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RESEARCH LETTER

A BOX-SCAR fragment for the identification of *Actinobacillus pleuropneumoniae*

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Abstract

Bacterial respiratory diseases are responsible for considerable mortality, morbidity and economic losses in the swine industry. *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumonia, is one of the most important disease agents, but its identification and surveillance can be impaired by the existence of many other related bacteria in normal swine microbiota. In this work, we have evaluated a BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) sequence characterised amplified region (SCAR) marker for the specific identification of *A. pleuropneumoniae* and its use in a multiplex PCR to detect additionally *Haemophilus parasuis* and *Pasteurella multocida*, two other major respiratory pathogens of pigs that are members of the family *Pasteurellaceae*. PCRs based on the BOX-SCAR fragment developed were rapid, sensitive and differentiated *A. pleuropneumoniae* from all swine-related members of the *Pasteurellaceae* family tested. Single and multiplex BOX-SCAR fragment-based PCRs can be used to identify *A. pleuropneumoniae* from other bacterial swine pathogens and will be useful in surveillance and epidemiological studies.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of swine pleuropneumonia, a disease responsible for substantial economic losses in the porcine industry. There are 15 serotypes of the bacterium known whose prevalence varies between countries (Bosse *et al.*, 2002). The rapid and accurate identification of the pathogen at the species level is important for both detection and disease surveillance to implement the appropriate control strategy. In routine laboratories, *A. pleuropneumoniae* is identified by staining and/or biochemical and/or molecular tests. When cultivated, *A. pleuropneumoniae* can exhibit small colonies with irregular staining and has a very low viability, dying within a few days (Garrity, 2005). Biochemical tests can take days to process. Thus rapid molecular methods have been increasingly used to enable rapid identification of *A. pleuropneumoniae* (Versalovic & Lupski, 2002).

The most widely used molecular marker to identify the *A. pleuropneumoniae* is the *apxIVA* gene, which encodes an RTX exotoxin specific to the bacterium (Costa *et al.*, 2004; Yang *et al.*, 2009; Urbaniak & Markowska-Daniel, 2011). However, there are some situations in which the detection of *A. pleuropneumoniae* based on the *apxIVA* gene or its product may fail. One such instance led to the discovery of an insertion element named IS*Apl1*, which prevented the ApxIV-based detection of *A. pleuropneumoniae* due to its stable insertion within its cognate gene (Tegetmeyer *et al.*, 2008). Additionally, a Trp to stop (TGG to TGA) mutation in the coding region of the *apxIVA* gene that may lead to a truncated protein has been described in some serotype three strains (O'Neill *et al.*, 2010). Discrepancies between the identification of *A. pleuropneumoniae* isolates by biochemical tests and PCR detection have also been reported (Urbaniak & Markowska-Daniel, 2011). Thus, new molecular markers are necessary for the identification of *A. pleuropneumoniae* isolates. In a previous study, we identified a prominent BOX (BOX-A1R-based repetitive extragenic palindromic)-PCR amplicon that was specific for *A. pleuropneumoniae*, suggesting its potential as a molecular marker (Rossi *et al.*, 2013).

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The aim of this study was to determine whether the BOX-PCR amplicon could be used firstly to derive a simple PCR to identify *A. pleuropneumoniae*, and secondly, a multiplex PCR to differentiate the bacterium from *Haemophilus parasuis* and *Pasteurella multocida* (the two other major pig pathogens of the *Pasteurellaceae* family).

Materials and methods

Microorganisms and culture conditions

A total of 139 microorganisms were employed: 110 clinical isolates of *A. pleuropneumoniae* of serotypes 1 ($n = 1$), 2 ($n = 25$), 3 ($n = 4$), 4 ($n = 2$), 5 ($n = 2$), 7 ($n = 45$), 8 ($n = 28$) and undetermined cross-reactive serotypes ($n = 3$) obtained in various farms in south-eastern Brazil between the years 2003 and 2010; 16 *A. pleuropneumoniae* serotype reference strains and other 13 strains including six different species of the *Actinobacillus* genus (*A. porcintonsillarum*, *A. lignieresii, A. suis, A. indolicus, A. porcinus, A. equuli*), *P. multocida* and *H. parasuis*. All microorganisms were grown at 37 °C for 24 h at 5% CO_2 in Brain Heart Infusion (BD, Becton, Dickinson and Company, Germany) with NAD (10 μg mL^{-1}; Sigma-Aldrich).

