Original article

Application of real-time PCR for detection of *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* in fecal samples from pigs

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Abstract

The aim of the study was to develop and validate real-time PCR method for the quantification of *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* in porcine feces. Before the optimization process was performed two different extraction methods were compared to select the more efficient one. Based on the results achieved at this stage the boiling procedure was rejected and a commercially available silica-membrane based method was chosen for further analysis. The primers and the Taqman probe for *B. hyodysenteriae* and *L. intracellularis* were based on the sequence of NADH oxidase gene and 16S rDNA gene, respectively. The detection limit of the real-time PCR for suspension of feces inoculated with *B. hyodysenteriae* and *L. intracellularis* was determined to be 1.5×10^3 CFU/ml and 6.5×10^1 CFU/ml, respectively. The results of this study demonstrate that our real-time PCR is able to detect low number of *B. hyodysenteriae* and *L. intracellularis* cells which is satisfying in routine diagnosis of swine dysentery and proliferative enteropathy. Therefore, it is possible to identify both subclinically infected pigs and those representing an acute form of mentioned diseases. In summary, the quantitative real-time PCR is useful for routine diagnosis of *L. intracellularis* and *B. hyodysenteriae*. Compared to conventional PCR, the new validated quantification method based on real-time PCR is fast and with reduced risk of laboratory contamination. The novel technique is specific and even more sensitive than the previously used one. Furthermore, the new real-time PCR enables quick detection and quantification of both pathogens in fecal samples, which helps to estimate the health status of a pig herd.

Key words: Real-time PCR, DNA extraction, *Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, feces

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Introduction

Proliferative enteropathy (PE) caused by *Lawsonia intracellularis* (*L. intracellularis*) and swine dysentery (SD) with causative agent *Brachyspira hyodysenteriae* (*B. hyodysenteriae*) are considered to be one of the most important intestinal diseases in pig production worldwide. The diseases are mainly characterized by hemorrhagic diarrhea affecting pigs after weaning and in all phases of finishing (Hampson et al. 2006, McOrist et al. 2006).

Previously, the diagnosis of PE and SD was mainly based on PCR. Although conventional PCR is a specific and highly sensitive method, processing a large number of samples is time consuming. Furthermore, the detection of the PCR product by gel electrophoresis requires additional time to obtain the final result. Also, several steps in the PCR analysis may lead to laboratory contamination.

L. intracellularis and *B. hyodysenteriae* are quite difficult to cultivate and the methods used to grow the bacteria are not adaptable for routine procedures. In case of *L. intracellularis in vitro* cultivation is possible only in cell culture (most recommended is mouse fibroblasts McCoy cell line – ATTC CRL 1696) (Yeh et al. 2006). The other *ante mortem* methods confirming the presence of *L. intracellularis*, such as dot blot hybridization, histopathology or IFT (immunofluorescence testing) using specific monoclonal antibodies (Jensen et al. 1997), cannot be applied to fecal samples.

The isolation of *B. hyodysenteriae* is performed in anaerobic conditions, on TSA agar plates supplemented with 5% defibrinated ovine blood, spectinomycin and vancomycin. This method is the only alternative to PCR for detection of *B. hyodysenteriae* in fecal specimens (Szynkiewicz and Binek 1986). Laboratory identification of both bacteria is laborious and often takes several days or even weeks to receive the definitive result.

To reduce the cost and time for identification of the above pathogens related with conventional bacteriological procedures and to shorten the time of PCR procedure by eliminating labour-intensive electrophoresis, a real-time PCR was implemented to replace conventional PCR technique. Therefore, the aim of the study was to develop and validate real-time PCR method for the quantification of *L. intracellularis* and *B. hyodysenteriae* in porcine feces.

