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J Vet Res 60, 253-256, 2016
DOI:10.1515/jvetres-2016-0038

SHORT COMMUNICATION

Real-time quantitative PCR for detection and identification of *Actinobacillus pleuropneumoniae* serotype 2

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Received: February 2, 2016 *Accepted: July 18, 2016*

Abstract

Introduction: Porcine pleuropneumonia inflicts important economic losses on most commercial herds. Detection of subclinical or chronic infection in animals still remains a challenge, as isolation and identification of *A. pleuropneumoniae* serotypes is difficult and quantification of the bacteria on agar plates is often almost impossible. The aim of the study was to develop and evaluate a serotype-specific quantitative TaqMan probe-based PCR for detection of serotype 2 in pig lungs, tonsils, and nasal swabs. **Material and Methods:** The primers were designed from the capsular polysaccharide biosynthesis genes of *A. pleuropneumoniae* serotype 2. PCR specificity and sensitivity were evaluated using reference strains and several other bacterial species commonly isolated from pigs. **Results:** The real-time qPCR for detection of *A. pleuropneumoniae* serotype 2 was highly specific and gave no false positives with other serotypes or different bacterial species of pig origin. The detection limit for pure culture was 1.2×10^4 CFU/mL, for lung tissue and nasal swabs it was 1.2×10^5 CFU/mL, and for tonsils - 1.2×10^5 CFU/mL. **Conclusion:** The method can be used to serotype *A. pleuropneumoniae* isolates obtained during cultivation and to detect and identify *A. pleuropneumoniae* serotype 2 directly in nasal swabs and tonsil scrapings obtained from live pigs or lung tissue and tonsils collected post-mortem.

Keywords: pigs, *Actinobacillus pleuropneumoniae*, identification, quantitative real-time PCR, TaqMan probe.

Introduction

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) is a worldwide disease responsible for significant economic losses in most commercial pig herds. To date, 15 serotypes of *A. pleuropneumoniae* have been classified into two biotypes based on nicotinamide dinucleotide (β-NAD) requirement for *in vitro* growth. Infection with *A. pleuropneumoniae* in the pig may be subclinical or result in severe clinical signs and even sudden death (7).

Isolation and identification of *A. pleuropneumoniae* serotypes are still difficult and require a lot of knowledge and laboratory experience. Colonies suspected of being *A. pleuropneumoniae* tend to be easily overgrown by other bacteria and it can be difficult to differentiate them from other *Pasteurellaceae* (6, 10). Serotyping, which is usually provided by specialised reference laboratories, poses another difficulty. In addition, some serotypes cannot be correctly identified due to cross-reactions observed during serotyping with antisera (12). To overcome these problems, several molecular techniques have been developed to identify *A. pleuropneumoniae* serotypes from pure cultures (1, 2, 8, 9, 14, 15). PCR tests for individual identification of *A. pleuropneumoniae* serotype 2 have also been described (8, 9, 11, 14), but no real-time quantitative TaqMan probe-based PCR has been developed. The qPCR test is based on amplification of serotype-specific DNA regions involved in the biosynthesis of the capsular polysaccharide (cps) genes (9).

The aim of the study was to develop a serotype - specific quantitative TaqMan probe-based PCR assay for detection of *A. pleuropneumoniae* serotype 2, which is one of the most prevalent serotypes in Europe (7). This serotype is responsible for a great part of the clinical pleuropneumonia in Polish pig herds (unpublished data). The specificity and sensitivity of

the quantitative TaqMan probe-based PCR test were also examined. A serotype-specific qPCR might have practical importance for diagnostic laboratories and for control of porcine pleuropneumonia.

Material and Methods

Bacterial strains and growth conditions. In the study, the ATCC 27089 strain of *A. pleuropneumoniae* serotype 2 was used as a reference material. Evaluation of PCR specificity was carried out using *A. pleuropneumoniae* reference strains of serotypes 1 and 3–15 and 70 field strains from the collection of the National Veterinary Research Institute, previously identified by serological tests. The PCR used for specificity evaluation was that described by Marois-Créhan *et al.* (11). Eleven other bacterial species frequently found in swine were tested. All strains used in the study are listed in Table 1. DNA extracts from *Mycoplasma hyopneumoniae*, *Actinobacillus indolicus*, and *Actinobacillus porcinus* were also included in the studies.

All V-factor-dependent strains were grown on pleuropneumonia-like organism (PPLO) agar (BD BBL, Becton Dickinson, USA), supplemented with 10 µg/mL of β-NAD, 1 mg/mL of glucose, and 5% horse serum. Other strains tested were grown on Columbia agar (Oxoid, UK) supplemented with 5% sheep blood. The strains were incubated for 24 h at 37°C in 8% CO_2, except *C. perfringens* which was incubated anaerobically.