DNA extraction

Genomic DNA was isolated with the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The DNA samples obtained were analysed by agarose gel electrophoresis (0.8%).

BOX-PCR

Oligonucleotides used in this work are presented in Table 1. The BOX-PCR was performed using the oligonucleotide BOXA1R as described elsewhere (Rossi *et al.*, 2013). The amplified fragments were separated by electrophoresis in a 2.0% agarose gel.

BOX fragment isolation

The prominent DNA fragment of approximately 1.3 kb obtained for *A. pleuropneumoniae* with BOX-PCR for five different strains (573, 653, 710, 718 and Shope4074) was purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions and cloned into the pGEM®-T Easy vector (Promega). Different clones of each strain library were selected, their plasmids isolated with the Wizard® SV Minipreps DNA Purification System (Promega) and the DNA sequence of the BOX-PCR determined after Sanger sequencing using standard primers M13F and M13R, which are specifically designed to complement the polylinker of the vector.

BOX-Sequence Characterised Amplified Region (SCAR) fragment primer design

The DNA sequences of the BOX-PCR fragments were subjected to a similarity search in the GenBank database (NCBI) using BLASTN. Sequences were aligned using CLUSTALW (Larkin *et al.*, 2007). The conserved region for *A. pleuropneumoniae* was used to design the oligonucleotide pair AppscarF/AppscarR for the amplification of the 233 bp BOX-SCAR fragment.

BOX-SCAR amplification

PCR was performed for all of the 139 microorganisms using 1 U of GoTaq™ DNA polymerase (Promega) in a final volume of 25 μL of enzyme buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 50 ng of DNA in a C1000™ thermal cycler (Bio-Rad). The DNA was initially denatured at 95 °C for 3 min, followed by 35 cycles (94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s) and a final extension step at 72 °C for 5 min. The amplicon generated was analysed using 1.0% agarose gel electrophoresis. As a positive

Table 1. Oligonucleotides used in this work

Primers	Sequence 5′–3′	Amplicon	Source
AppscarF	TCCTAGCTAATGACAAGCGGT	233 bp	This work
AppscarR	GAGCTGACGTGCCACTTCTAA		
HPS-forward	GTGATGAGGAAGGGTGGTGT	821 bp	Oliveira *et al.* (2001)
HPS-reverse	GGCTTCGTCACCCTCTGT		
Pm0762F	TTGTGCAGTTCCGCAAATAA	567 bp	Liu *et al.* (2004)
Pm0762R	TTCACCTGCAACAGCAAGAC		
BOXA1R	CTACGGCAAGGCGACGCTGACG	Variable	Versalovic *et al.* (1991)
16S-do	AGTGGCGGACGGGTGAGTAA	417 bp	Costa *et al.* (2004)
16S-up	CACGGAGTTAGCCGGTGCTT		
APXIV-Do	GAATTCACCTGAGTGCTCACCACC	388 bp	
APXIV-Up	GACGTAACTCGGTGATTGAT		

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control, all of the DNAs were also subjected to a PCR for the detection of the 16S rDNA with the primer pair 16S-Up and 16S-Do using the same reaction parameters and cycles described above. The 16S gene was used as internal amplification control.

BOX-SCAR sensitivity

The limit of detection of the BOX-SCAR oligonucleotide pair was assessed by performing the reaction for five different strains with DNA concentrations ranging from 200 ng to 1 pg. The sensitivity was also evaluated using different concentrations of cells. Bacterial cell cultures were washed twice with PBS, and a tenfold dilution series was made. PCR reactions were performed with 10^6–10^0 CFU mL^{-1}.