Materials and Methods

Clinical samples and reference strains. For optimization of real-time PCR clinical material (feces and sections of intestines: ileum, colon and caecum) from a pig herd considered to be free from *B. hyodysenteriae* and *L. intracellularis* infections was collected. The status of the farm was confirmed by conventional PCR as described earlier (Żmudzki et al. 2004). A reference strain of *B. hyodysenteriae* B204 (ATCC 31212) at concentration 1.5×10^8 CFU/ml and a filtrate of reference strain *L. intracellularis* (ATCC 55783) containing 6.5×10^6 CFU/ml, kindly provided by National Veterinary Institute (DTU) in Copenhagen, Denmark, were also used in the study.

Optimization of sample preparation. The optimization was performed on the samples of swine feces contaminated with *B. hyodysenteriae* reference strain B204. In parallel, feces were mixed with a filtrate of *L. intracellularis.* For extraction of DNA from fecal samples two different methods were tested. In 1st method bacterial culture and fecal samples were boiled in 99ºC for 10 minutes. In the 2nd method the same kind of samples was processed using commercial isolation kit (Genomic Mini, A&A Biotechnology, Gdynia, Poland), according to the manufacturer's recommendations. In the beginning, for both methods, 10 tubes containing 90 µl of 10% suspension of feces in physiological salt solution were prepared. Next, one tube was inoculated with 2 fool loops of *B. hyodysenteriae* B204, corresponding to the concentration of 1.5×10^8 CFU/ml (Kunkle and Kinyon 1988). In the last step 10-fold dilutions of the mixture were prepared for DNA extraction. Analogically, feces suspension was inoculated with a filtrate of *L. intracellularis* containing 6.5×10^6 CFU/ml and 10-fold dilutions were prepared as described above.

To define the initial concentration of *B. hyodysenteriae* and to determine standard curve plot, a calculation of the number of colony forming units of *B. hyodysenteriae* per milliliter (CFU/ml) was performed as follows: 2 ml of physiological salt solution was inoculated with 2 well-filled plastic loops (1 µl) of *B. hyodysenteriae* B204 suspension and 10-fold dilutions of the bacterial suspension were prepared. The samples were stained with trypan blue (Sigma-Aldrich, St. Louis, Missouri, USA) in a proportion of 1:2 and transferred to Burker's chamber. The number of *B. hyodysenteriae* cells was counted under microscope (100x magnification).

In case of *L. intracellularis* the optimization procedure was identical as for *B. hyodysenteriae*, except for estimation of the number of cells. A ready-to-use filtrate of *L. intracellularis* containing 6.5×10^6 CFU/ml was thoroughly mixed with 10% suspension of feces and then 10-fold dilutions of the mixture were further processed for DNA isolation using the 1st and the 2nd method. After DNA extraction, samples were

assayed in the Stratagene detection system (Stratagene, Mx3005P QPCR, La Jolla, California, USA) and the differences in Ct values between method I and II were determined.

Sensitivity of the real-time PCR. The detection limit of the PCR assay was estimated on the basis of the diluted samples prepared as above. For the determination of detection limit and repeatability of the assay the dilution experiments were repeated 5 times (Table 1). The following range of dilutions was tested: 10^2-10^4 for *B. hyodysenteriae* and 10^1-10^3 for *L. intracellularis*. Analogically, 10-fold dilutions of pure culture and feces suspension inoculated with *B. hyodysenteriae* and *L. intracellularis* were analyzed by conventional PCR (Żmudzki et al. 2004) and its results were compared to real-time PCR.

Specificity of the real-time PCR. The evaluation of specificity was tested with the use of the following bacterial strains: *Lawsonia intracellularis* (ATCC 55783), *Brachyspira hyodysenteriae* B204 (ATCC 31212), *Brachyspira innocens* (ATCC 29796), *Brachyspira pilosicoli* P43/6/78T (ATCC 51139), *Brachyspira murdochii* 155-20T (ATCC51284), *Brachyspira intermedia* PWS/AT (ATCC 51140), *Clostridium perfringens* (ATCC 13124), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), *Escherichia coli* (clinical isolate, identified by multiplex PCR – Osek 2001).

Primers and probe. The primers and the Taqman probe for *B. hyodysenteriae* were based on the sequence of NADH oxidase gene (GenBank accession no. U19610), previously described by Akase et al. (2009). The primers and the probe for *L. intracellularis* used in the study were previously designed by Lindecrona et al. (2002) and based on the sequence of 16S rDNA gene of *L. intracellularis* available in GenBank (accession No. L15739).

