Application of quantitative PCR for detection of \textit{Mycoplasma suis} in blood samples

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Received: June 3, 2014 Accepted: October 27, 2014

Abstract

The aim of the study was to develop and validate a real-time PCR method, using a TaqMan probe, for quantification of \textit{Mycoplasma suis} in porcine blood. No PCR signals with closely related non-haemotrophic mycoplasmas were obtained. The detection limit of PCR for plasmid combined with blood DNA was determined to be $10^3$/reaction (5 µL of DNA) ($1.2 \times 10^5$ target copies in 1 mL of blood). The linearity of real-time PCR (near 1) indicates its use as a quantitative method. Real-time and quantitative PCR were sensitive and specific for the detection and quantification of \textit{M. suis} in the blood of animals with acute and chronic form of eperythrozoonosis. Developed quantitative PCR cannot be used to detect carrier animals with a small amount of \textit{M. suis} in their blood. The validity of real-time PCR used in the studies was confirmed by the low inter- and intra-assay coefficients of variation. This fact confirms the applicability of the assay in other laboratories.

Keywords: swine, \textit{Mycoplasma suis}, porcine eperythrozoonosis, blood, real-time PCR.

Introduction

Haemotrophic mycoplasmas (HM) do not grow \textit{in vitro} in traditional bacteria culture media. They grow well, however, \textit{in vivo} on the surface of red blood cells of many animals. This results in deformation and destruction of red blood cells, accompanied by the process of lysis and anaemia of the infected animals.

\textit{Mycoplasma suis} (\textit{M. suis}), also known as \textit{Mycoplasma haemosuis}, renamed due to reclassification of mycoplasmas, along with \textit{Eperythrozoon suis} (11), are contributing factors of porcine eperythrozoonosis (PE) among domestic pigs. Infections caused by these microorganisms have a considerable impact on the agricultural and livestock sector of the economy (2, 9, 17, 18).

The primary symptoms of PE are closely related to haemolytic anaemia, which affects pigs of various age, and which is sometimes fatal for young pigs (6, 7). Infection might have subclinical course. It is also possible that many infected animals will never reveal any symptoms at all.

The inability to cultivate \textit{M. suis} \textit{in vitro} is a significant obstacle in understanding their physiological properties and metabolism, as well as their pathogenic mechanisms. It is also problematic for laboratory diagnostics. Diagnosis of infection is based on clinical symptoms and the observation of microorganisms on the surface of red blood cells in blood smears. Another recently developed method involves the detection of microorganisms by the use of molecular methods (4, 10, 12, 14). Blood smear methods, however, are too imprecise when compared to the molecular methods used for detecting infections. Over the last ten years, molecular methods, especially those based on PCR, have improved diagnostic possibilities in respect of HM diseases. The conventional PCR methods are still successfully used to diagnose \textit{M. suis} infections in pigs (5, 10, 14).

To minimise the time necessary for conducting the PCR procedure, by eliminating labour-intensive electrophoresis, a real-time PCR was used to replace the conventional PCR technique. Therefore, the aim of the study was to develop and validate a real-time PCR method for the quantification of \textit{M. suis} in porcine blood.
Material and Methods

A total of eight *M. suis* isolates were used for the study. They were collected from naturally infected pigs with haemorrhagic form of PE and clinical symptoms of fever and anaemia. *Mycoplasma suis* strain 2009/a (obtained from naturally infected pig suffering from acute PE, and maintained in splenectomised pig by subsequent artificial infection) was established as a positive control and used for experimental infection in splenectomised piglets.

Porcine mucosal mycoplasmas (*Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, and *Mycoplasma hyorhinis*) were obtained from the National Veterinary Institute (DTU) in Copenhagen, Denmark and grown in Mycoplasma medium (Friis) at 37°C for 7-10 d. DNA was extracted from bacterial cultures using a commercial isolation kit (Genomic Mini, A&A Biotechnology, Poland).

**Standard DNA.** In order to prepare a recombinant plasmid for PCR quantification, the entire *M. suis* 16S rRNA gene was amplified using a set of primers described previously (14). Further on, the amplicon was cloned in the plasmid TOPO TA Cloning (Invitrogen) according to the manufacturer's guidelines. The DNA plasmid was purified from the *E. coli* transformants using the PureLink Quick Gel Extraction Kit (Invitrogen). The concentration and the purity of the DNA plasmid were specified by optical density measurements (Biophotometer, Eppendorf). A plasmid size was used to calculate the concentrations in plasmid copies per microlitre. The test’s detection limit was measured by testing tenfold dilutions of the recombinant plasmid containing the 16S rRNA gene of *M. suis* (10⁹ to one copy plasmid/reaction). A spectrophotometer (NanoDrop, ThermoScientific) was used to quantify the plasmid concentration. The number of plasmid copies was calculated and adjusted similarly serially diluted in buffer TE alone and in DNA extracted from the blood of a carp (heterologous DNA standard without *M. suis*). The number of target copies per millilitre of blood was calculated based on 100% DNA extraction efficiency and considering one copy of 16 rRNA gene (one target copy corresponds to one organism).

