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Detection of *Actinobacillus pleuropneumoniae* in pigs by real-time quantitative PCR for the *apxIVA* gene

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ABSTRACT

A real-time quantitative PCR (qPCR) for detection of the *apxIVA* gene of *Actinobacillus pleuropneumoniae* was validated using pure cultures of *A. pleuropneumoniae* and tonsillar and nasal swabs from experimentally inoculated Caesarean-derived/colostrum-deprived piglets and naturally infected conventional pigs. The analytical sensitivity was 5 colony forming units/reaction. In comparison with selective bacterial examination using tonsillar samples from inoculated animals, the diagnostic sensitivity of the qPCR was 0.98 and the diagnostic specificity was 1.0. The qPCR showed consistent results in repeatedly sampled conventional pigs. Tonsillar brush samples and *apxIVA* qPCR analysis may be useful for further epidemiological studies and monitoring for *A. pleuropneumoniae*.

Introduction

Actinobacillus pleuropneumoniae is one of the major causes of respiratory disease in pigs and is established as an endemic infection on most commercial pig farms (Sorensen et al., 2006). Infection with *A. pleuropneumoniae* is often subclinical, but sometimes results in severe clinical signs, growth retardation or mortality, causing welfare problems and substantial economic losses (Gottschalk and Taylor, 2006). An accurate diagnosis is essential for proper treatment and for implementation of disease control programmes.

Selective bacterial examination (SBE) can be used for detection of *A. pleuropneumoniae* in tonsillar or nasal swab samples collected from live pigs. However, suspected *A. pleuropneumoniae* colonies tend to be overgrown by other bacterial species and it can be difficult to differentiate between *A. pleuropneumoniae* and other *Pasteurellaceae* from the oropharyngeal cavity (Moller et al., 1996). Thus, the sensitivity and specificity of tests based on culture for detection of *A. pleuropneumoniae* are low.

A number PCR targets have been described for identification or serogrouping of *A. pleuropneumoniae* (Chiers et al., 2001; Jessing et al., 2003; Schuchert et al., 2004; Tonpitak et al., 2007; Angen et al., 2008; Zhou et al., 2008; Ito, 2010). Several conventional PCRs have been described for qualitative detection of *A. pleuropneumoniae* DNA in nasal or tonsillar swabs, or from tissue samples, such as tonsil or lung (Lo et al., 1998; Savoye et al., 2000; Fittipaldi et al., 2003; Serrano-Rubio et al., 2008). When studying the epidemiology or pathogenesis of *A. pleuropneumoniae*, it is often useful to obtain quantitative data on the pathogen load in individual pigs. Furthermore, the use of real-time PCR would enable high throughput sample analysis compared to SBE or conventional PCR.

The aim of this study was to develop a real-time PCR for direct quantitative detection of *A. pleuropneumoniae* DNA in live pigs and to evaluate the test with samples from experimentally infected pigs or conventionally reared pigs originating from *A. pleuropneumoniae* infected farms.

Materials and methods

PCR primers, probes and amplification protocol

The *apxIVA* gene was chosen as a suitable qPCR target gene because this gene is specific for all *A. pleuropneumoniae* serotypes and because information on this gene is available from qualitative PCR tests (Cho and Chae, 2003; Fittipaldi et al., 2003). PCR was performed using two primers described previously, APXIVANEST1-F and APXIVANEST1-R (Schaller et al., 2001). A conserved sequence within the predicted PCR product, identified with BLAST,[1] was used to design a TaqMan probe (Table 1). The real-time PCR was performed in a total volume of 25 μL per well with 0.2 μM each primer and probe in Tris ethylene diamine tetraacetic acid (10 mM Tris HCl, 1 mM EDTA, pH 8.0), 2× Premix Ex Taq (TaKaRa), 0.5 μL PCR grade water and 10 μL template (from 200 μL Instagene purification, as indicated below) or 10 μL saline as a negative control. The template consisted of DNA extracts (from samples or standards) or negative controls. A BioRad iQ5 thermocycler was used for qPCR analysis. The PCR programme consisted of an initial denaturation (60 s at 95 °C), followed by 40 cycles of 10 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C.

Quantification of *A. pleuropneumoniae* colony forming units (CFUs) in samples was performed using the iQ5 algorithm. A standard curve was included by duplicate testing of three standard samples of a dilution of *A. pleuropneumoniae* serotype

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[1] See: http://blast.ncbi.nlm.nih.gov/blast.