Real-time reaction mixture and parameters of the assay. The assay was carried out in the Mx3005P QPCR System (Stratagene, La Jolla, California, USA). The amplification mixture consisted of 5 µl template DNA and a QuantiTect mastermix (Qiagen, Hilden, Germany) containing 8 mM $MgCl_2$, dNTP mix and HotStarTaq DNA polymerase, in a total volume of 25 µl. The concentration of the primers and the probes for both bacteria were the same and equaled to 20 µM. Amplification of *B. hyodysenteriae* was performed in a 96-well plate with optical caps at the following settings: 10 min at 95ºC, 50 cycles of 15 s at 95ºC and 1 min of annealing and extension at 52ºC. For *L. intracellularis* the parameters were as follows: 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min of annealing at 52ºC and 1 min of extension at 62ºC.

Statistical analysis. Difference in Ct values between sample preparation methods were determined by Student's *t*-test with a significance threshold of $P < 0.05$. Linear regression was used to determine sensitivity of the real-time PCR.

Results

Optimization of sample preparation. A comparison of the efficacy of DNA extraction methods showed no significant differences between 1st and 2nd method for both bacteria. The average Ct values for samples prepared by the 1st method (boiling: bacterial culture and boiling: bacterial culture + feces) for *B. hyodysenteriae* were 3.39 (SD, 1.16) and 3.56 (SD, 1.31), respectively. In 2nd method (mini spin column: bacterial culture and mini spin column: bacterial culture + feces) average Ct values were 3.58 (SD, 1.39) and 3.56 (SD, 0.83), respectively. Analogically, average Ct values for *L. intracellularis* for 1st method were: 2.97 (SD, 0.42) for pure bacterial culture and 2.69 (SD, 1.01) for bacterial culture with feces. In the 2nd method the average Ct values were 3.95 (SD, 1.66) and 3.01 (SD, 1.09), respectively. Considering the optimal average Ct values (Ct 3.56 for *B. hyodysenteriae* with linear regression R2≥0.99 and Ct 3.01 for *L. intracellularis* with R2≥1.0) of analyzed bacteria, 2nd method was selected for the further studies (Fig. 1).

Sensitivity of the real-time PCR. The detection limit of *B. hyodysenteriae* in bacterial culture was 1.5×10^2 CFU/ml, with Ct value of 38.4. However, the linearity of reaction was achieved only up to 1.5×10^3 CFU/ml, with Ct value of 38.8 and linear regression $R^2 \geq 0.99$ (Fig. 2). In suspension of feces inoculated with *B. hyodysenteriae* the detection limit was $1.5 \times 10_3$ CFU/ml (Ct value 37.3) and linearity was maintained ($R^2 \geq 0.99$). Similarly, the detection limit of *L. intracellularis* in bacterial filtrate was 6.5×10^1 CFU/ml (Ct value 38.9) and linearity was accomplished at concentration 6.5×10^2 CFU/ml (Ct value 35.0; $R^2 \geq 1.0$). In suspension of feces inoculated with *L. intracellularis* the detection limit was 6.5×10^1 CFU/ml (Ct value 36.9) and linearity was obtained at concentration 6.5×10^2 CFU/ml (Ct value 35.5; $R^2 31.0$). For conventional PCR the detection limit in pure bacterial culture and feces suspension inoculated with *B. hyodysenteriae* was 1.5×10^3 CFU/ml (Fig. 3). Analogically, the detection limit for bacterial filtrate and feces

Table 1. Sensitivity of real-time PCR for *B. hyodysenteriae* and *L. intracellularis* identification.