Multiplex PCR

A multiplex PCR was developed by combining the BOX-SCAR with the primer pairs HPS-forward/HPS-reverse and Pm0762F/Pm0762R, which were previously shown to be specific for *H. parasuis* and *P. multocida*, respectively (Oliveira *et al.*, 2001; Liu *et al.*, 2004). The reaction parameters were the same as for the SCAR marker, except for the addition of 4% dimethyl sulfoxide and the doubling of dNTP (0.4 μM) and enzyme (2 U). The DNA mixture (25 ng from each pathogen) was initially denatured at 94 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 54 °C for 45 s and 72 °C for 1.5 min) and a final extension step at 72 °C for 4 min.

Results and discussion

SCAR fragments are routinely used for the molecular characterisation and identification of specimens in several fields of life sciences (Dias *et al.*, 2007; Devaiah *et al.*, 2011; Dutta *et al.*, 2012). These molecular markers were originally developed from fragments obtained with random amplification of polymorphic DNA (RAPD) reactions, but criticism related to the lack of reproducibility of such reactions led to the use of oligonucleotides for the amplification of a SCAR, that was reproducible and specific.

Currently, other techniques such as BOX-PCR and ERIC-PCR are used to obtain SCAR (Couillerot *et al.*, 2010). Using BOX-PCR as a genomic fingerprinting method, we identified a prominent and *A. pleuropneumoniae*-specific amplicon of approximately 1300 bp that was present in all clinical isolates and the reference strains (Rossi *et al.*, 2013).

The DNA sequences of the *A. pleuropneumoniae* BOX-PCR amplicon obtained from five different *A. pleuropneumoniae* isolates were identical. The amplicon was 1301 bp, and homology searching established that it included part of the *obgE* gene (coding for a GTPase), an intergenic region and part of the gene for a hypothetical protein. A BLASTP search found 66% identity (e-value 3e-94) of the predicted 204 amino acids available of the *A. pleuropneumoniae* hypothetical protein with a putative glucose-6-phosphate dehydrogenase, present in an *H. influenzae* genome (GenBank sequence ID YP_008543336). However, further work is required to establish whether the homologous *A. pleuropneumoniae* or *H. paraphrohaemolyticus* gene encodes proteins with glucose-6-phosphate dehydrogenase activity. The *A. pleuropneumoniae* BOX-PCR amplicon organisation is shown for strain L20 (Fig. 1d), whose complete genome is available from GenBank under the reference access NC_009053. The same BOX-PCR DNA fragment organisation was observed in all publicly available *A. pleuropneumoniae* complete genomes.

A BLASTN search showed that the *obgE* gene is conserved among some *Pasteurellaceae*, unlike the remainder of the BOX fragment sequence. An *A. pleuropneumoniae*-specific fragment was selected to constitute the BOX-SCAR fragment to allow for precise and fast identification (Fig. 1d). The SCAR fragment is 233 bp, so the PCR reaction for its detection only takes approximately 50 min to complete using the parameters described herein.

The oligonucleotide pair AppscarF/AppscarR produced a strong amplicon with all of the *A. pleuropneumoniae* reference strains (Fig. 1b) and clinical isolates tested. Validation of the PCRs and DNA integrity was performed by amplifying the 16S gene (Fig. 1c). Among the other *Pasteurellaceae* tested, only *A. lignieresii* had a similar but weaker band, which could not be eliminated by modifying the reaction parameters. The inability of PCR-based methods to distinguish these species, which are phenotypically related and can only be separated based on their source of isolation or haemolytic reaction (Christensen & Bisgaard, 2004), has been previously observed (Gram *et al.*, 1996). However, *A. lignieresii* is not a member of swine microbiota and therefore should not interfere with *A. pleuropneumoniae* detection, as it is a commensal of the human oropharynx, and diseases involving this microorganism occur mainly in ruminants (Christensen & Bisgaard, 2004).

