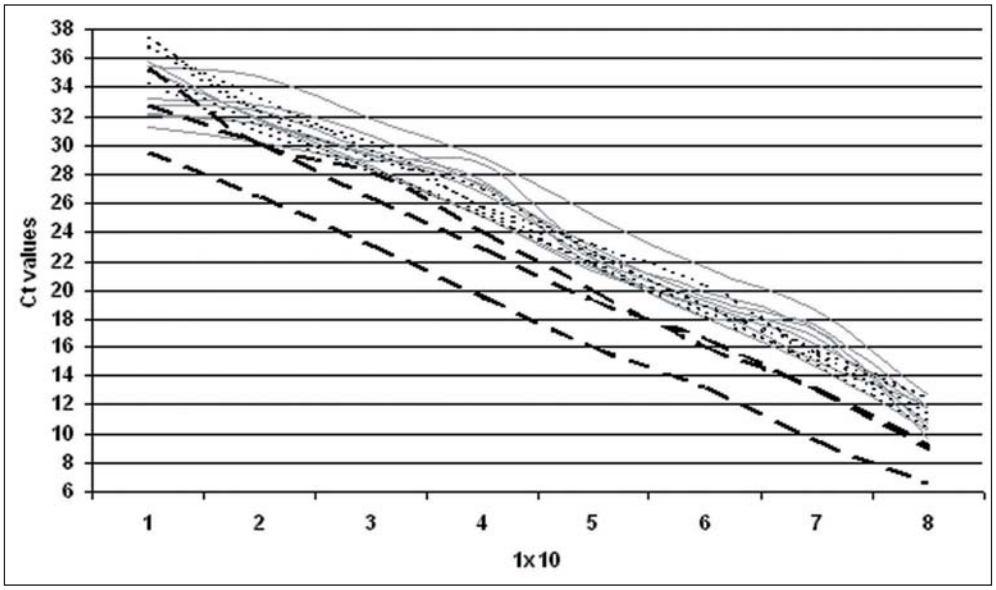

Fig. 1: Ct values of plasmid standards (different preparations) from 10^1-10^8 copies; thin, grey, lines: circular plasmid; thin dotted black lines: circular plasmid with transfer RNA (tRNA); black, thick broken lines: linearised plasmid with tRNA (average values from duplicates)

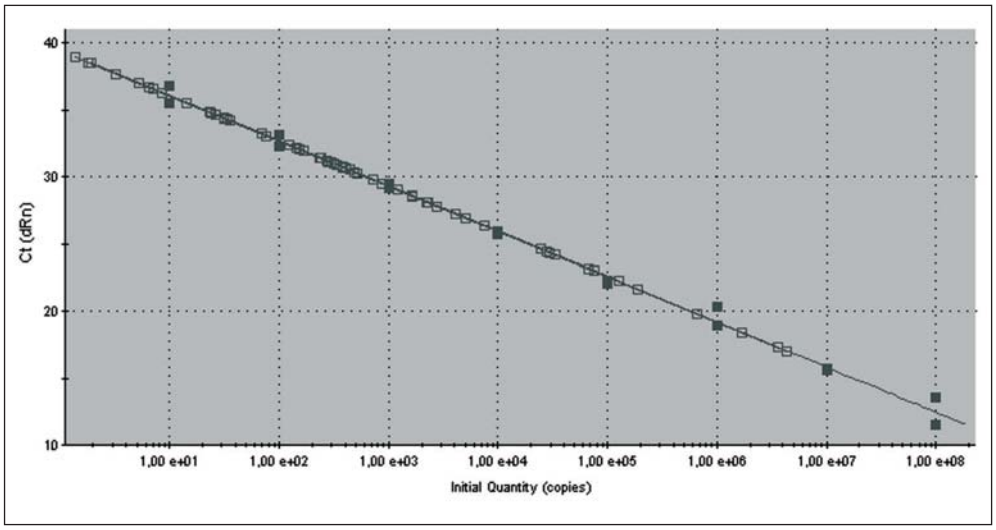

Fig. 2: Example of a standard curve generated with the MX 3000 P® computer software; closed squares: standard samples of known concentrations, open squares: unknown samples; RSq: 0.995, efficiency: 98.0 %, slope: -3.372

Specificity with non-haemotrophic porcine *Mycoplasma* species

The real-time PCR (both SYBR®-Green and probe-based) never yielded a positive result with any of the other species tested.