Biological material	Linearity ($R^2 \geq 0.98$)		Detection limit: CFU/ml; (number of positive results/number of repetitions)	
	B. hyodysenteriae	*L. intracellularis*	*B. hyodysenteriae*	*L. intracellularis*
Bacterial culture	Tested concentrations: 10^8-10^3 (Ct 18.4-38.8) $R^2 \geq 0.99$	Tested concentrations: 10^6-10^1 (Ct 21.2-38.9) $R^2 \geq 1.00$	10^4; (5/5) 10^3; (5/5) 10^2; (1/5)	10^3; (5/5) 10^2; (5/5) 10^1; (3/5) 10^0; (0/5)
Feces + bacterial culture	Tested concentrations: 10^8-10^3 (Ct 19.5-37.4) $R^2 \geq 0.99$	Tested concentrations: 10^5-10^2 (Ct 24.0-35.5) $R^2 \geq 1.00$	10^4; (5/5) 10^3; (5/5) 10^2; (0/5)	10^3; (5/5) 10^2; (5/5) 10^1; (2/5) 10^0; (0/5)
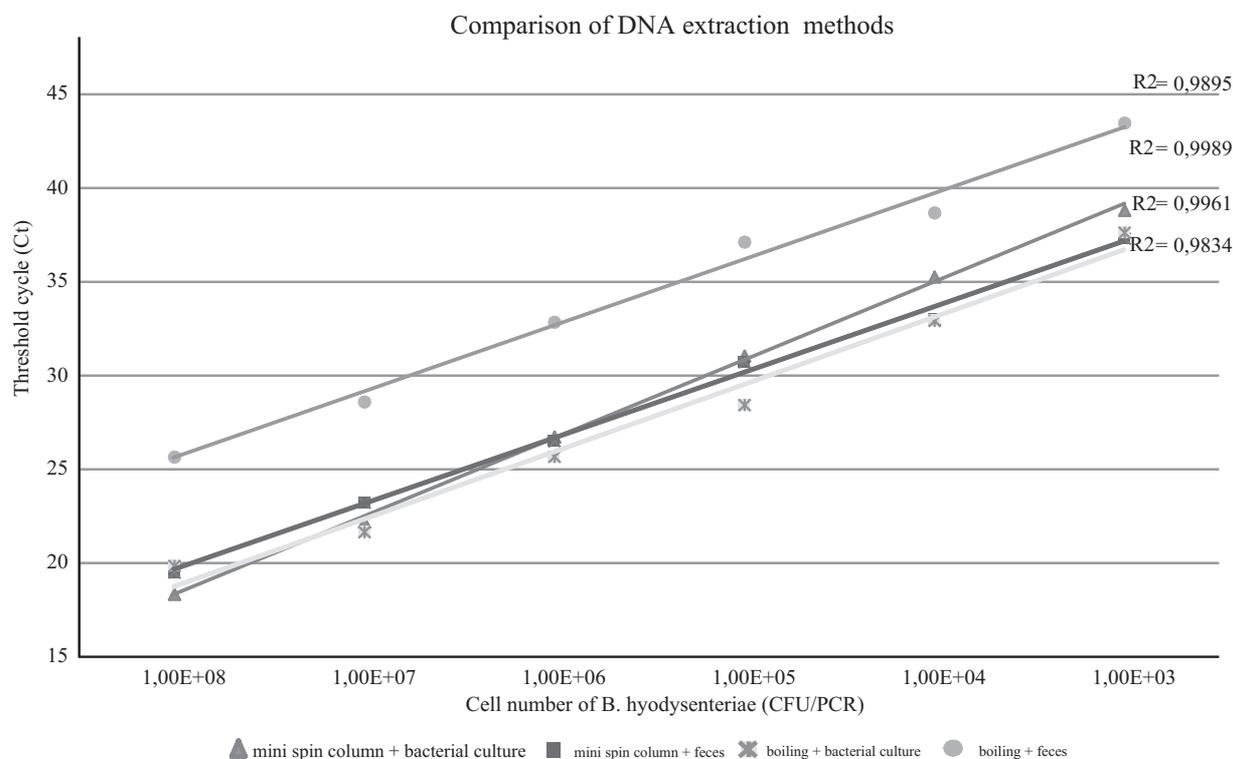
Fig. 1. Comparison of DNA extraction methods: boiling (1st method) and mini spin column procedure (2nd method) using 10-fold dilutions of *B. hyodysenteriae* (10^8-10^3).
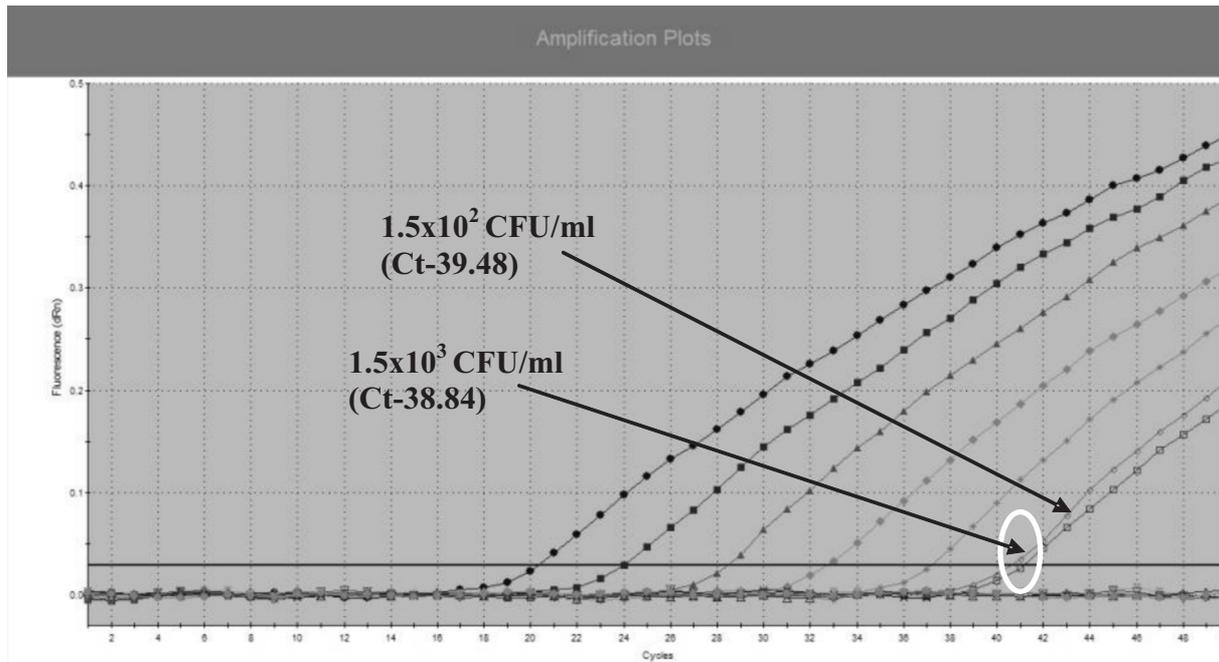
Fig. 2. Standard curve generated by analysis of a 10-fold dilution series of *B. hyodysenteriae* spiked in feces solution by real-time PCR.
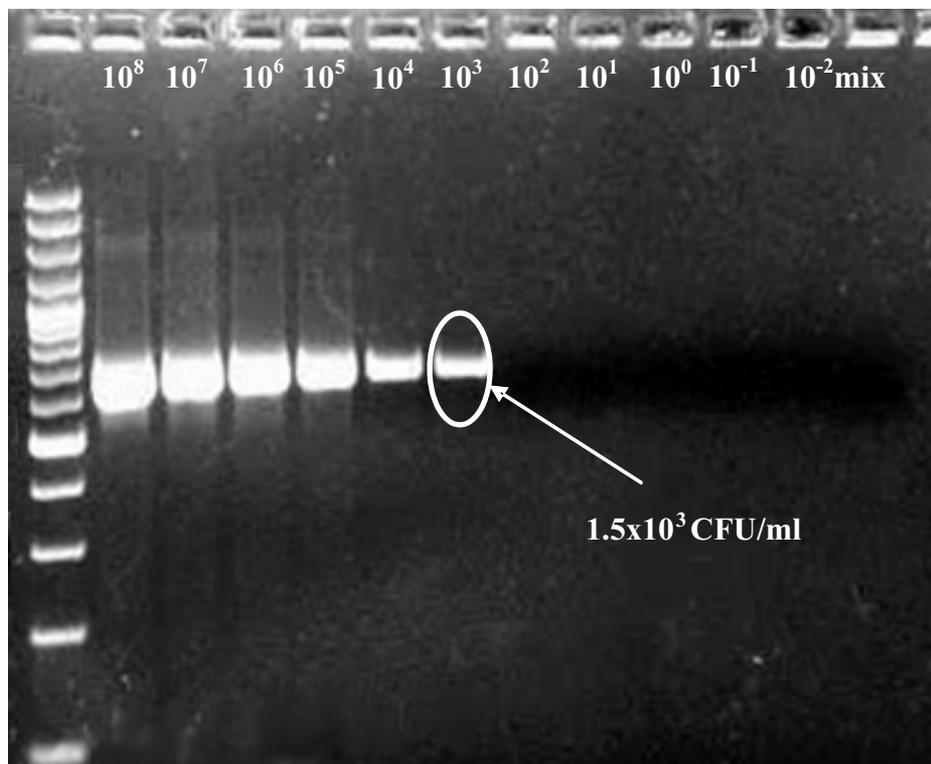