The *apxIVA* gene and its product have been frequently used as one of the most important molecular markers in the detection and serodiagnosis of *A. pleuropneumoniae* (Dreyfus *et al.*, 2004; Yang *et al.*, 2009; Urbaniak & Markowska-Daniel, 2011). The *omlA* and *cpx* genes have also been used as markers for *A. pleuropneumoniae*, but we opted not to use these genes in the present work due to serotype variability and previous problems with nonspecific amplifications, respectively (Gram *et al.*, 2000;

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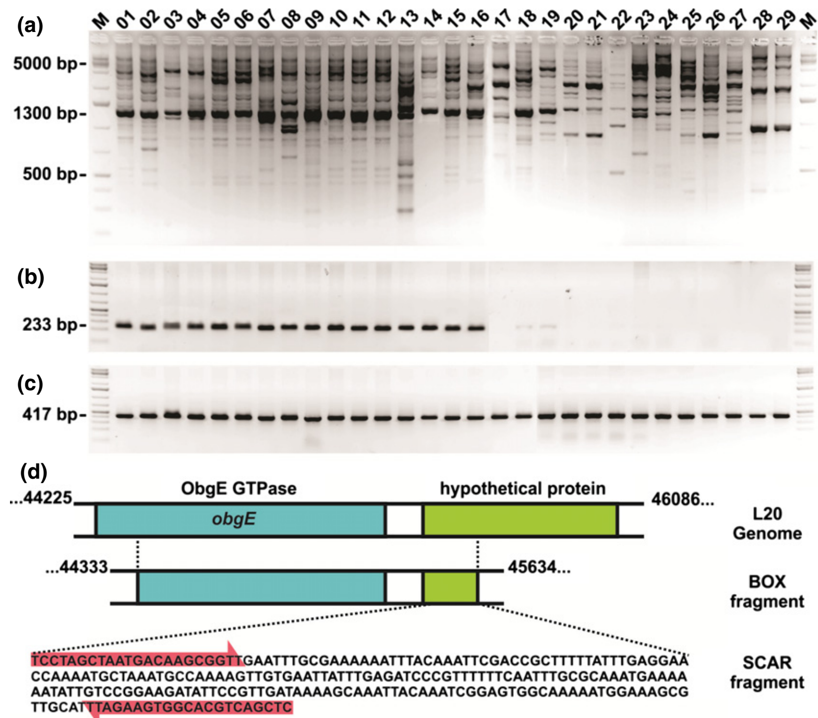

Fig. 1. PCR for the validation of a SCAR fragment for the detection of *Actinobacillus pleuropneumoniae*. Fragments obtained for BOX-PCR (a), BOX-SCAR (b) and 16S (c) are shown. M: molecular marker GeneRuler® DNA Ladder Mix (Fermentas, Thermo Fisher Scientific); 1–16: *A. pleuropneumoniae* reference strains of serotypes Shope4074, S1536, S1421, M62, K17, L20, FemΦ,WF83, 405, CVJ13261, D13039, 56153, 8329, N273, 3906 and HS153, respectively. 17: *Actinobacillus porcintonsillarum* 46996, 18–19: *Actinobacillus lignieresii* 3155 and 3156, 20–21: *Actinobacillus suis* 3157 and 3159, 22: *Actinobacillus porcinus* 3160, 23–24: *Actinobacillus indolicus* 3164 and 3165, 25–26: *Actinobacillus equuli* 8674 and 2667, 27: *Haemophilus parasuis* 8988, 28–29: *Pasteurella multocida* 5875 and 6199. (d) Structure of the BOX-PCR fragment sequenced and the BOX-SCAR marker. Arrows show primer annealing sites.