Comparison of PCR results of the 3 different methods

596 samples (372 from blood, 224 from spleen) were evaluated with SYBR® Green PCR and probe-based PCR and compared to the nested PCR results previously obtained (STRIMITZER et al., 2004). 67.5 % showed the same results for nested PCR and SYBR® Green (11.4 % were positive, 56.0 % were negative), while 5.5 % were positive for SYBR® Green but negative for nested PCR and in 27.0 % of the cases vice versa. 227 samples were compared for nested PCR vs. probe-based PCR and 31.7 % were concordant (27.8 % positive, 3.9 % negative), while 13.7 % were positive only with the probe and 54.6 % were only positive in the nested PCR (Tab. 1).

Between the 2 real-time assays 236 samples were compared, 36.4 % double positives, 49.2 % double negatives, 6.4 % probe-only positive samples and 8.1 % SYBR® Green-only positive ones.

Sequencing

From the samples with different results in the nested PCR and the real-time PCR 37 (21 blood and 16 spleen samples) were chosen for sequencing with primers M.s.287_for and M.s.1287_rev; however, only 4 samples yielded data that could be evaluated.

One sample was positive with nested PCR and SYBR® Green PCR but negative with the probe and 99 % identical with „*Aeromonas salmonicida* subsp.“ (AM296510). Sequence comparison regarding the PCR/nested PCR priming region showed 100 % identity, whereas the probe region was diverse enough to yield a negative result.

The other 3 samples were all SYBR® Green negative, whereas one of these samples was probe positive and they all were 95 % identical to “uncultured bacterium clone” Genbank accession numbers AY977784, DQ905599, AF371797.

For the remaining samples with different results in the nested PCR and real-time PCR sequences could not be obtained, despite PCR-amplification with the chosen primers (n=5 positive with real-time PCR but negative in the nested PCR, n=22 negative with real-time PCR but positive in the nested PCR and 6 with variable results).

2 samples that were positive in all 3 PCR assays including probe-based PCR were also chosen for sequencing. One of these 2 samples was 98 % identical to the *M. suis* strain "Guangdong" and 96 % identical to the strains "Illinois" and "Zachary" and the other sample was 98 % identical to the strains "Illinois" and "Zachary" and 95 % identical to "Guangdong".

Probe-based qPCR

Positive results were obtained with 10 or more copies. RSq values and efficacies were similar among circular templates, linearised templates and linearised templates with transfer RNA (tRNA), although results were less variable when tRNA was added, and linearisation produced slightly lower Ct values (Tab. 2; Fig. 1).

Positive field samples (n=74) with unknown concentrations of bacterial DNA were compared to a standard dilution series of plasmid DNA. According to the Ct values obtained for these samples the calculated amount of target DNA ranged from $1.74x10^0$ to $3.45x10^7$ copies/sample (Fig. 2). Since 200 µl blood were used for each extraction and DNA was eluted in a volume of 200 µl and used in 5 µl aliquots and considering that 16S rRNA sequences are contained in the small *Mycoplasma* genomes in 1-2 copies (AMIKAM et al., 1984), the lowest calculated concentration of 1.74 copies in 5 µl of DNA corresponds to 0.87-1.74 organisms/5 µl of blood. Evaluation of DNA from negative blood samples spiked with cloned bacterial DNA gave comparable results (data not shown).