Fig. 3. Identification of 10-fold dilutions of *Brachyspira hyodysenteriae* culture using conventional PCR technique.

suspension inoculated with *L. intracellularis* was 6.5×10^2 CFU/ml.

Specificity of the real-time PCR. None of the bacterial species other than *B. hyodysenteriae* and *L. intracellularis* has given signal up to 40th and 50th PCR cycle, respectively.

Discussion

Real-time PCR has several advantages over conventional PCR. 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 reagents. In addition, it has an increased dynamic range for quantification of target sequence (at least 5 orders of magnitude). Furthermore, with no post-PCR manipulation of the samples, the danger of cross-contamination between samples is greatly reduced. Finally, real-time PCR results can be obtained within 1 h.

The real-time PCR for identification of *B. hyodysenteriae* and *L. intracellularis* developed in this study proved to be faster and more efficient compared to the conventional PCR for *ante mortem* diagnosis of SD and PE. In contrast to some commonly used conventional PCR protocols, performed inseparably in combination with time-consuming gel electrophoresis, this method enhances a reliable quantification and substantially shortens the time of laboratory work.

One of the most important advantages of PCR for the identification of *B. hyodysenteriae* and *L. intracellularis* is lack of limitations associated with standard bacteriological techniques and complicated *in vitro* cultivation methods. Moreover, detection of these microorganisms with the use of a 5' nuclease assay is not affected by viability of bacterial cells, which is always a concern of standard cultivation procedures due to antimicrobial residues present in intestinal specimens. In this context, real-time PCR, compared to conventional PCR, offers even wider spectrum of benefits, like reduced risk of contamination, quantification of pathogens in a sample based on data collected in exponential growth phase and quick results with no-post PCR processing. Taking into account all above advantages we decided to implement a new diagnostic tool to routine laboratory diagnostic of intestinal infections in pigs.

Comparing to conventional PCR, real-time PCR is more precise tool for the quantification of fecal bacterial levels, including greater resolution and reproducibility of results. However, both techniques require the efficient extraction of DNA from fecal samples which is problematic due to the complexity of gut microflora and the presence of PCR inhibitors. Moreover, DNA isolation from clinical material can be inefficient because fecal samples are usually not homogeneous and the inhibitory substances in feces can affect the amplification process (Jacobson et al. 2004). Therefore, in the first step of this project, two extraction methods were tested in order to select the more efficient one. Although average Ct values for both analyzed variants of extractions were similar and statistically no differences were observed, a commercially available Genomic Mini kit, based on silica-membrane, was chosen for the further analysis. The choice was supported by the results obtained by other authors (Song et al. 2004, Freschi et al. 2005, Nelson