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Table 1
Sequences of primers and fluorescent probe for detection of *Actinobacillus pleuropneumoniae* by quantitative PCR (qPCR).

Name	Sequence	Location[a]	Product size (bp)	Reference
apxIVANEST1-F	5′-GGGGACGTAACTCGGTGATT-3′	6050–6069	377	Schaller (2001)
apxIVANEST1-R	5′-GCTCACCAACGTTTGCTCAT-3′	6427–6407		Schaller (2001)
apxIVAPr (probe)	5′-FAM-CGGTGCGGACACCTATATCT-BHQ1-3′	6162–6182		This study

bp, base pairs.
[a] Nucleotide location in AF021919.

1536, consisting of 5×10^1, 5×10^3 and 5×10^5 CFUs, respectively. The optimal threshold of fluorescence was determined as a cut-off for optimal reaction efficiency within the assay. Samples were considered to be positive when duplicates showed comparable results and the increase in fluorescence had a sigmoid curve.

Bacterial strains and growth conditions

Bacteria were grown at 37 °C in an atmosphere of 5% CO_2 on sheep blood agar (SBA) (Biotrading) or heart infusion agar with 5% sheep blood (HIS) and, depending on their β-nicotinamide adenine dinucleotide (NAD) requirement, supplemented with 0.05% NAD (AppliChem GmbH) (HIS-V plates). Casitone–glucose–vitamin HIS agar plates supplemented with NAD and Clindamycin, Gentamycin, Vancomycin and Amphotericine B (CGVA). were employed for selective culture of β-NAD-dependent *Pasteurellaceae* (Velthuis et al., 2003), using three 10-fold serial dilutions per sample. After detection and quantification of *A. pleuropneumoniae*-like colonies with SBE, two colonies suspected to be *A. pleuropneumoniae* on the basis of colony morphology were selected from each primary plate and subcultured on HIS-V plates. Isolates were confirmed as *A. pleuropneumoniae* by positive satellite growth, a positive Christie–Atkins–Munch–Petersen (CAMP) reaction near a streak of *Staphylococcus aureus* on SBA, urease activity and mannitol fermentation in the appropriate test medium with added β-NAD (Frey, 2003).

Samples and sample handling

Validation tests were performed with samples from pure cultures, tonsillar and nasal samples from Caesarean-derived/colostrum-deprived (CD/CD) piglets and tonsillar samples from conventional pigs. Tonsillar samples from live pigs were obtained by brushing the surface of the tonsil for 10 s with a sterile toothbrush, which was immersed in 10 mL 0.9% sterile NaCl (saline) solution for 20 min. After removing the toothbrush, 100 μL were used for 10-fold serial dilutions. Samples were concentrated by centrifugation (1500 *g* for 15 min) and 500 μL saline were added to the pellet, which was stored at −20 °C before DNA isolation.

Nasal samples from CD/CD piglets were derived from each nostril by twirling a cotton swab (Applimed SA) for 5 s and then the swab was immersed in 1 mL saline for 20 min. After removing the swab, 100 μL sample were used for 10-fold serial dilutions and the remaining sample was stored at −20 °C.

DNA isolation

Instagene Matrix kit (BioRad) was used for DNA isolation. DNA isolation from bacterial cultures was performed according to the manufacturer's instructions. A minor modification of Frey's protocol was used for DNA isolation from tonsillar samples (200 μL) and nasal samples (400 μL). After centrifugation (5 min at 12,000 *g*), the supernatant was discarded and 200 μL Instagene Matrix were added to each pellet, then samples were heated at 56 °C for 30 min. After mixing for 10 s, samples were heated at 100 °C for 8 min, mixed again for 10 s, centrifuged for 5 min at 12,000 *g* and then the DNA was stored at −20 °C. Prior to qPCR analysis, samples were thawed, briefly mixed and centrifuged at 12,000 *g* for 5 min. Pure saline samples were used as negative controls.

