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Development and Application of an ERIC-PCR Method for Genotyping and Differentiating *Actinobacillus pleuropneumoniae* Isolates

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ABSTRACT

***Background*:** The pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is one the most important swine respiratory diseases. Biochemical and serological tests are widely applied for the diagnosis and characterization of this bacterium. However, in some isolates, conflicting results are found. There are at least 15 serotypes with significant differences in virulence that have been identified until now. Moreover, cross reactions between serotypes are not uncommon. The serotype determination from isolates occurring in outbreaks is an important procedure in prophylaxis and control of the disease. The present work focuses on the application of an ERIC-PCR technique for genotyping and differentiation of *A. pleuropneumoniae* isolates.

***Materials, Methods & Results*:** Fifteen reference strains for the recognized *A. pleuropneumoniae* serotypes were analyzed in this work, alongside with 27 field isolates that had been previously characterized regarding biochemical, serological and molecular features. Total DNA from each sample was purified and subjected to PCR amplification using ERIC-specific primers (ERIC1R and ERIC2). The resulting amplicons were analyzed by agarose gel electrophoresis and their sizes were estimated from the gel images. Bands with similar sizes were identified and used to construct a binary matrix that took into account the presence or absence of individual bands in all lanes. Pair-wise similarity coefficients were computed from the binary matrix and the similarity matrices obtained were utilized to construct an UPGMA-based dendrogram. The amplicons obtained from the *A. pleuropneumoniae* reference strains generated a very distinctive pattern for each one of the tested strains. Moreover, all samples presented a large enough number of amplicons (bands) as to enable an unequivocal differentiation of each sample. Reproducibility of the developed ERIC-PCR method was assessed by means of duplicate PCR reactions. All duplicate reactions presented exactly the same pattern. Furthermore, amplicon abundance for bands with the same size in each duplicate was also very similar. When applied to *A. pleuropneumoniae* field isolates, collected from clinical cases of the disease, we were able to differentiate all samples from each other, even those belonging to the same serotype.

***Discussion*:** In the present work we have analyzed *A. pleuropneumoniae* strains isolated from a wide spread geographical area in Brazil, covering outbreaks that occurred over a period of more than a decade. The ERIC-PCR technique was standardized using DNA from the serotyped *A. pleuropneumoniae* reference strains, generating distinctive amplification patterns for each sample, which were not serotype specific. It is expected that the discriminatory power of the method would be enhanced by the large numbers of amplicons obtained for each sample. We have also analyzed the reproducibility of the ERIC-PCR method by performing several experiments where DNA from the same sample was amplified in duplicate independent PCR reactions and the PCR amplification patterns obtained were reproducible in all tested experiments. Also, very little variation in amplification efficiency was detected for the individual amplicons within a given sample. The application of the ERIC-PCR genotyping technique to *A. pleuropneumoniae* isolated from animals with clinical signs of the disease allowed the differentiation of each individual sample. A very distinctive ERIC-PCR pattern was obtained even for samples belonging to the same serotype, indicating that there is no association between serotype and amplification pattern. These results suggest that the method could be useful to discriminate between isolates even when applied to a larger population. The results presented in this work suggest that ERIC-PCR is a promising genotyping technique which could be successfully applied to differentiating *Actinobacillus pleuropneumoniae* isolates and could be important for epidemiological studies.

Keywords: enterobacterial repetitive intergenic consensus, DNA fingerprinting, swine, respiratory diseases.

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INTRODUCTION

The bacterium *Actinobacillus pleuropneumoniae* is one of the most important pathogens of the swine respiratory tract, occurring widely in pig-producing countries and causing pleuropneumonia [7,17,19]. The acute form of the disease causes fibrinous pleuritis, resulting in high mortality and significant economic losses. In chronic and subclinical states the morbidity is low and the losses are mainly due to reduced growth rates and increased costs with medication and/or vaccination [19].