Discussion

M. suis, the causative agent of porcine eperythrozoonosis, still represents a challenge for specific diagnosis, since it cannot be cultured from samples and its appearance in peripheral blood is irregular. Antibodies against *M. suis* can be detected in serum for 2 months or more after infection (SCHULLER et al., 1990; HÖLZLE et al., 2006) and correlations to latent infections are not possible. The development of a PCR assay for the diagnosis of chronic infections is therefore of great value for the evaluation of the herd health status on the basis of individual samples. In comparison to classic (nested) PCR the real-time PCR is less time-consuming, less susceptible to contamination (because the tubes are not opened after amplification) and it also gives quantifiable results. The latter may be of importance for the evaluation of any correlations between the level of bacteriaemia and clinical outcome of the infection. HÖLZLE and coauthors (2007c) developed a probe-based quantitative real-time PCR for the detection of *M. suis* based on a previously characterised conserved gene, MSG1 (HÖLZLE et al., 2007b), which could reliably detect 100 or more genome equivalents of *M. suis* with a sensitivity of 100 % and a specificity of 96.7 %. The good performance of this assay should facilitate the establishment of a routine protocol for diagnostic procedures.

In the present study samples previously tested by nested PCR were re-evaluated using both a SYBR® Green and a probe-based PCR assay. Currently, splenectomy to provoke an acute infection is considered as the gold standard of diagnosis (HÖLZLE, 2007); however, this is an impracticable method for routine diagnosis and diagnosing the bacteria in blood smears is considered to be of low sensitivity and specificity (STRIMITZER et al., 2004; HÖLZLE, 2007). In the absence of a gold standard for the 3 PCR-techniques we evaluated the specificity using sequencing data (where available). Since false positive results were obtained by nested-PCR and (to a lesser extent) by SYBR® Green PCR, whereas the results from the probe-based PCR were always substantiated by sequencing, it was concluded that probe-based PCR is the most specific technique. Unfortunately the lack of material prevented the sequence analysis of some of the presumably false positive samples from the nested and SYBR® Green PCR after cloning, therefore their evaluation was extrapolated from the sequencing results that could be obtained from direct PCR product sequencing. The sensitivity of the assay is of great importance for low levels of infection as can be expected in carrier animals. In this respect the real-time PCR using the probe could reliably detect 10 copies of a cloned plasmid or a calculated number of <2 copies/PCR, compared to STRIMITZER et al. (2004) who could detect only 0.25 pg of a partially purified DNA (corresponding to roughly 12,000 copies based on the assumption that the *M. suis* genome is 745 kb in size; MESSICK et al., 2000). Known positive samples (n=74) were compared to a dilution series of the cloned standard (10^1 bis 10^8 copies) which contained a calculated amount of $1.74x10^0$ - $3.45x10^7$ 16S rRNA-gene copies. It should be mentioned that samples with high Ct values, i.e. low concentrations of target DNA, occasionally remained negative in the repetitive samples (but were still considered positive in the evaluation).

Undiluted samples were occasionally negative in the PCR for the detection of porcine DNA. This always occurred in combination with SYBR® Green negative *M. suis* PCR results and variable probe-based PCR, indicating inhibition of PCR (to which the probe appeared to be less prone) as previously described by HÖLZLE et al. (2003). Dilutions of 1:10 alleviated this problem, therefore this step is recommended in order to improve sensitivity.

Another point is the general choice of target sequences; since in contrast to other organisms the 16S RNA-gene in mycoplasms is not a high-copy gene (AMIKAM et al., 1984), other single-copy genes such as heat shock protein (HÖLZLE et al., 2007a) or adhesion protein genes (HÖLZLE et al., 2007b) might provide equal or even better targets, provided the specificity can be verified not only with other porcine *Mycoplasma* species but with any bacterial or viral agent available, at least in silico.

Conclusions

The usefulness of real-time PCR for the detection and quantification of *M. suis* in various samples could be demonstrated. With regard to specificity and speed this technique is superior to conventional nested PCR and allows for the evaluation of the bacterial load. Further studies using this method should include data on the clinical and epidemiological status of the affected animals to detect possible correlations between the bacterial load and clinical features of infection. Although the use of SYBR® Green for fluorescence detection is easier and less expensive, the minor groove binder probe that was developed gave the best performance in terms of specificity. The finding that false positive reactions remained undetected in the previously used nested PCR despite sequence determina-

tion of the products should give rise to further studies on the inter- and intra-specific variability of haemophilic *Mycoplasmas* in pigs and other animals.

