Prevalence of *Mycoplasma suis* in slaughter pigs, with correlation of PCR results to hematological findings

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Received 4 March 2008; received in revised form 21 June 2008; accepted 26 June 2008

**Abstract**

Porcine infectious anemia is a well-known disease that occurs worldwide and is caused by the unculturable hemotrophic bacterium *Mycoplasma suis*. The actual prevalence and impact of *M. suis* infections, however, remain fairly unknown. This study examined the prevalence of *M. suis* in post-weaning pigs by employing a quantitative real-time LightCycler PCR. *M. suis* infections were detected in 164 out of 1176 feeder pigs (20–30 kg; 13.9%) as well as on 79 out of 196 pig farms (40.3%). The comparison of PCR results with microscopic investigation of acridine-orange-stained blood smears revealed a considerably lower sensitivity of the microscopic method: only 35 out of 1176 blood smears were microscopically positive. The microscopic detection of *M. suis* was shown to be closely linked to the bacterial load in the blood. *M. suis* infection is associated with significantly decreased hematocrit, erythrocyte counts and hemoglobin concentrations as well as significantly higher bilirubin concentrations. Furthermore, *M. suis* blood loads were significantly associated with erythrocyte count, hematocrit, hemoglobin, glucose and iron concentrations indicating that high *M. suis* loads are connected with clinical anemia. In conclusion, this study has shown, that *M. suis* infections are often under-diagnosed in pig husbandry and can therefore lead to considerable economic profit losses in pig husbandry. Furthermore, our study has shown that the LightCycler PCR could be an appropriate tool for a sufficiently coherent identification of *M. suis* in latent carrier animals in view of introducing effective treatment and disease control measures.

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Available online at www.sciencedirect.com

**Keywords:** *Mycoplasma suis*; Infectious anemia; Prevalence; Anemia; Quantitative real-time PCR

1. Introduction

*Mycoplasma suis*, a hemotrophic pathogen of pigs, causes economic losses in swine production through-
bacteremia is low and clinical signs vary, ranging from mild icteroaemia, general unthriftiness, poor growth rates or bad reproductive performance to increased susceptibility for other infectious diseases (Henry, 1979; Brownback, 1981; Zinn et al., 1983; Schweighardt et al., 1986). Hemotrophic mycoplasmas can successfully establish chronic and persistent infections despite an intense immune response and antibiotic treatment (Smith, 1992). Pigs infected with *M. suis* probably remain chronic carriers even after clinical signs have been resolved.

Although first recognized in the United States in 1932 (Kinsley, 1932), little is known about the epidemiology of *M. suis* infections. To date, *M. suis* has not been cultivated in vitro and traditional diagnostic methods, e.g. the microscopic examination of acridine-orange- or Giemsa-stained blood smears have proven to be of low sensitivity and specificity. Therefore, the development of molecular methods has greatly facilitated the identification of these agents (Gwaltney et al., 1993; Messick et al., 1999; Hoelzle et al., 2003). Today, PCR analysis is the method of choice for the diagnosis of *M. suis* infections and a sensitive and specific real-time PCR assay for the detection and quantification of *M. suis* has been introduced (L.E. Hoelzle et al., 2007). This diagnostic PCR assay can be used to detect accurately acute and chronic infections in pigs, which is essential to developing control measures.

The present study was undertaken to investigate the prevalence of *M. suis* infections in pigs in Germany and to determine the correlation between positive PCR results and clinical–hematological findings. Diagnosis and quantification were performed by means of a quantitative LightCycler (LC) PCR. PCR results were compared with microscopic results investigating acridine-orange-stained blood smears.

### 2. Materials and methods

#### 2.1. Sample collection

In order to determine the definitive sampling scheme, a preliminary experiment was performed using the blood samples of 160 pigs (20–30 kg) from a slaughterhouse. The pigs originated from 46 different farms (average of 3.48 blood samples/farm). The number of PCR-positive animals and the number of PCR-positive pig farms were used to carry out the statistical evaluation (i) of how many farms needed to be tested per region in order to identify – with a 95% confidential index (CI) – at least one positive farm as well as (ii) how many animals per farm needed to be tested to detect at least one positive animal with a CI of 95%.

The main experiment involved collecting a total of 1176 EDTA-anti-coagulated blood samples as well as corresponding serum samples from slaughterhouse pigs (20–30 kg) across a period of 6 months (in 2006 and 2007). The pigs originated from 196 pig farms in Germany (eight German federal states representing the southern, the western, and the eastern parts of Germany; Table 1). Six animals were tested per farm. In order to rule out a pre-selection, the animals were chosen solely on the basis of their origin and their weight with possible clinical symptoms not being taken into account.