The virulence of *A. pleuropneumoniae* is associated to several factors as capsule, outer major proteins, lipopolysaccharides (LPS), proteases, adhesins and the RTX protein toxins. The RTX family of toxins is widely spread among gram negative pathogenic bacteria [7,18] and *A. pleuropneumoniae* serotypes vary in RTX (Apx I, II and III) production, which have distinct citotoxic and hemolytic effects. Also, all serotypes present a fourth RTX toxin, designated ApxIV. Previously, we have used PCR for the *apxIVA* gene and 16S rDNA sequencing to characterize field isolates of *A. pleuropneumoniae* and solve conflicting classification results [5].

A. pleuropneumoniae serotyping is one of the most important tools used in epidemiological studies and control programs of swine pleuropneumonia. It is mainly based on serological variations of the capsular antigens. There are at least 15 serotypes with significant differences in virulence that have been identified until now [2,6].

In order to differentiate *A. pleuropneumoniae* strains and/or isolates from outbreaks, we describe the developed and application of an ERIC-PCR (enterobacterial repetitive intergenic consensus) method based on the amplification of repetitive elements present in the *Actinobacillus pleuropneumoniae* genome.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The 15 reference strains for the recognized *Actinobacillus pleuropneumoniae* serotypes used in this work are listed in Table 1. The field isolates of *A. pleuropneumoniae* (Table 2) were from Centro Nacional de Pesquisa de Suínos e Aves (CNPSA), EMBRAPA, Brazil, and had been previously characterized regarding biochemical and serological features [9,14,15] as well has molecularly [4,5]. Culture conditions have been previously described [10].

Table 1. *A. pleuropneumoniae* reference strains.

Serotype	Sample	Source
1	Shope 4074	ATCC 27088[a]
2	1536	Dr. R. Ross[b]
3	1421	Dr. R. Ross[b]
4	M62	ATCC 33378[a]
5a	K17	ATCC 33377[a]
5b	L20	Dr. R. Ross[b]
6	Femo SCI-A	Dr. R. Petersen[c]
7	WF83 SCI-A	Dr. R. Petersen[c]
8	F384	Dr. R. Petersen[c]
9	F60	Dr. R. Petersen[c]
10	13039	Dr. R. Ross[b]
11	56153	Dr. R. Ross[b]
12	1096	Dr. R. Ross[b]
13	-	CNPSA-EMBRAPA[d]
14	-	CNPSA-EMBRAPA[d]

[a]ATCC: American Type Culture Collection; [b]Dr. Richard Ross, Iowa State University, USA; [c]Dr. Ruth Petersen, Intervet, Denmark; [d]Centro Nacional de Pesquisa de Suínos e Aves, Empresa Brasileira de Pesquisa Agropecuária, Concórdia, SC, Brazil

Table 2. *A. pleuropneumoniae* field isolates, apx genes profiles and serotypes.

Isolate	*apx* genes			Serotype
	I	II	III	
6635		●	●	3
6636		●	●	3
6735		●	●	NT
6740	●	●		5b
6742		●	●	3
6743	●	●		5b
6744	●	●		5b
6745	●	●		5b
6746	●	●		5b
6747		●		7
6751	●	●		5b
6754	●	●		5b
6778	●	●		5b
6789	●	●		5b
6790	●	●		5b
6791		●	●	3
6792	●	●		5b
6796	●	●		5b
6800	●	●		5b
6801	●	●		5b
6802	●	●		5b
6803		●		7
6804		●		7
6805	●	●		5b
6806	●	●		5b
6807	●	●		5b
6936		●	●	3

All samples were obtained from swines with clinical disease. The apx genes profiles and serotypes had been previously characterized. NT: nontypeable.

DNA isolation and PCR conditions

Total DNA purification has been previously described [5,13]. Briefly, a loopful of colonies from Columbia agar plates were washed in distilled water, resuspended in water with 1.7% SDS, 50 mg/mL of proteinase K and incubated at 65°C for 1 h. Total DNA was purified by repeated phenol-chloroform extractions and precipitation from the aqueous phase by adding 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol. The DNA was dried and resuspended in distilled water.

ERIC-PCR

PCR amplification was performed using primers ERIC1R and ERIC2 [20]. The PCR assays were performed in a Veriti DNA thermal cycler[3]. PCR mixture contained the reaction buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3), 3.0 mM $MgCl_2$, 20 pmol of each primer, 2.5 U of *Taq* polymerase, 200 µM of each dNTP, template DNA and distilled water for a final volume of 25 µL. The reaction mixture was subjected to the following PCR cycling conditions: 95ºC for 2 min, followed by 35 cycles at 94ºC for 30 s, 50ºC for 1 min and 72ºC for 2.5 min, and a final extension at 72ºC for 20 min.