et al. 2010). Pilot studies performed by Song et al. (2004) have confirmed that the use of commercial kit for DNA extraction produces high-quality DNA free of PCR-inhibiting substances. The same author in several experiments proved that silica membrane-based detection methods are strongly recommended due to their high specificity, sensitivity and reproducibility. Also, in opinion of Nelson et al. (2010) commercial fecal DNA extraction kits, developed particularly to overcome problems associated with incompletely removed fecal inhibitors, are effective and guarantee highly repeatable results. These findings are in agreement with our experience (data not shown) that bacterial DNA isolated by silica membrane-based methods is better purified and of higher quality. Finally, according to studies performed by Freschi et al. (2005) the efficiency of PCR performed on samples subjected to the boiling-centrifugation DNA extraction method was very similar to those achieved by using the commercial kit. However, the speed and low cost of the boiling-centrifugation technique must be emphasized. In contrast, in opinion of this author DNA samples prepared by boiling-centrifugation were more easily degraded and could not be stored for long periods at 4°C. This indicates that such samples are of limited value and cannot be included in retrospective studies. For this reason commercial kits for DNA isolation should be recommended when long-term storage of the extracted DNA is required.

Comparing the sensitivity of conventional and real-time PCR for identification of *B. hyodysenteriae*, the difference of one logarithm in favor of the second method was only observed in samples containing pure culture. For samples representing clinical material, where *B. hyodysenteriae* culture was mixed with feces suspension, the detection limit (1.5×10^3 CFU/ml) was on the same level in both PCR methods. To compare these results with the infection status of pigs it should be emphasized that the number of spirochetes in dysenteric feces comes to 2×10^6 CFU/ml (Elder et al. 1994). Also, in a challenge study performed by Neef et al. (1994) where gnotobiotic pigs were infected with *B. hyodysenteriae*, *B. innocens* and P43 strain of *B. pilosicoli*, the number of spirochetes in feces from 5 to 10 days post infection varied from 1×10^5 to 2×10^9 per gram of feces. The numbers did not differ significantly in pigs with and without clinical signs of the disease. Kunkle and Kinyon (1988) reported similar numbers of *B. hyodysenteriae* at the onset of swine dysentery.

The results of this study suggest that our real-time PCR is able to detect lower number of *B. hyodysenteriae* than excreted by clinically ill animals, therefore it is possible to identify both subclinically infected pigs and those representing an acute form of the disease.

It must be emphasized that Ct values of clinical samples can be easily converted to number of bacterial cells, which provides an approximate information concerning the health status of an animal. This data is important for field veterinarians, suggesting further strategy of medication or vaccination on a farm (Nathues et al. 2009).

The detection limit of the real-time PCR for suspension of feces inoculated with *L. intracellularis* was determined to be 6.5×10^1 CFU/ml, which is comparable to the results presented by Lindecrona et al. (2002). In the opinion of this author, the sensitivity of the 5; nuclease assay is equal to a fecal excretion of approximately 4×10^4 *L. intracellularis* cells per 1 g of feces. For comparison, challenge experiments performed by Smith et al. (1997) have shown that pigs may excrete *L. intracellularis* from 2 to 10 weeks after infection, in the range of 5×10^4 to 7×10^8 organisms per 1 g of feces. Having in mind the above range of *L. intracellularis* cells excreted in fecal samples, our real-time PCR is able to detect a relatively low number of *L. intracellularis* cells which is satisfying in routine diagnosis of PE.

The results of this study demonstrated that quantitative real-time PCR is useful for routine diagnosis of *L. intracellularis* and *B. hyodysenteriae*. Compared to conventional PCR, the new validated quantification method based on real-time PCR is fast and offers reduced risk of laboratory contamination. The novel technique is specific and sensitive. Furthermore, the real-time PCR enables quick detection and quantification of both pathogens in fecal samples, which helps to estimate the health status of a pig herd.

Acknowledgements

References

Akase S, Uchitani Y, Sohmura Y, Tatsuta K, Sadamasu K, Adachi Y (**2009**) Application of real time PCR for diagnosis of Swine Dysentery. J Vet Med Sci 71: 359-362.