Table 1. Results of testing specificity of quantitative TaqMan probe-based PCR for detection of *A. pleuropneumoniae* serotype 2

Species	Strain	qPCR results
Actinobacillus pleuropneumoniae serotype 1	4074 and 1 field strain	−
Actinobacillus pleuropneumoniae serotype 2	ATCC 27089*, 4226, and 31 field strains	+
Actinobacillus pleuropneumoniae serotype 3	1421 and 3 field strains	−
Actinobacillus pleuropneumoniae serotype 4	M62 and 8 field strains	−
Actinobacillus pleuropneumoniae serotype 5	K17 and 9 field strains	−
Actinobacillus pleuropneumoniae serotype 6	Fe/71D and 9 field strains	−
Actinobacillus pleuropneumoniae serotype 7	WF83	−
Actinobacillus pleuropneumoniae serotype 8	405	−
Actinobacillus pleuropneumoniae serotype 9	CVJ13261 and 6 field strains	−
Actinobacillus pleuropneumoniae serotype 10	13039	−
Actinobacillus pleuropneumoniae serotype 11	56153 and 2 field strains	−
Actinobacillus pleuropneumoniae serotype 12	9499/84	−
Actinobacillus pleuropneumoniae serotype 13	N273	−
Actinobacillus pleuropneumoniae serotype 14	3905	−
Actinobacillus pleuropneumoniae serotype 15	HS-143 and 1 field strain	−
Haemophilus parasuis	ATCC 19417	−
Bordetella bronchiseptica	ATCC 4617	−
Escherichia coli	ATCC 25922	−
Klebsiella pneumoniae	ATCC 13883	−
Pasteurella multocida	ATCC 12945	−
Streptococcus suis	PIWet 5135	−
Staphylococcus aureus	ATCC 25923	−
Salmonella typhimurium	ATCC 14028	−
Trueperella pyogenes	field strain	−
Clostridium perfringens	ATCC 13124	−
Erysipelothrix rhusiopathiae	ATCC 19414	−
Mycoplasma hyopneumoniae	DNA extract only	−
Actinobacillus indolicus	DNA extract only	−
Actinobacillus porcinus	DNA extract only	−

* – type strain
ATCC: American Type Culture Collection; strains of *A. pleuropneumoniae*, serotypes 1–15, were kindly provided by Professor M. Gottschalk from Université de Montréal, Canada

Table 2. Primers and probe used for quantitative TaqMan probe-based PCR for detection of *A. pleuropneumoniae* serotype 2

Name	Function	Sequence (5' → 3')	% GC*	Location	Product size (bp)
AP307F	Forward primer for cps region of serotype 2	AGCAAAGGATTTTGGCATTG	40.0	4857–4876	210
AP516R	Reverse primer for cps region of serotype 2	GCCATTCTGGTTTTTGTTT	40.0	5065–5047	
PR427AP	Probe	FAM-AGGCTTTGCTTATGATCGCA-BHQ	45.0	4977–4996	

* – percentage of guanine-plus-cytosine

DNA isolation. Bacterial DNA from the colonies was isolated using a Genomic Mini DNA isolation kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The DNA was eluted in 100 µL of Tris buffer (10 mM Tris HCl, pH 8.5) and stored at −70°C for further analysis. Before analysis, the DNA was thawed at room temperature, mixed, and briefly centrifuged.

Primers and amplification protocol. Sequences of the primers and probe used are listed in Table 2. Primers were designed from the capsular polysaccharide biosynthesis genes (9) of *A. pleuropneumoniae* serotype 2 by using Primer Express software (Applied Biosystems, part of Thermo Fisher Scientific, USA). The primers and probe were analysed by BlastN (NCBI, USA) for specificity. For the real-time PCR, a commercial Master Mix QuantiTect Probe PCR kit (Qiagen, USA) at total volume of 20 µL per reaction was used. The reaction mix contained: 7.64 µL of H_2O, 10 µL of 2x QuantiTect Probe PCR Master Mix, 100 nM of each primer, 80 nM of probe, and 2 µL of DNA template per well. The Stratagene Mx3005P PCR system (Agilent Technologies, USA) was used to perform the real-time PCR amplification with initial incubation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 62°C for 1 min. Fluorescence data were collected at the end of the annealing phase of every cycle.

Assay validation. Evaluation of the PCR specificity was conducted using DNA from pure cultures of the range of different bacterial strains, including *A. pleuropneumoniae* serotype 2 type (Table 2). The sensitivity of the PCR was tested using 10-fold serial dilutions. A six hour culture of *A. pleuropneumoniae* serotype 2 type strain (ATCC 27089) from PPLO agar was suspended in saline solution in order to obtain a dilution series containing 10^8 to 10^1 colony-forming units (CFU)/mL. The concentration was confirmed by a viable count and DNA was extracted from 100 µL aliquots using the protocol mentioned above. The same dilution series of *A. pleuropneumoniae* serotype 2 type strain was used to spike samples of homogenised lung and tonsil samples as well as nasal swabs. Uninfected status of the examined pigs had been previously determined regarding *A. pleuropneumoniae* in these samples by conventional PCR as described by Schaller *et al.* (13).