#### 2.2. Hematological and biochemical blood analysis

Hematological parameters were evaluated in EDTA-anti-coagulated blood using the Scil Vet ABC tool (Scil Animal Care Company GmbH, Viernheim, Germany) and including erythrocyte, leucocyte, and thrombocyte counts as well as hematocrit, hemoglobin, mean cellular volume, mean cellular hemoglobin, and mean cellular hemoglobin concentration. Biochemical parameters (blood glucose, bilirubin, iron) in blood sera were analyzed using the Hitachi 911 Chemistry Analyzer (Roche, Mannheim, Germany). The reference ranges employed were 5% and 95% quantiles. Blood smears were stained acridine-orange and investigated under a fluorescence microscope (Olympus BX50; 1000-fold magnification).

#### 2.3. DNA extraction

200 μl-volumes of whole anti-coagulated blood (EDTA) were mixed with equal volumes of lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 320 mM sucrose, 1% (v/v) Triton X-100) and centrifuged (8,000 × g, 22 °C, 60 s). The pellet was suspended in 400 μl lysis buffer and then centrifuged again. After repeating this step once, the pellet was
used to extract whole DNA by using the GenElute Bacterial Genomic DNA Kit (Sigma, Buchs, Switzerland), with an elution volume of 200 μl. Samples were stored at −20°C until use.

2.4. Quantitative real-time PCR

M. suis DNA was detected and quantified with the LightCycler 2.0 System (Roche Diagnostics, Rotkreuz, Switzerland), as described previously (L.E. Hoelzle et al., 2007). Primers (msg1-Fw 5'-ACAAC-TAATGCACTAGCTCCTATC-3' and msg1-Rv 5'-GCTCCTGTAGTTGATAGGAATAATTGA-3') as well as probes (5'-TTCACGCTTCTCCTGACCAAAGAC-Fluorescein-3' and 5'-LCRed-640-CAAGACTCTCCTGACCAAAGAGC-3') were purchased from Metabion (Martinsried, Germany). Real-time PCR was performed by means of the LightCycler Fast Start DNA MasterPLUS Hybridisation Probe Kit (Roche Diagnostics) with 0.5 μM of each primer as well as 0.2 μM of each probe. For quantification purposes genomic M. suis DNA was extracted from experimentally infected pigs and quantified, as described previously (L.E. Hoelzle et al., 2007).

2.5. Data analysis

95% confidence intervals (CI) were calculated for observed PCR prevalence. Data (PCR results, hematological data, regional distribution) was compiled and analyzed with Excel and SPSS 14.0 for Windows (Microsoft, Wallisellen, Switzerland), Analyze-it (standard edition Software ONE AG), and Origin (Redacom) software. Mean values, median, CIs, and quantiles were calculated for each parameter. Parameters between PCR-positive and -negative pigs were compared using the Mann–Whitney test. The correlations between M. suis blood loads and blood parameters were assessed by the Pearson correlation coefficient. Differences were considered to be significant, if the P value was ≤0.05. Possible connections between PCR results and the microscopic examination were evaluated on the basis of the contingency table and Cohen’s kappa coefficient analysis. The kappa value was interpreted according to the method suggested by Landis and Koch (1977). Kappa values of 0.20 or less indicate a slight compliance; 0.21–0.40 a fair agreement; 0.41–0.60 a moderate agreement; 0.61–0.80 a substantial agreement; 0.81 an almost perfect agreement, and 1.00 a perfect agreement between tests.

3. Results

3.1. Prevalence

The required number of samples and farms which were essential for a substantive prevalence study were determined by performing a preliminary experiment. Of the 160 preliminary samples from 46 farms, 17 (10.6%) animals from 6 farms (13.3%) reacted as PCR-positive for M. suis, with a mean in-house prevalence of 41.5% and a mean bacterial load of $5.31 \times 10^5$ M. suis copies/ml blood. Depending on regional differences in pig farm size and frequency, a
corresponding sampling procedure (Table 1) was selected in order to identify – with a 95% CI – at least one positive farm per region and at least one positive animal per farm.

In the main experiment 164 (13.9%; 95% CI: 12.0–16.1%) out of 1176 pigs reacted as PCR-positive (Table 1). These PCR-positive pigs came from 79 out of a total of 196 pig farms (40.3%; 95% CI: 33.9–48.0%). Within the positive pig farms, the percentage of infected animals varied between 25.0 and 46.2%. The highest prevalence of individual animals was found in the southern part of Germany (17.5%) and the highest prevalence within the farms was determined in Lower Saxonia (West Germany; 48.0%; Table 1). However, M. suis positive farms were found in all investigated parts of Germany with at least 33.3% PCR-positive farms without significant regional distinctions.