Assay validation with pure cultures

The specificity of the qPCR was evaluated using DNA from pure cultures of a range of reference and field strains of *A. pleuropneumoniae*, other *Pasteurellaceae* and other porcine bacterial pathogens (see Appendix A: Supplementary Table 1). To evaluate the within and between test accuracy, duplicate serial dilution series of a 6 h culture of *A. pleuropneumoniae* 1536 in saline, from 5×10^0 to 5×10^6 CFUs/well, were tested in triplicate on two different PCR plates on the same day using a threshold of 200 relative fluorescence units as a cut-off. The limit of detection of the qPCR was determined as the lowest number of CFUs from the same 10-fold serial dilution in which all three replicates displayed a positive result. A standard curve was created with a serial dilution series, tested in triplicate.

Assay validation with samples from Caesarean-derived/colostrum-deprived piglets

To validate test specificity, tonsillar samples from 77 uninfected CD/CD piglets were collected. The correlation between quantitative results obtained by qPCR and SBE was determined in tonsillar and nasal samples from 10 CD/CD piglets inoculated with *A. pleuropneumoniae*. The inoculum was prepared by growing *A. pleuropneumoniae* 1536 overnight on a HIS-V plate. Thereafter, one colony was suspended in 200 μL saline and another HIS-V plate was inoculated with 50 μL suspension. After 6 h, the plate was rinsed with 5 mL saline and an appropriate concentration (2.5×10^6 CFUs/mL) was prepared, guided by optical density measurements.

Pigs were inoculated intranasally at 28 days of age with 1.0 mL inoculum in each nostril. Tonsillar and nasal samples were collected at 1, 2, 4, 6, 8, 11, 13, 15, 18 and 21 days post-inoculation (dpi). Pigs were examined daily for signs of pleuropneumonia (elevated body temperature and abnormal respiration). On day 21, piglets were euthanased with pentobarbital (Euthanimal, Alfasan). In total, 65 nasal and 65 tonsillar samples were tested using SBE and qPCR.

Serum samples were collected at −1 and 21 dpi and tested in the complement fixation test (CFT; Nielsen, 1974) at the Animal Health Service (Deventer, The Netherlands). At postmortem examination, pneumonia and pleurisy were scored as described by Hannan et al. (1982). Homogenised tonsils and pneumonic lesions were sampled for bacterial growth on CGVA, SBA, HIS-V and chocolate agar plates, and growth of *A. pleuropneumoniae* was confirmed, as described above.

The experiments with CD/CD piglets were authorised by the Animal Care and Ethics Committee (AEC) of Utrecht University, according to the Dutch Experiments on Animals Act (licence numbers DEC2009.III.10.099, DEC2010.II.02.025 and DEC2010.II.02.027). An analgesic (Fentanyl, B. Braun Melsungen AG) was administered to pigs showing clinical signs. Pigs were euthanased when the humane endpoint was reached, as accorded by the AEC.

Assay validation with samples from conventional pigs

To provide data on the specificity of the qPCR, 70 gilts of at least 14 weeks of age, housed on three *A. pleuropneumoniae* free farms, were randomly selected and sampled. The farms were considered to be free from *A. pleuropneumoniae* on the basis of the following criteria: no reports of *A. pleuropneumoniae* outbreaks during the previous 5 years, absence of lesions consistent with *A. pleuropneumoniae* upon slaughterhouse monitoring and negative test results on serological monitoring for *A. pleuropneumoniae* by ApxIV-ELISA (Dreyfus et al., 2004).

An observational cohort study was performed on two *A. pleuropneumoniae* infected wean-to-finish farms with pigs originating from the same farrowing herd. The prevalence and the change in *A. pleuropneumoniae* load in tonsillar samples over time were investigated. Tonsillar samples were collected at 4, 10, 16 and 24 weeks of age from 20 pigs per farm on the same day. The presence of *A. pleuropneumoniae* was confirmed by clinical signs, postmortem examination and serology. qPCR results for *A. pleuropneumoniae* were compared between the two farms and between points in time, expressed by age and time since the first positive sample.

The tonsillar samples used in field validation experiments were obtained in compliance with the Dutch Act on the Practice of Veterinary Medicine, as agreed by the Institutional Animal Care and Ethics Committee.