Acknowledgements

We cordially thank Dr. Schmoll, Clinic of Swine, Institute of Parasitology and Zoology, Clinical Department for Farm Animals and Herd Health Management, University of Veterinary Medicine Vienna, and Dr. Strimitzer, formerly Department of Pathobiology, University of Veterinary Medicine Vienna, for the DNA samples.

References

AMIKAM, D., GLASER, G., RAZIN, S. (1984): *Mycoplasmas* (*Mollicutes*) have a low number of rRNA genes. J. Bacteriol. **158**, 376-378.

DOYLE, L.P. (1932): A rickettsia-like or anaplasmosis-like disease in swine. J. Am. Vet. Med. Ass. **81**, 668-671.

GWALTNEY, S.M., HAYS, M.P., OBERST, R.D. (1993): Detection of *Eperythrozoon suis* using the polymerase chain reaction. J. Vet. Diagn. Invest. **5**, 40-46.

HEINRITZI, K. (1983): Klinik, Hämatologie und Metabolismus des eperythrozoonotischen Anfalls. Prakt. Tierarzt **65** „Colloquium veterinarium XIV", 40-44.

HEINRITZI, K. (1984): Beitrag zur Splenektomie beim Schwein. Tierärztl. Prax. **12**, 451-454.

HEINRITZI, K. (1989): *Eperythrozoon*-Infektion beim Schwein als Faktorenkrankheit. Berl. Münch. Tierärztl. Wschr. **102**, 337-342.

HEINRITZI, K. (1990): Zur Diagnostik der *Eperythrozoon-suis*-Infektion. Tierärztl. Prax. **18**, 477-481.

HEINRITZI, K. (1992): Untersuchungen zur Übertragbarkeit von *Eperythrozoon suis*. Tierärztl. Umschau **47**, 588-599.

HENRY, S.C. (1979): Clinical observations on eperythrozoonosis. J. Am. Vet. Ass. **174**, 601-603.

HÖLZLE, L.E. (2007): Zur Bedeutung der haemotrophen Mycoplasmen in der Veterinärmedizin unter besonderer Berücksichtigung der *Mycoplasma suis*-Infektion beim Schwein. Berl. Münch. Tierärztl. Wochenschr. **120**, 34-41.

HÖLZLE, L.E., ADELT, D., HÖLZLE, K., HEINRITZI, K., WITTENBRINK, M.M. (2003): Development of a diagnostic PCR assay based on novel DNA sequences for the detection of *Mycoplasma suis* (*Eperythrozoon suis*) in porcine blood. Vet. Microbiol. **93**, 185-196.

HÖLZLE, L.E., HELBLING, M., HÖLZLE, K., RITZMANN, M., HEINRITZI, K., WITTENBRINK, M.M. (2007c): First LightCycler real-time PCR assay for the quantitative detection of *Mycoplasma suis* in clinical samples. J. Microbiol. Meth. **70**, 346-354.

HÖLZLE, L.E., HÖLZLE, K., HARDER, A., RITZMANN, M., AUPPERLE, H., SCHOON, H.A., HEINRITZI, K., WITTENBRINK, M.M. (2007a): First identification and functional characterization of an immunogenic protein in unculturable haemotrophic *Mycoplasmas* (*Mycoplasma suis* HspA1). FEMS Immunol. Med. Microbiol. **49**, 215-223.

HÖLZLE, L.E., HÖLZLE, K., HELBLING, M., AUPPERLE, H., SCHOON, H.A., RITZMANN, M., HEINRITZI, K., FELDER, K.M., WITTENBRINK, M.M. (2007b): MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes. Microbes Infect. **9**, 466-474.

HÖLZLE, L.E., HÖLZLE, K., RITZMANN, M., HEINRITZI, K., WITTENBRINK, M.M. (2006): *Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs. Clin. Vaccine Immunol. **13**, 116-122.

KINSLEY, A.T. (1932): Protozoan like body in blood of swine. Vet. Med. **27**, 196.