**Real-time reaction mixture and test parameters.** The test was performed in the Mx3005P QPCR System (Stratagene, USA). The PCR mixture consisted of 5 µL of template DNA and a QuantiTect probe set (Qiagen, Germany) containing 8 mM MgCl₂, dNTP mix, and HotStartTaq DNA polymerase, in a total volume of 25 µL. The concentration of the primers was 0.45 µmol 1⁻¹ and the probe was 0.40 µmol 1⁻¹. Amplification of *M. suis* was performed in a 96-well plate with optical caps at the following settings: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 45 s of annealing at 59°C.

**Sensitivity of the real-time PCR.** The detection limit of the PCR test was estimated on the basis of the diluted samples prepared as above. For the determination of a detection limit and repeatability of the test, the dilution experiments were repeated five times (Table 1). The following range of dilutions was tested: 10⁹ to one copy plasmid/reaction. Analogically, to assess the impact of polymerase inhibitors, 10-fold dilutions of pure plasmid and plasmid with blood extract were analysed by real-time PCR.

**Specificity of the real-time PCR.** The following bacterial strains were used to verify the specificity of the test: *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, *Mycoplasma hyorhinis*, and *Escherichia coli* (clinical isolate).

**Primers and probe.** The primers and Taqman probe for *M. suis* were based on the sequence of 16S rRNA gene (3), previously described by Guimares et al. (4).

**Intra- and inter-assay repeatability.** The intra-assay repeatability was determined by running five replicates of the plasmid dilution in DNA extracted from the blood of a carp in the same run. The inter-assay repeatability was tested by running the same plasmid dilution in five separate runs. These replicates were used to determine the mean, standard deviation and, coefficient of variation in Ct (threshold cycle) values for each plasmid dilution.

In the study, six 4-week-old piglets were taken from a farrow to finish and a high health status farm. Before the experiment started, the animals were in quarantine for two weeks. After this period, three piglets were splenectomised. Directly after the operations, the animals were divided into two groups in two separate pens (pen 1 – the control group (piglets 1-3) and pen 2 – the experimental group, which underwent a splenectomy (piglets 4-6). All animals were given oxytetracycline (20 mg/kg b.w.) for 7 d. After three weeks, when the animals were 10 weeks old, the experiment started. The control group of animals was given PBS intravenously as a placebo, while experimental group of animals from pen 2, was infected with *M. suis* (10⁹ target copies/mL of strain 2009/a, i.v.).

In the course of the infection, the animals were subjected to daily clinical state evaluation (body temperature, breathing, appetite). Blood for the tests was taken 7 d before the infection, on the day of infection, as well as on days 4, 7, 14, 21, 28, 35, 42, 49, and 56 after the infection. Blood was tested for the quantity of *M. suis* in 1 mL using real-time PCR, and blood smears were stained using the Hemacolor staining kit. DNA was extracted from blood using a commercial isolation kit (QIAamp DNA Blood Mini Kit).
Results

The real-time PCR had a linear regression $R^2 = 0.997$ (E = 95.1%) and consistently detected $10^2$ of plasmid/reaction when using the plasmid standards diluted in TE (Fig. 1). When the plasmid dilutions contained DNA extracted from blood of carp, the linear regression was $R^2 = 0.999$ (E = 94.8%) and the test detected $10^2$ of plasmid/reaction when using the plasmid standards diluted in TE (Fig. 1). When the plasmid dilutions contained DNA extracted from blood of carp, the linear regression was $R^2 = 0.999$ (E = 94.8%) and the test detected $10^3$ of plasmid/reaction (Fig. 2, Table 1). The test also detected $10^2$ of plasmid/reaction in one of five tested samples at this dilution but with a high Ct = 39.5. The $M. suis$ positive swine blood sample, serially diluted and used as a template for both tests, had $1.2 \times 10^{11}$ target copies in 1 mL based on a plasmid-generated standard curve. The newly developed real-time PCR was able to detect $3 \times 10^3$ target copies per 5 µL ($1.2 \times 10^5$ target copies in one mL of blood) of extracted genomic DNA. This exactly corresponded to the results obtained with plasmid dilutions contained in the DNA of carp blood. The real-time PCR successfully detected all 8 $M. suis$ isolates tested. None of the bacterial species other than $M. suis$ was identified during the 40-cycle PCR run. The intra- and inter-assay repeatability of the real-time PCR is shown in Table 2.