Amplicons were submitted to electrophoresis in 1.5% Ultrapure agarose[4] at 85 V (8.5 V/cm) for 3 h in a BioRad Sub-Cell model 192 apparatus[5] containing 1x TBE buffer. Following electrophoresis the gels were stained with Sybr Gold[4] and photographed.

Phylogenetic analysis

Initially, the amplicons' sizes obtained from each sample were estimated from the gel images using the program Gel-Pro Analyzer version 4[6]. Bands occupying the same position in the different lanes of the gel (amplicons with similar sizes) were identified and used to construct a binary matrix that took into account the presence or absence of individual bands in all lanes. The binary matrix, scored as band presence (1) or absence (0), was used to compute pair-wise similarity coefficients, and the similarity matrices obtained were utilized to construct an UPGMA-based dendrogram [1,12].

RESULTS

ERIC-PCR of A. pleuropneumoniae reference strains

DNA from *A. pleuropneumoniae* reference strains, serotypes 1 to 14, was amplified using ERIC1R/ERIC2 primers and the resulting amplicons were separated by agarose gel electrophoresis (Figure 1). A distinctive pattern could be obtained for each one of the tested strains. Moreover, all samples presented a large enough number of amplicons (bands) as to enable an unequivocal differentiation of each sample.

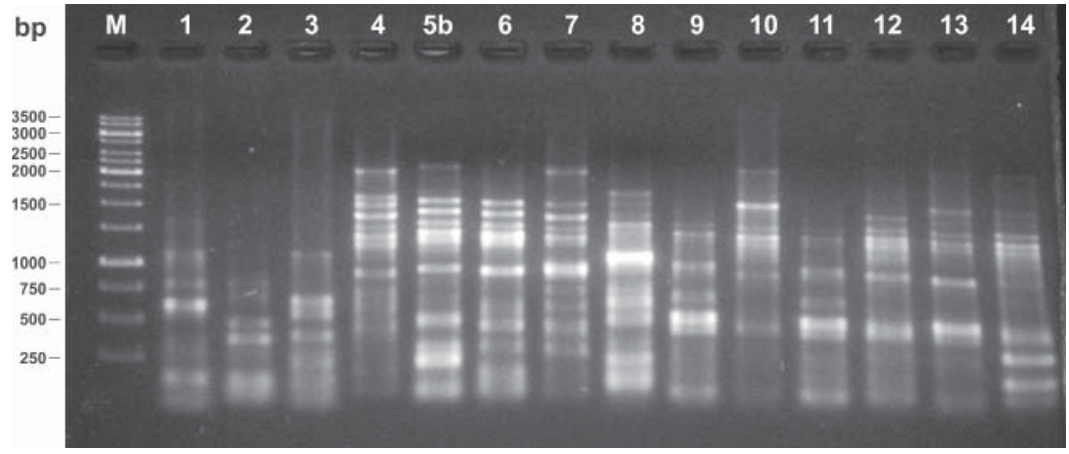

Figure 1. ERIC-PCR of *A. pleuropneumoniae* reference strains. DNA from reference strains was amplified using ERIC1R/ERIC2 primers and the amplicons submitted to electrophoresis on a 1.5% agarose gel. 1 to 14: amplicons from serotypes 1 to 14, respectively. M: 250 bp ladder (size marker). bp: base pairs.

ERIC-PCR reproducibility

In order to assess the reproducibility of the ERIC-PCR method, DNA from all *A. pleuropneumoniae* reference strains was amplified in duplicate PCR reactions and the amplicons submitted to agarose gel electrophoresis. A representative result is presented in Figure 2. All duplicate reactions presented exactly the same pattern. It is worth noting that the relative intensity (amplicon abundance) for bands with the same size in each duplicate is also very similar.