Elder RO, Duhamel GE, Schafer RW, Mathiesen MR, Ramanathan M (**1994**) Rapid detection of *Serpulina hyodysenteriae* in diagnostic specimens by PCR. J Clin Microbiol 32: 1497-1502.

Freschi CR, Carvalho LF de Oliveira CJ (**2005**) Comparison of DNA-extraction methods and selective enrichment broths on the detection of *Salmonella typhimurium* in swine feces by polymerase chain reaction (PCR). Braz J Microbiol 36: 363-367.

Hampson DJ, Fellström C, Thomson JR (**2006**) Swine dysentery. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (eds) Diseases of Swine. 9th ed., Wiley-Blackwell Publishing, USA, pp 785-805.

Jacobson M, Aspan A, Königsson MH, Segerstad CH, Wallgren P, Fellström C, Jensen-Waern M, Gunnarson A (**2004**) Routine diagnostics of *Lawsonia intracellularis* performed by PCR, serological and post mortem examination, with special emphasis on sample preparation methods for PCR. Vet Microbiol 102: 189-201.

Jensen TK, Möller K, Leser TD, Jorsal SE (**1997**) Comparison of histology, immunohistochemistry and polymerase chain reaction for detection of *Lawsonia intracellularis* in natural porcine proliferative enteropathy. Eur J Vet Pathol 3: 115-123.

Kunkle RA, Kinyon JM (**1988**) Improved selective medium for the isolation of *Treponema hyodysenteriae*. J Clin Microbiol 26: 2357-2360.

Lindecrona RH, Jensen TK, Andersen PH, Möller K (**2002**) Application of a 5' nuclease assay for detection of *Lawsonia intracellularis* in fecal samples from pigs. J Clin Microbiol 40: 984-987.

McOrist S, Gebhart CJ (**2006**) Proliferative enteropathies. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (eds) Diseases of Swine. 9th ed., Wiley-Blackwell Publishing, USA, pp 727-737.

Nathues H, Holthaus K, Beilage E (**2009**) Quantification of *Lawsonia intracellularis* in porcine faeces by real-time PCR. J Appl Microbiol 107: 2009-2016.

Neef NA, Lysons RJ, Trott DJ, Hampson DJ, Jones PW, Morgan JH (**1994**) Pathogenicity of porcine intestinal spirochetes in gnobiotic pigs. Infect Immun 62: 2395--2403.

Nelson EA, Palombo EA, Knowles SR (**2010**) Comparison of methods for the extraction of bacterial DNA from human faecal samples for analysis by real-time PCR. In: Méndez-Vilas A (ed) Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex, Microbiology Series Nº 2 Vol. 1, C/ Zurbarhn 1, 2º – Oficina 106002 Badajoz, Spain, pp 1479-1485.

Osek J (**2001**) Multiplex polymerase chain reaction assay for identification of enterotoxigenic Escherichia coli strains. J Vet Diagn Invest 13: 308-311.

Smith SH, McOrist S (**1997**) Development of persistent intestinal infection and excretion of *Lawsonia intracellularis* by piglets. Res Vet Sci 62: 6-10.

Song Y, Liu C, Finegold SM (**2004**) Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol 70: 6459-6465.

Szynkiewicz ZM, Binek M (**1986**) Evaluation of selective media for primary isolation of *Treponema hyodysenteriae* and *Treponema innocens*. Comp Immunol Microbiol Infect Dis 9: 71-77.

Yeh JY, Kim TJ, Park SY, Song CS, Yoon YD, Kim SK, Lee JB, Choi IS (**2006**) Isolation of *Lawsonia intracellularis* in Korea and reproduction of proliferative enteropathy in pigs and hamsters. J Vet Med Sci 68: 499-501.

Żmudzki J, Osek J, Stankevicius A, Pejsak Z (**2004**) Rapid detection of *Brachyspira hyodysenteriae* and *Lawsonia intracellularis* in swine faecal and mucosal specimens by multiplex PCR. Bull Vet Inst Pulawy 48: 207-214.