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Original article

Usefulness of PCR/RFLP and ERIC PCR techniques for epidemiological study of *Haemophilus parasuis* infections in pigs

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Abstract

Haemophilus parasuis belongs to opportunistic microorganisms of undefined virulence. The purpose of the studies was to compare suitability of PCR/RFLP in our modification and ERIC PCR for epidemiological study of domestic strains of *H. parasuis*. The results were evaluated taking into account two different aspects: suitability of the tests for isolating the highest possible number of clone groups and subjective evaluation of the method judged with respect to the following criteria: difficulty, availability of equipment and reagents as well as time and cost of the study. The results obtained in the present study show that the two methods used for typing of *H. parasuis* had high discriminatory power. Taking into account this parameter it can be concluded that ERIC PCR is more suitable than PCR/RFLP. This justifies the use of ERIC PCR for routine epidemiological analyses of mentioned pathogen. Taking into account the complexity of method used, ERIC-PCR based on random amplification of DNA, proved to be comparable to PCR/RFLP. The last mentioned technique is relatively less expensive and labour-consuming, especially when diagnostic PCR method is used for the epidemiological studies.

Key words: *Haemophilus parasuis*, PCR/RFLP, ERIC PCR, genotyping

Introduction

Haemophilus parasuis (*H. parasuis*) infections are mainly associated with Glässer's disease (Amano et al. 1994). Generally, the infection develops in serosa, joints and meninges. Infections with this microorganism are particularly serious in specific pathogen free (SPF) herds (Wiegand et al. 1997, Marois et al. 2008) and in herds with a good health status, where they are usually connected with high morbidity and mortality

rates. *Haemophilus parasuis* may cause infections of the respiratory tract, but its role as a causative factor of pneumonia still remains unclear (Cai et al. 2005, Palzer et al. 2007).

Haemophilus parasuis belongs to opportunistic microorganisms of undefined virulence (Oliveira and Pijoan 2004). Many authors (Oliveira et al. 2003, Olvera et al. 2006, Olvera et al. 2007a) reports that various strains of *H. parasuis* are quite likely to occur in a herd, colonizing the upper respiratory tract, but

usually only one of them causes a disease. For this reason, apart from epidemiological studies on a “macro” scale, comprising populations of a region or a country, it is important to distinguish the strains of *H. parasuis* in a herd.

H. parasuis in a herd can be distinguished by different typing techniques. At present, epidemiological studies are based on such serotyping methods as: agarose gel immunodiffusion (ID) (Kielstein et al. 1991) and indirect hemmagglutination (IHA) (Tadjine et al. 2004). However, despite improvements in the procedure, these techniques are still too labour-consuming, as they require special serotyping sets obtained from reference strains. Furthermore, there are limitations due to a large percentage of strains not belonging to any known serotype (Kielstein et al. 1991, Bak and Riising 2002).

The purpose of the studies was to compare suitability of PCR/RFLP (Polymerase Chain Reaction / Restriction Fragments Length Polymorphism) in our modification and ERIC PCR (Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction) for epidemiological study of domestic strains of *H. parasuis*.

Materials and Methods

Bacterial strains. The material taken for the study consisted of 52 field strains of *H. parasuis*, isolated from 52 pig farms located in 15 regions of Poland, in the years 2003-2006. The strains were divided into 3 groups, according to pathogenicity, determined on a basis of an interview about the disease and site of the isolation.

Group I – strains isolated from animals with symptoms typical for Glässer’s disease,

Group II – strains isolated from animals with diagnosed pneumonia,

Group III – strains isolated from healthy animals and herds in which no clinical signs characteristic of *H. parasuis* infections were found.

Procedure. A single bacterial colony grown on PPLO medium at 37°C for 24-48 h was suspended in 1 ml of sterile redistilled water and centrifuged at 13 000 g for 3 min. The supernatant was removed and the precipitate was washed with 1 ml of redistilled water. After recentrifugation, the precipitate was re-suspended with 100 µl of 10 mM Tris buffer. DNA was extracted from the suspension, using a Genomic Mini Kit (AA Biotechnology, Gdańsk). Concentration of the nucleic acid obtained was determined, using a BioPhotometer (Eppendorf). DNA purity was determined from OD₂₆₀/OD₂₈₀ coefficient. The samples with the values of this coefficient ranging from 1.5 to 1.9 were taken for further analysis.