Quantification of the bacterial loads in PCR-positive blood samples revealed a mean value of $7.62 \times 10^7$ M. suis copies/ml blood (95% CI: $6.68 \times 10^7$–$2.19 \times 10^8$; range: minimum load $1.4 \times 10^5$; maximum load $1.1 \times 10^{10}$).

### 3.2. Hematological findings in LightCycler PCR-positive pigs

Of the hematological parameters evaluated, four parameters were associated with an M. suis infection: M. suis LC PCR-positive pigs had significantly lower PCV, hemoglobin, erythrocyte values and significantly higher bilirubin concentrations ($P \leq 0.05$; Fig. 1). The mean values, 95% CI, and ranges for these parameters are provided in Table 2. No significant association between erythrocyte indices, i.e. mean cellular volume, mean cellular hemoglobin, and mean cellular hemoglobin concentration and the M. suis infection status of the pigs could be determined. Similarly, no significant association could be observed between the leucocyte, thrombocyte, serum glucose, and iron values and the PCR status of the pigs examined (Table 2).

When the 164 LC PCR-positive samples were analyzed, significant correlations between M. suis loads in the blood and the erythrocyte number ($r_s = -0.35$; 95% CI: $-0.49$ to $-0.21$; $P \leq 0.0001$), the hemoglobin value ($r_s = -0.24$; 95% CI: $-0.38$ to $-0.08$; $P = 0.0031$), the hematocrit value ($r_s = -0.35$; 95% CI: $-0.48$ to $-0.21$; $P \leq 0.0001$), the iron ($r_s = -0.15$; 95% CI: $-0.30$ to $-0.00$; $P = 0.005$), and the glucose concentration ($r_s = -0.16$; 95% CI: $-0.30$ to $-0.00$; $P = 0.043$) could be determined (Fig. 2). Contrary to these findings, no significant correlation could be observed between the M. suis blood load and the bilirubin concentration ($r_s = -0.03$; 95% CI: $-0.01$ to $-0.08$; $P = 0.36$) or other hematological parameters, i.e. leucocyte and thrombocyte counts, glucose, and iron values (data not shown).

### 3.3. Comparison of PCR results with the microscopic examination

In addition to PCR testing, blood smears of all pigs were examined independently. Out of 164 PCR-positive samples, 31 blood smears proved to be positive under
the microscope. Contrary to this, out of 1012 PCR-negative samples, only 4 blood smears proved to be microscopically positive. The statistical comparison of both methods revealed an almost perfect agreement (Cohen’s kappa coefficient of 0.88; \(P < 0.0001\)). The microscopic result was strongly associated with the bacterial load, as shown in Fig. 3: All 31 microscopically positive samples had an \textit{M. suis} load \(>1.00 \times 10^5\) and all samples with an \textit{M. suis} load \(>1.00 \times 10^7\) were determined to be microscopically positive.

4. Discussion

This is the first large scale study investigating the prevalence of \textit{M. suis} in randomly sampled post-weaning pigs using PCR and correlating the PCR results with hematological findings and microscopic results. Although \textit{M. suis} pig infections have been continuously reported worldwide over the past 75 years (findings in Europe, the United States, South America, i.e. Brazil, Africa, i.e. Nigeria, and Asia, i.e. China; Dipeolu et al., 1982; Schuller et al., 1990; Hoelzle et al., 2003; Messick, 2004; Wu et al., 2006; Guimaraes et al., 2007; L.E. Hoelzle et al., 2007), the actual prevalence of \textit{M. suis} in pig populations was not evaluated and no extensive studies have been published to date. The present study focused on the prevalence of \textit{M. suis} in healthy post-weaning pigs (20–30 kg) from slaughterhouses because feeder pigs are known to be the most affected (Messick, 2004). The pigs were chosen to represent the main swine producing areas of Germany. The DNA samples were analyzed by means of a recently introduced quantitative LightCycler PCR assay that has proven to be highly sensitive and specific and thus accurate with regard to the diagnosis of \textit{M. suis} infections (L.E. Hoelzle et al., 2007).