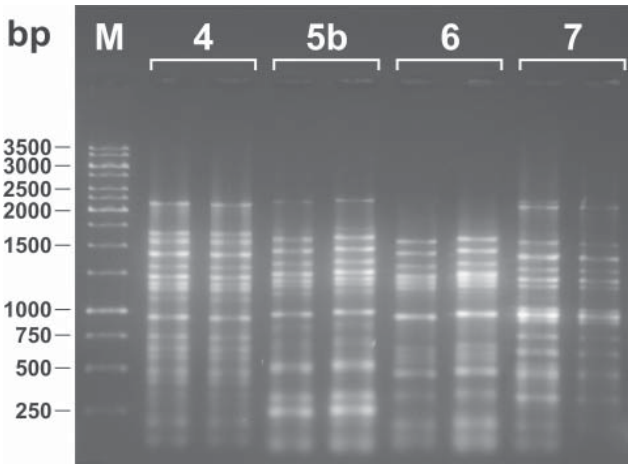

Figure 2. ERIC-PCR reproducibility. DNA from *A. pleuropneumoniae* reference strains, serotypes 4, 5b, 6 and 7, was amplified using ERIC1R/ERIC2 primers in independent PCR reactions. The resulting amplicons were submitted to electrophoresis on a 1.5% agarose gel. M: 250 bp ladder (size marker). bp: base pairs.

Application of ERIC-PCR to field strains

The ERIC-PCR method was applied to several *A. pleuropneumoniae* field strains. All samples were collected from clinical cases of the disease and are listed in Table 2. The resulting ERIC-PCR pattern is presented in Figure 3. Analysis of the patterns using reconstructed phylogenetic trees based on binary matrices allows differentiating all samples from each other, even those belonging to the same serotype.

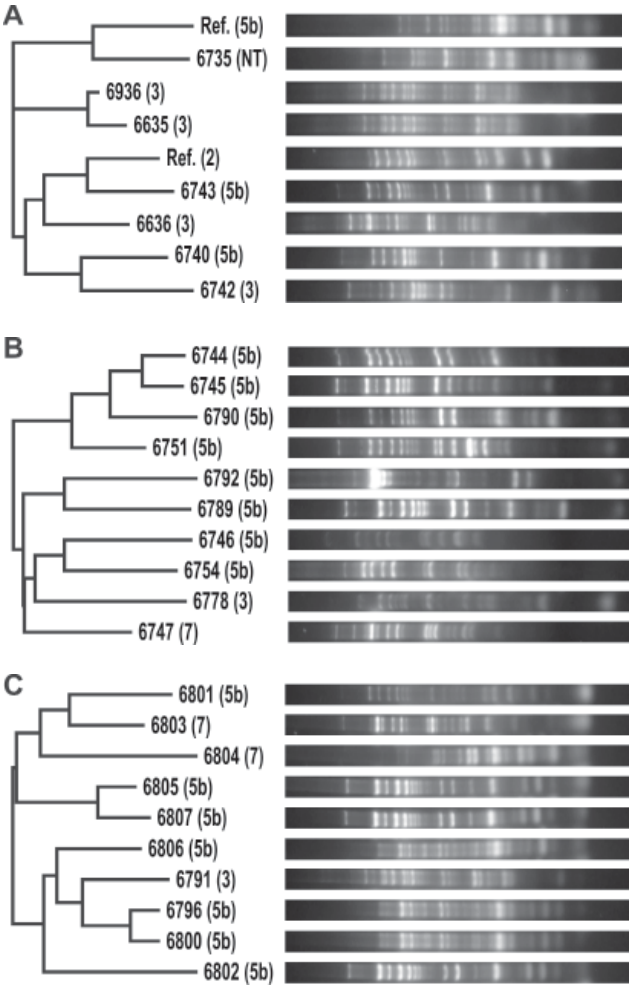

Figure 3. Phylogenetic analyses of ERIC-PCR derived patterns from *A. pleuropneumoniae* samples. Three groups containing 9 (panel A) or 10 (panels B and C) *A. pleuropneumoniae* DNA samples were analyzed by ERIC-PCR. Each group was PCR amplified and submitted to gel electrophoresis separately. The reconstructed phylogenetic tree is presented on the left alongside with the corresponding ERIC-PCR pattern (on the right). The numbers correspond to the samples listed on Table 2 (with their respective serotypes). Ref.: reference strain (listed on Table 1). NT: nontypeable.