MESSICK, J.B., COOPER, S.K., HUNTLEY, M. (1999): Development and evaluation of a polymerase chain reaction assay using the 16SrRNA gene for detection of *Eperythrozoon suis* infection. J. Vet. Diagn. Invest. **11**, 229-236.

MESSICK, J.B., SMITH, G., BERENT, L., COOPER, S. (2000): Genome size of *Eperythrozoon suis* and hybridization with 16S rRNA gene. Can. J. Microbiol. **46**, 1082-1086.

NEIMARK, H., JOHANSSON, K.E., RIKIHISA, Y., TULLY, J.G. (2001): Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of 'Candidatus *Mycoplasma haemofelis*', 'Candidatus *Mycoplasma haemomuris*', 'Candidatus *Mycoplasma haemosuis*' and 'Candidatus *Mycoplasma wenyonii*'. Int. J. Syst. Evol. Microbiol. **51**, 891-899.

OBERST, R.D., HALL, S.M., JASSO, R.A., ARNDT, T., WEN, L. (1990b): Recombinant DNA probe detecting *Eperythrozoon suis* in swine blood. Am. J. Vet. Res. **51**, 1760-1764.

OBERST, R.D., HALL, S.M., SCHONEWEIS, D.A. (1990a): Detection of *Eperythrozoon suis* DNA from swine blood by whole organism DNA hybridizations. Vet. Microbiol. **24**, 127-134.

RIKIHSA, Y., KAWAHARA, M., WEN, B., KOCIBA, G., FUERST, P., KAWAMORI, F., SUTO, C., SHIBATA, S., FUTOHASHI, M. (1997): Western immunoblot analysis of *Haemobartonella muris* and comparison of 16S rRNA gene sequences of *H.muris*, *H.felis* and *Eperythrozoon suis*. J. Clin. Microbiol. **35**, 823-829.

RUTTKOWSKI, B. (2006): personal communication.

SCHULLER, W., HEINRITZI, K., AL-NUKTHA, S., KÖLBL, S., SCHUH, M. (1990): Serologische Verlaufsuntersuchungen mittels KBR und ELISA zum Nachweis von Antikörpern gegen die *Eperythrozoon suis*-Infektion des Schweines. Berl. Münch. Tierärztl. Wschr. **103**, 9-12.

SCHWEIGHARDT, H., FELLNER, A., PECHAN, P., LAUERMANN, E. (1986): Eperythrozoonose beim Schwein - ein Fallbericht. Wien. Tierärztl. Mschr. **73**, 250-253.

SMITH, A.R., RAHN, T. (1975): An indirect hemagglutination test for the diagnosis of *Eperythrozoon suis* infection in swine. Am. J. Vet. Res. **36**, 1319-1321.

SOGIN, M.L., GUNDERSON, J.H. (1986): Structural diversity of eukaryotic small subunit ribosomal RNAs - evolutionary implications. Ann. New York Acad. Sci. **503**, 125-139.

SPLITTER, E.J., WILLIAMSON, R.L. (1950): Eperythrozoonosis in swine. J. Am. Vet. Med. Assoc. **116**, 360-364.

STRIMITZER, S., OBWALLER, A., SCHMOLL, F., JOACHIM, A., WALOCHNIK, J., JUNCKER, M. (2004): Entwicklung und Evaluierung einer Polymerasekettenreaktion (PCR) zum Nachweis von *Mycoplasma* (syn. *Eperythrozoon*) suis. Vet. Med. Austria/Wien. tierärztl. Mschr. **91**, 120-126.

UILENBERG, G., THIAUCOURT, F., JONGEJAN, F. (2004): On molecular taxonomy: what is in name? Exp. Appl. Acarol. **32**, 301-312.

Authors' address:

Mag. Birgit Gattinger, Dr. Joachim Spergser, Walpurga Wille-Piazzai, Dipl.Ing.Dr. Jolanta Kolodziejek, Dr. Alexander G. Tichy, Univ.Prof.Dr. Anja Joachim, Veterinärplatz 1, A-1210 Vienna, Austria.

e-mail: Anja.Joachim@vu-wien.ac.at