The control group of animals did not reveal any disease symptoms during the whole period of observation. $M. suis$ genetic material was not detected in pig 2, unlike in animals 1 and 3. The detectable load of $M. suis$ target copies measured by real-time PCR were $5 \times 10^2$ on the 49th d after infection and $2.4 \times 10^5$ on the 56th d after infection for pig 1 and pig 3 respectively. Fig. 3 shows the concentration of the target copies in the blood of all pigs during the course of the experiment.

All animals tested were negative for $M. suis$ according to the real-time PCR test for the whole period prior to the experimental infection. Pigs 4 and 5 were negative for $M. suis$ infection in two samples (days 4 and 7) while pig 6 was negative in three samples (days 4, 7, and 14) after the experimental infection.

Pig 4 became positive for $M. suis$ infection by real-time PCR and an acute disease was confirmed by day 14. Two weeks after the infection, pig 4 displayed pale skin and mucous membranes, and an increase in temperature ranging from 40.6°C to 41.2°C. The largest number of $M. suis$ target copies measured 21 d after infection by real-time PCR for pig 4 was $1.2 \times 10^{11}$ target copies in 1 mL of blood, which then gradually decreased to $2.4 \times 10^7$ target copies/mL in 56 d after infection (the last day of observation).

Other pigs from the control group were not demonstrating clinical symptoms characteristic for $M. suis$ infection. Detectable levels of $M. suis$ in blood occurred two (pig 5) and three (pig 6) weeks after infection, in the quantity of $6.4 \times 10^5$ and $8 \times 10^6$ target copies/mL respectively. Above mentioned animals presented high concentration (peaks) of $M. suis$ target copies occurring within three-four weeks (Fig. 3). The peak loads of $M. suis$ target copies in pig 5 were observed 21 d ($8 \times 10^7$ target copies/mL of blood) and 49 d after infection ($8 \times 10^8$ target copies/mL of blood). The peak loads of $M. suis$ target copies in pig 6 were observed 28 d ($4 \times 10^8$ target copies/mL of blood) and 49 d after infection ($1 \times 10^9$ target copies/mL of blood).

At the same time when high levels of $M. suis$ were detected (bacteraemia) by real-time PCR (Fig. 3), blood smears were positive for $M. suis$ (pigs 4 and 6, Fig. 3 - marked with arrows, Fig. 4A).

Fig. 1. Standard curve generated by analysis of a 10-fold dilution of plasmid with $M. suis$ 16S rRNA gene in TE by real-time PCR.
Fig. 2. Standard curve generated by analysis of a 10-fold dilution of plasmid with M. suis 16S rRNA gene in TE combined with 30 µL of DNA extracted from blood of carp by real-time PCR.

Fig. 3. Load of target copies/mL of blood in the six experimental pigs (1-3 – control pigs; 4-6 – splenectomised and challenged pigs).

Fig. 4. Blood smears stained using the Hemacolor staining kit
A. + sign – observed in pig 4, 21 d after experimental infection; B. - sign – negative control pig
1, 14 d after beginning of the experiment.
Table 1. Sensitivity of real-time PCR for *M. suis* identification

<table>
<thead>
<tr>
<th>Material</th>
<th>Linearity</th>
<th>Detection limit: plasmid/reaction; (number of positive results/number of repetitions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid diluted in 1 x TE</td>
<td>R ≥ 0.997</td>
<td>10^3; (5/5)</td>
</tr>
<tr>
<td>Plasmid diluted in a carp blood DNA</td>
<td>R ≥ 0.999</td>
<td>10^3; (5/5)</td>
</tr>
</tbody>
</table>

Table 2. Intra-assay and inter-assay repeatability of real-time PCR

<table>
<thead>
<tr>
<th>Plasmid copies</th>
<th>Intra-assay repeatability</th>
<th>Inter-assay repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean-crossing point (Ct ± SD)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>10^3</td>
<td>36.95 ± 0.19</td>
<td>0.51</td>
</tr>
<tr>
<td>10^4</td>
<td>33.7 ± 0.12</td>
<td>0.36</td>
</tr>
<tr>
<td>10^5</td>
<td>30.22 ± 0.11</td>
<td>0.36</td>
</tr>
<tr>
<td>10^6</td>
<td>26.72 ± 0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>10^7</td>
<td>23.24 ± 0.05</td>
<td>0.21</td>
</tr>
<tr>
<td>10^8</td>
<td>19.62 ± 0.08</td>
<td>0.41</td>
</tr>
<tr>
<td>10^9</td>
<td>16.44 ± 0.27</td>
<td>1.64</td>
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CV – coefficient of variation  
SD - standard deviation

Discussion

The most common methods which enable detection of *M. suis* in diagnostic laboratories are techniques based on stained blood smears. However, due to their low sensitivity, specificity, and reproducibility they are often replaced with methods based on PCR.