The overall prevalence of \textit{M. suis} in German feeder pigs determined within the scope of our study was 13.9\% for all pigs and 40.8\% for the pig farms. Furthermore, our study clearly indicates the nationwide spread of \textit{M. suis} in Germany. On the basis of the identified prevalence of \textit{M. suis} in healthy post-weaning pigs, it can be assumed that undetected \textit{M. suis} infections can, in part, lead to significant economic losses in pig production. Extended feeding periods, higher feed costs, greater susceptibility to contract infectious viruses of the respiratory and intestinal tracts as well as the premature slaughtering of the animals can lead to considerable profit losses (Henry, 1979; Heinritzi, 1989; Gresham et al., 1994; Hoelzle et al., 2003). The fact that this study was able to identify a correlation between PCR results and the clinical–hematological manifestations of the anemia is further proof of these findings. The mean values of the group of PCR-positive pigs were compared with the mean values of PCR-negative pigs as well as with reference values. The reference values used for the comparison were those determined by Kixmoeller et al. (2006) for this specific breed and age group of pigs, because the hematological reference values of pigs vary tremendously and depend largely on age and breed. The mean values found for all parameters in both groups (PCR negative, PCR positive) were within the selected reference ranges or approached the ranges. However, we were able to determine that the mean values of blood

<table>
<thead>
<tr>
<th>Blood parameter</th>
<th>PCR positive ((n = 164))</th>
<th>PCR negative ((n = 1012))</th>
<th>Reference range(^a)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>Median 32.27 95% CI 31.56–32.98</td>
<td>Median 34.16 95% CI 33.69–34.65</td>
<td>32–42</td>
<td>0.0001(^*)</td>
</tr>
<tr>
<td>Hemoglobin (mmol/l)</td>
<td>6.24 6.12–6.37</td>
<td>6.68 6.58–6.78</td>
<td>6.6–8.3</td>
<td>0.0001(^*)</td>
</tr>
<tr>
<td>Erythrocytes ((10^{12}/\mu l))</td>
<td>6.26 6.11–6.42</td>
<td>6.89 6.73–6.91</td>
<td>5.8–7.8</td>
<td>0.05(^*)</td>
</tr>
<tr>
<td>Serum glucose (mmol/l)</td>
<td>3.97 3.75–4.19</td>
<td>4.08 3.99–4.17</td>
<td>3.8–6.7</td>
<td>0.15</td>
</tr>
<tr>
<td>Bilirubin ((\mu)mol/l)</td>
<td>5.89 5.18–6.59</td>
<td>4.34 4.08–4.61</td>
<td>&lt;4.3</td>
<td>&lt;0.0001(^*)</td>
</tr>
<tr>
<td>Iron ((\mu)mol/l)</td>
<td>16.84 15.71–17.97</td>
<td>16.05 15.56–16.55</td>
<td>&gt;17.2</td>
<td>0.83</td>
</tr>
</tbody>
</table>

\(^a\) Reference ranges according to Kixmoeller et al. (2006).
\(^*\) \(P\) values of \(\leq 0.05\) were considered as significant.
parameters, i.e. erythrocyte count, hematocrit value, and hemoglobin concentration were significantly lower in PCR-positive animals in comparison to PCR-negative animals. This is in contrast to the study carried out by Guimaraes et al. (2007) that was unable to determine any significant differences between the hematological parameters of animals infected and animals not infected with M. suis, although anemia is one of the most prevalent and common clinical symptoms of M. suis induced diseases (Heinritzi, 1999). Moreover, the present study was able to determine a significant correlation between the bacterial load in the blood of the animals and the severity of anemia (i.e. erythrocyte count, hematocrit value, and hemoglobin concentration). However, the coefficients of correlation ($r_s$ values) are rather low indicating weak correlations between hematological findings and the bacterial loads. Therefore, hematological findings do certainly not reflect the M. suis infection status and can, therefore, not be useful as an indicator of an infection. The lack of significant differences in erythrocyte indices (mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration) within this context, is an indication for a normochromic, normocytic anemia caused by intravascular hemolysis. However, it remains insufficiently investigated that which form of hemolysis the M. suis infected anemic animals actually suffer from (Zachary and Smith, 1985; K. Hoelzle et al., 2007).

By systematically comparing microscopy results and quantitative PCR, this study has clearly shown that the microscopic evaluation of pathogen quantities of $10^6$ M. suis copies/ml blood allows for a sufficiently coherent diagnosis in acutely diseased pigs. In the case of lower bacterial loads, however, sensitivity and specificity are too low in order to be employed within the scope of a control or eradication program. Furthermore, false positive microscopic results are possible due to the difficulty to distinguish M. suis from immature DNA harboring erythrocytic structures (Henry, 1979; Hoelzle et al., 2003).

In conclusion, our study has clearly shown that M. suis infections are often under-diagnosed or even not diagnosed in disease complexes leading to preterm slaughtering at a weight of 20–30 kg. In future, greater importance should be given to the unambiguous diagnosis and efficient treatment of porcine infectious anemia. The quantitative LightCycler PCR is a highly
sensitive and specific diagnosis procedure that allows for a sufficiently coherent identification of *M. suis* in latent carrier animals in view of introducing effective treatment and disease control measures.

**References**