Statistical analysis

Statistical analysis was performed using R version 2.11.1 and SPSS 16.0.2 for Windows. To evaluate assay accuracy, linear models with threshold cycle as outcome and $\log_{10}$ of the bacterial cell concentration, assay and their interactions as explanatory variables were evaluated. Triplicates within assays were analysed separately as a random effect. Proportions were determined with left one-sided confidence intervals (CIs) based on the binomial distribution. The correlations between quantitative results of SBE and qPCR were obtained by performing a conditional analysis on test positive samples. Partial correlation analysis was performed for the $\log_{10}$ of quantitative SBE and qPCR results for both nasal and tonsillar samples, with piglet number as the controlled variable to adjust for repeated measurements within the same animal.

Table 2
Relation between dichotomous quantitative PCR (qPCR) and selective bacterial examination (SBE) results of nasal and tonsillar samples in Caesarean-derived/colostrum-deprived pigs experimentally inoculated with *Actinobacillus pleuropneumoniae*.

	Nasal samples SBE		Total	Tonsillar samples SBE		Total
	+	–		+	–	
qPCR						
+	42	5	47	56	7	63
–	2	16	18	1	1	2
Total	44	21	65	57	8	65

For comparison of qPCR results in samples from *A. pleuropneumoniae* infected farms, linear mixed models with the individual pig as a random intercept and farm, age, time since the first positive sample and their interactions as explanatory variables were evaluated. The Akaike Information Criterion was used to select the best models.

Results

Assay validation with pure cultures

The qPCR for *apxIVA* produced positive results only when testing DNA from *A. pleuropneumoniae*, whereas samples containing DNA from other bacterial species tested negative (see Appendix A: Supplementary Table 1). A standard curve indicated that the qPCR had a reaction efficiency of 93.0% (see Appendix A: Supplementary Fig. 1). No random effect of intra-assay variability was found. A random effect of inter-assay variability was observed on the intercept, but not on the regression coefficient, indicating the need to incorporate a standard series for the quantification of samples in each assay. The analytical sensitivity was 5×10^0 CFUs per reaction. This resulted in an analytical sensitivity of 250 CFUs per tonsillar sample.

Assay validation with samples from Caesarean-derived/colostrum-deprived piglets

All 77 tonsillar samples from uninfected CD/CD piglets tested negative by qPCR, resulting in an estimated test specificity of 100% (CI: 0.96–1.00). In total, 56/57 SBE positive tonsillar samples from *A. pleuropneumoniae* inoculated pigs tested positive by qPCR (Table 2), resulting in an estimated sensitivity of 98% (CI: 0.92–1.00). Of the nasal samples, 42/65 (64.6%) samples tested positive (Table 2), but numbers of CFUs decreased over time in SBE and qPCR (see Appendix A: Supplementary Fig. 2).

Four of five surviving pigs showed negative SBE and qPCR results for nasal samples at 21 days. Seven of eight tonsillar samples and 5/21 nasal samples were SBE negative but qPCR positive. Sequence analysis of these qPCR products showed 98–100% similarity to GenBank AF021919. One tonsillar and 16 nasal samples were negative in both SBE and PCR. Partial correlation analysis of test positive samples demonstrated a good correlation between the $\log_{10}$ of SBE and qPCR for tonsillar (0.72) and nasal (0.88) samples (see Appendix A: Supplementary Fig. 3).

Five pigs survived until 21 days. Clinical signs varied considerably between pigs. Body temperature was temporarily elevated in all pigs, but respiratory signs were observed in some pigs more often than in others (see Appendix A: Supplementary Fig. 2). Partial correlation analysis showed that abnormal respiration correlated better with the quantitative test results from nasal ($\rho \sim 0.5$) than from tonsillar samples ($\rho \sim 0.4$). All CFT titres at day −1 were <40 and all surviving pigs seroconverted (Table 3). In two pigs, no lesions were found, although one of the lungs was positive for *A. pleuropneumoniae* by culture.

Assay validation with samples from conventional pigs

All 70 tonsillar samples from the three *A. pleuropneumoniae* free farms tested negative and the test specificity was estimated to be 100% (CI: 0.96–100). In the study on two *A. pleuropneumoniae* positive farms, 11 pigs remained negative on qPCR analysis of tonsillar samples, six pigs tested positive once, nine tested positive twice, four were positive three times and 10 pigs tested positive at all consecutive sampling times. Five pigs were temporarily lost to follow-up during the second or third sampling. No pig became test negative once it had tested positive.