Among the classic methods of PCR, which allow detection of *M. suis*, the use of KSU-2 DNA (4), 16S rRNA gene (2, 14, 16), and 1.8 kb EcoRI (10) was previously reported. Some of these molecular methods have been implemented into a routine diagnostic assay. Although the sensitivity of these methods, as declared by their authors, is often satisfactory, they still do not allow estimation of the initial quantity of *M. suis* in a sample. Classical PCR methods do not provide the possibility to distinguish between animals with acute form of the disease from its chronic form or the carrier pigs. Real-time PCR has several advantages over conventional PCR. The quantification of numbers of *M. suis* loads in the different phases of infection is an important value to correlate with severity of PE. It focuses on the logarithmic phase of product accumulation rather than on the end product abundance. Therefore, it is more accurate since it is less affected by amplification efficiency or depletion of a reagent. In addition, it has an increased dynamic range for quantification of target sequences.

The present study focused on the development of the quantitative PCR assay, for which the TaqMan MGB probe was used. The MGB moiety affects an increase in specificity and sensitivity of real-time PCR (13). The detection limit of the real-time PCR for plasmid combined with blood DNA was determined to be 10^3/reaction (5 µL of DNA) (1.2 × 10^5 target copies in one mL of blood), which is comparable to the results presented by Guimaraes et al. (4) when the template DNA came from blood. The linear regression of real-time PCR, especially with DNA extracted from blood, was very close to the value 1. This indicates a high linearity of real-time PCR method and the possibility of its use as a quantitative method. The correspondence between the quantification results obtained with use of genomic (serially diluted positive sample of experimental pig) and plasmid DNA (serially diluted plasmid in blood DNA) was found.

Due to PCR inhibitors during the extraction procedure, the comparison of positive control - plasmid spiked with DNA extracted from the blood and solely...
plasmid samples - indicated that the efficiency of real-time PCR applied in our studies is affected by inhibitors derived from the blood. The sensitivity of the real-time PCR was ten-fold lower when plasmid combined with blood DNA was used.

The positive advantage of real-time PCR used in the studies was confirmed by the low intra- and inter-assay coefficients of variation of about 0.15 to 1.64 and 0.27 to 1.92 respectively. These parameters allow the applicability of the assay in other laboratories.

On the basis of the study with experimental animals, identification of the pathogen and defined quantities of M. suis in 1 mL of blood were confirmed. Splenectomised and infected animals were characterised by the presence of high and medium quantities of M. suis, whereas only one pig presented clinical symptoms and the other two were defined as infected without visible clinical symptoms of the disease.

The detection of carrier animals is correlated with sensitivity of the method (1, 4, 8, 12, 15, 17). In the present study, it was possible to detect a massive presence of M. suis in blood by examination of stained blood smears. On the basis of worldwide knowledge and due to the use of sensitive PCR methods (4, 15), the widespread prevalence of asymptomatic carriers were confirmed. This provides an explanation why two blood samples derived from control animals (not experimentally infected) from the standard farm were the ones with low positive results available for this method.

Our real-time PCR method can be useful for quantification of M. suis and identification of acutely infected pigs. The peak of bacteraemia, the acutely infected animal in this study showed total load of $1.2 \times 10^{11}$ target copies/mL$^-1$ of blood. In the blood smear evaluations, this pig had a mean of ~13 organism/erythrocyte (based on analysis of five blood smears of samples taken from pig 4 (21 d after infection) – Fig. 4A). Given the known total erythrocyte count in this sample – $4.95 \times 10^{10}$ mL$^-1$, an approximate number of organisms/mL$^-1$ of blood, was estimated at $-6.5 \times 10^{10}$. This calculated value is lower than the value obtained by real-time PCR. This value does not include free organisms in the background (not attached to red blood cells) thus this can explain the difference in the estimated amount of M. suis. The results of this study are much closer to the previous one (4), which reported an average load of $6.3 \times 10^{10}$ organisms/mL$^-1$ of blood than the results obtained by Hoelzle et al. (12) – with $1.5 \times 10^{10}$ organisms/mL$^-1$ of blood in acute form of the disease.

In conclusion, real-time PCR was sensitive and specific for the detection and quantification of M. suis in the blood of animals. As M. suis infection can cause non-specific clinical symptoms. However, the developed real-time PCR cannot be used to detect carrier animals with a small amount of M. suis in blood.

In addition, this technique may be also used in the further studies on cultivation of M. suis in vitro.

Acknowledgements: This work was supported by a grant N N308 096537 from the Polish Ministry of Science and Higher Education.

References