The detection limit was defined as the lowest number of CFU/mL with 95% positive qPCR results. The samples were analysed in 10 replicates for each concentration.

Results

Reference strains of *A. pleuropneumoniae* serotype 2 were correctly identified by qPCR. Good differentiation from other serotypes of *A. pleuropneumoniae* and closely related bacterial species was also obtained. Moreover, other bacteria that are commonly found in pig were all negative in the qPCR assay. The PCR specificity results are presented in Table 1.

The slope of the standard curve was 3.421 and indicates 96.0 % amplification efficiency of the qPCR. The correlation coefficient (R^2) was 0.992. Ct value ≥38 was selected as the positive cut-off point.

The detection limit of the qPCR assay for DNA derived from pure bacterial culture was 1.2×10^4 CFU/mL. Analysis of results obtained from spiked samples of lung tissue revealed a detection limit of 1.2×10^5 CFU/mL. When spiked nasal swabs and tonsils were tested, the detection limits were 1.2×10^5 CFU/mL and 1.2×10^5 CFU/mL, respectively.

Discussion

The isolation of *A. pleuropneumoniae* is a difficult procedure due to its specific cultivation requirements (3) and the fact that *A. pleuropneumoniae* can be easily overgrown by other faster-growing bacteria, especially in samples from live pig including tonsil scrapings or nasal swabs (7). Moreover, overgrowth by other bacteria and presence of other *Pasteurellaceae* make quantification of *A. pleuropneumoniae* on agar plates practically impossible. Bacteriological detection of *A. pleuropneumoniae* also is more difficult in chronic infection and in healthy carrier pigs (5). In general, the isolation rate is very low (3–5). Therefore, PCR assays are adequate alternatives to culture methods for detection and identification of specific serotypes of *A. pleuropneumoniae*.

In recent years, several PCR assays have been described for detection of specific serotypes of *A. pleuropneumoniae* (1, 8, 14, 15); however, only one quantitative PCR based on SYBR Green was evaluated for detection of serotype 2 (11). The use of TaqMan

probes ensures higher reaction specificity and does not require additional analysis of melting curves to identify nonspecific amplification products (*e.g.* PCR primer dimers), which could also be a source of fluorescence in SYBR Green PCR. Therefore, the aim of the study was to develop and evaluate a serotype-specific quantitative TaqMan probe-based PCR for detection of *A. pleuropneumoniae* serotype 2 in lung tissue, tonsils, and nasal swabs from pigs.

The real-time PCR for detection *A. pleuropneumoniae* serotype 2 reported in the study is highly specific and does not give positive reactions with other serotypes or non-target species. From these findings, it can be concluded that qPCR is suitable for *A. pleuropneumoniae* serotype 2 identification and differentiation between serotype 2 and other serotypes as well as non-target bacterial species. The analytical sensitivity (detection limit) of the PCR was comparable to that previously described by Marois-Créhan *et al.* (11), who reported a sensitivity of 2.9×10^5 CFU/mL for pure culture. Furthermore, the detection limits as regards the other experimentally spiked samples were similar to those of that study.

Lower analytical sensitivities was linked to samples of experimentally spiked tonsils, lungs, and nasal swabs than to those obtained from pure cultures. These results are in line with the study cited above, which reported lower sensitivity for tonsils than for pure culture (11). It can be related to the presence of inhibitors which are not eliminated during DNA extraction and may inhibit the reaction. To overcome the problem, adding an internal control as an indicator of the amplification efficiency in each reaction could improve the PCR. Further analysis using clinical samples from infected and uninfected (SPF) herds should be conducted to validate the assay in the field.

In conclusion, the specific quantitative TaqMan probe-based PCR for detection of *A. pleuropneumoniae* serotype 2 has been proven highly specific and sensitive and can be used both to serotype *A. pleuropneumoniae* isolates obtained using cultivation methods and to detect and identify *A. pleuropneumoniae* serotype 2 directly in samples obtained from live pigs (nasal swabs, tonsil scrapings) or collected during post-mortem examination (lung tissue, tonsils).

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This work was supported by the National Science Centre (Poland) grant no. 2014/13/B/NZ6/02566.

Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Acknowledgements: The authors thank Professor Marcello Gottschalk and Sonia Lacouture from Université de Montréal, Canada for providing bacterial strains of *A. pleuropneumoniae*.

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