Both *A. pleuropneumoniae* positive farms reported variable signs of respiratory distress from 10 to 24 weeks of age. One pig on farm 1 died during the study and *A. pleuropneumoniae* was cultured from lesions found at postmortem examination.

Evaluation of statistical models showed that 'time since first positive sample' could be excluded, meaning that the number of CFUs was not affected by time since infection. At 24 weeks of age, mean CFUs in samples from the lower incidence farm ($10^{4.5}$ CFUs, CI: $10^{3.9}$–$10^{5.1}$) differed significantly ($P = 0.001$) from the higher incidence farm ($10^{5.9}$ CFUs, CI: $10^{5.3}$–$10^{6.3}$) (see Appendix A: Supplementary Table 2). There were no significant differences in mean numbers of CFUs between points in time or between farms at other sampling times.

Discussion

The aim of this study was to develop and evaluate a quantitative PCR for detection of *A. pleuropneumoniae apxIVA* DNA in diagnostic samples from live pigs. The qPCR test performed well with samples from CD/CD and conventional pigs. The test sensitivity and specificity applied to tonsillar samples from CD/CD pigs were 98% and 100%, respectively. Samples from *A. pleuropneumoniae* free pigs tested negative and the qPCR showed consistent test results in

Table 3
Results of complement fixation test (CFT), postmortem scores and culture of lesions and homogenised tonsils from Caesarean-derived/colostrum-deprived piglets experimentally inoculated with *Actinobacillus pleuropneumoniae*.

Pig number	CFT titre	Pneumonia score	Pleurisy score	Lung sample bacteriology	Tonsillar sample bacteriology
1	ND	20.7	6.6	A	A
2	ND	19.3	1.0	A	A
3	ND	33.9	33.2	A	A
4	640	0.0	0.0	ND	A
5	320	5.9	0.3	A	A
6	ND	6.9	4.7	A	A
7	1280	7.1	8.6	No growth	A
8	160	0.0	0.0	A	A
9	1280	9.2	15.0	A	A
10	ND	28.1	26.6	A	A

ND, not determined; A, *A. pleuropneumoniae* confirmed by culture.

samples from *A. pleuropneumoniae* infected farms. From these findings, it can be concluded that the qPCR is suitable for diagnosis of *A. pleuropneumoniae* in field studies.

The analytical sensitivity of the qPCR per reaction was similar to the qualitative nested PCR (Schaller et al., 2001). The minimal detection limits for tonsillar samples from CD/CD pigs tested with SBE and qPCR were similar (200 and 250 CFUs), whereas for nasal samples qPCR had a higher detection limit (20 vs. 250 CFUs). However, the performance of SBE in samples from conventional pigs might be worse because of the presence of other bacterial species. In our experience, agar plates were overgrown by *Pseudomonas* or *Proteus* spp. and the presence of other *Pasteurellaceae* made quantification of *A. pleuropneumoniae* virtually impossible.

In total, 56/65 (86.1%) tonsillar and 42/65 (64.6%) nasal samples tested positive in both qPCR and SBE. Seven tonsillar and five nasal samples were positive by qPCR but negative by SBE, of which one tonsillar and three nasal samples had low quantities of DNA (see Appendix A: Supplementary Fig. 3). The regression line of the nasal samples has the same slope, but a higher intercept than the line of the tonsillar samples. These results suggest that the qPCR detects relatively more DNA in the nasal sample, possibly originating from dead or non-growing bacteria. Bacterial loads in nasal samples may decrease over time, but it can also be hypothesised that *A. pleuropneumoniae* does not survive well in the nasal cavity.

Since CD/CD piglets were inoculated intranasally, no conclusions can be drawn about the optimal sampling location in naturally exposed piglets. Comparison of the quantitative results of qPCR with SBE resulted in a partial correlation coefficient of 0.72 for tonsillar samples and 0.88 for nasal samples, i.e. more than 50% of the variance of qPCR results could be explained by the variance of SBE results. This implies that quantification of *A. pleuropneumoniae* in samples by qPCR is a good alternative to SBE.

Conclusions

The qPCR for *apxIVA* developed in this study has good sensitivity and specificity compared to SBE using tonsillar samples for detection and quantification of *A. pleuropneumoniae* DNA in samples from live pigs. This qPCR may be a useful tool for herd monitoring and epidemiological studies of *A. pleuropneumoniae*.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2012.02.004.

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