DISCUSSION

Swine pleuropneumonia is one of the most important respiratory diseases in swine breeding herds around the world. To determine epidemiological aspects and to monitor prophylactic programs the precise characterization of the etiologic agent is required [17]. The standard methods to detect *A. pleuropneumoniae* infection are culture and serology [3]. According to some authors [8,16] the wide variability of the results from biochemical tests, difficulties in serotyping and the occurrence of bacteria that mimic the phenotypic and antigenic patterns impose the necessity of new, rapid and sensitive methods to solve doubts on the classification of *A. pleuropneumoniae*.

To the extent of our knowledge this is the first work applying ERIC-PCR to differentiate among isolates of the bacterium *A. pleuropneumoniae*. The present study revealed that whole genome analysis could be a satisfactory approach for genotyping *A. pleuropneumoniae* strains. It is worth mentioning that the strains analyzed in this work were isolated not from a single outbreak but over a period of more than a decade and also from wide spread geographical area in Brazil [11].

In order to standardizing the ERIC-PCR technique applied to *A. pleuropneumoniae*, we have used DNA samples from serotyped reference strains (Figure 1). All samples analyzed presented a distinctive amplification pattern. It is worth noting that the patterns were not serotype specific, as further analyses indicated. Also important, it is expected that the large numbers of amplicons for each sample would enhance the discriminatory power of the method.

Another key aspect to any genotyping method is its reproducibility. We conducted several experiments where DNA from the same sample was amplified in duplicate independent PCR reactions. A representative result from such an experiment is presented in Figure 2. The PCR amplification patterns obtained for reference serotypes were reproducible in all tested experiments. Besides the number and size of bands for each sample, the band intensities where also reproducible, indicating very little variation in amplification efficiency for the individual amplicons within a given sample.

When the developed ERIC-PCR genotyping technique was applied to DNA from *A. pleuropneumoniae* isolated from animals with clinical signs of the disease, a specific amplification pattern could also be visualized for each individual sample (Figure 3). In order to analyze and compare the resulting patterns we have used reconstructed phylogenetic trees based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean), using similarity matrices derived from the binary matrices corresponding to the band pattern. Reconstructed trees analyses indicate that all samples presented enough differences as to allow the differentiation of every sample. It must be pointed out that a maximum of ten samples could be analyzed at a single electrophoresis run due to limitations imposed by the gel system we have used. Despite most of the samples belonging to serotype 5b, none presented the same ERIC-PCR pattern. Also, there is no association between serotype and amplification pattern, indicating that the method could be useful to discriminate between isolates even when applied to a larger population.

Since the use of ERIC-specific primers enables PCR amplification from a plethora of bacterial genomes other than the Enterobacteriaceae family [21], a major drawback in applying ERIC-PCR to differentiate among *A. pleuropneumoniae* strains is the requirement for previously isolated and identified pure colonies of this bacterium. Currently, we are working on a different genotyping strategy based on the amplification of unique repetitive sequences of *A. pleuropneumoniae*, enabling PCR genotyping from clinical samples such as nasal swabs or bronchoalveolar lavage without any prior sample characterization.

CONCLUSION

Taken together, our results suggest that ERIC-PCR is a promising genotyping technique which could be successfully applied to differentiating *Actinobacillus pleuropneumoniae* isolates. This genotyping technique could be instrumental in epidemiological studies aiming at comparing the strains occurring at different outbreaks of the disease which in turn would have an impact on vaccination and other control programs.

SOURCES AND MANUFACTURERS
[1]Oxoid, Basingstoke, UK.
[2]Sigma Chemical Co., St. Louis, MO, USA.
[3]Applied Biosystems (Life Technologies), São Paulo, SP, Brazil.
[4]Invitrogen (Life Technologies), São Paulo, SP, Brazil.
[5]Bio-Rad Laboratórios Brasil Ltda, São Paulo, SP, Brazil.
[6]MediaCybernetics, Rockville, MD, USA.

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Declaration of interest. The authors report no conflicts of interest.

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