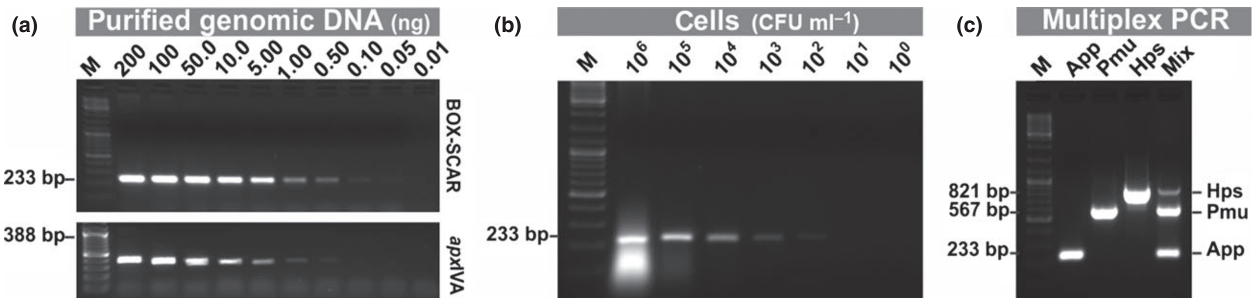

Fig. 2. Sensitivity tests for the validation of the BOX-SCAR fragment using either (a) purified DNA or (b) washed cells. (c) Multiplex PCR developed with the BOX-SCAR for three important *Pasteurellaceae* respiratory swine pathogens: *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Pasteurella multocida*. M: molecular marker GeneRuler® DNA Ladder Mix (Fermentas, Thermo Fisher Scientific).

Souza *et al.*, 2008). When using the primer pair APXIV-Up and APXIV-Do and the amplification conditions suggested by Costa *et al.* (2004), only 84% of our clinical isolates, which had been previously identified by biochemical tests and characterised by multiplex PCR (Rossi *et al.*, 2013), were positive for the *apxIVA* DNA fragment. Similarly, 94.5% of isolates that had previously been identified as *A. pleuropneumoniae* using biochemical tests were positive for the *apxIVA* gene in a study by Urbaniak & Markowska-Daniel (2011). In contrast, the BOX-SCAR obtained in this work detected 100% of the 110 clinical isolates tested.

Using purified DNA and the reaction conditions described, the BOX-SCAR PCR was 10 times more sensitive than the *apxIVA* PCR with the APXIV-Up/APXIV-Do primer pair: specifically, the indicative fragment was detected using DNA concentrations as low as 50 pg (Fig. 2a). When using pure bacterial cell cultures, the BOX-SCAR primers generated a DNA fragment with as little as 10^2 CFU mL^{-1} (Fig. 2b).

The possibility of using the BOX-SCAR amplicon in a multiplex PCR designed to detect *A. pleuropneumoniae*, *P. multocida* and *H. parasuis* was evaluated. *P. multocida* and *H. parasuis* are both major pathogens of swine and members of the *Pasteurellacaeae*. *Pasteurella multocida* is frequently isolated from the lungs of pneumonic pigs and is believed to play a central role in porcine pneumonic pasteurellosis (Davies, 2003; Pors *et al.*, 2013). *Haemophilus parasuis* is one of the earliest and most prevalent colonisers of piglets in the farrowing house, and a common

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isolate from nasal secretions in pigs and the cause of Glässers disease (Rapp-Gabrielson *et al.*, 2006). The multiplex PCR was developed with two previously developed oligonucleotide pairs, one specific for the transcriptional repressor UlaR in *P. multocida* (Liu *et al.*, 2004) and the other for the 16S rDNA in *H. parasuis* (Oliveira *et al.*, 2001). The specificity of the oligonucleotides was validated by testing them with several different species of the *Pasteurellaceae* family. The BOX-SCAR can be successfully incorporated in a multiplex PCR reaction (Fig. 2c), thereby expending the potential use of this molecular marker.

The BOX-SCAR and multiplex-PCRs developed are potential tools to identify *A. pleuropneumoniae* and to distinguish it from related microorganisms that are either members of the normal or pathogenic microbiota in swine. These methods will be useful not only in isolation studies but also in surveillance and epidemiological research, because they are fast, efficient, sensitive and relatively inexpensive.

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