PCR-RFLP. The method used in the present study was based on duplicating a definite region of *H.*

parasuis genome and exposure of the subsequent amplicon to restriction endonucleases. The DNA fragments, obtained from cleavage with the enzymes, make up a pattern characteristic of a given bacteria species or strain. The PCR methodology was used as described (Oliveira et al. 2001), but amplification conditions were modified by the authors of the present study. Modifications included the introduction of new restriction endonucleases. The concentration of the DNA used in the study was 10 ng/µl. The PCR sequence of *H. parasuis* nucleotide product (Oliveira et al. 2001) was found in GenBank database. The nucleotide sequence was analysed using a Neb Cutter (New England Bio Labs). Polymorphism of the length of restriction fragments was analysed with the use of those restriction endonucleases which digested the amplified fragment of *H. parasuis* genome, max. in four sites: *Bsr*I and *Mbo*II (Biolabs). Digestion with restriction endonucleases was carried out in a water bath at 37°C for 16 h. The products of digestion were subject to electrophoretic separation in 12.5% polyacrylamide gels – “Excel Gel DNA Analysis Kit” (Amersham Biosciences). Horizontal electrophoresis was carried out in a Multiphor I (Amersham Biosciences). The buffer for electrophoresis contained in buffer stripes for the anode consisted of 0.45 mM Tris/acetate; 0.4% of 4 SDS; 0.005% orange G. The stripes for the cathode consisted of 0.08 mM Tris; 0.80 mM Tricine; 0.6% SDS. The gel was spread with 7 µl of the sample containing 2 µl of PCR product, 2 µl of loading buffer prepared according to the instructions of gel manufacturers and 3 µl of sterile deionized water. The electrophoretic separation was carried out at 25 mA for about 60 min. After electrophoretic separation, the gels were stained with silver, using a DNA Silver Staining Kit (Amersham Biosciences). The results of the restriction analysis were scanned, using an hp scanjet 4470 c.

ERIC-PCR was carried out according to the method described by Rafiee et al. (2000). In this method, the PCR reaction is carried out with the use of universal starters with long nucleotide sequences, complementary to conserved fragments in the genome of the *Enterobacteriaceae* family (ERIC1 – 5’ ATGTAAGCTCCTGGGATCAC 3’; ERIC2 – 5’ AAGTAAGTGACTGGGGTGAGCG 3’) (Versalovic et al. 1991). However, the distribution of the conserved fragments varies, depending on the bacteria species or strain.

The reaction mixture (25 µl) contained: 10 mM Tris-HCl, the pH 8.3; 50 mM KCl; 10 mM of oligonucleotides; 30 pmol of each starter; 2 µM Mg²⁺; 0.4 U Taq DNA polymerase; 1 µg of isolated DNA. The amplification process was carried out using a T3 Thermocycler (Biometra). Each of 35 reaction cycles included: denaturation at 95°C for 30 s, starter annealing at 50°C for 30 s and elongation of DNA strand at 72°C for 2 min.

Table 1. Assessment of molecular typing of *H. parasuis* by PCR/RFLP and ERIC PCR.

Method	Diversity coefficient*	Range*	Number of profiles*	Difficulty**	Availability of reagents**	Length of time**	Cost of study**
ERIC-PCR	0.988	2-8	45	++	++	++	+++
PCR/RFLP (MboII)	0.946	4-7	21	++	++	++	++

* Explanations for objective parameters: range – the smallest and the largest number of bands visible in gels; **Explanations for subjective parameters: +++ high; ++ medium; + low.

The ERIC-PCR products were subjected to electrophoretic separation in 1.5% agarose gel and TBE buffer at a constant voltage of 80 V/cm for 1.5 h. The sizes of DNA fragments resulting from amplification were determined with reference to a marker Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas). The results of the reaction were read in UV light, using a GelDoc for imaging and documentation (BioRad) with QuantityOne program (BioRad).

The analysis of genotypic affiliation of *H. parasuis* isolates was made using an UPGMA (Unweighted Pair-Group Method using Arithmetic averages), with regard to Dice's coefficient and 1% tolerance for the differences in the band location, using a BioNumerics software. Evaluation of the two techniques did not include the intensity of bands. In the ERIC PCR method, only the products longer than 500 base pairs (bp) were taken into account. The result was shown as a single dendrograph consisting of electrophoretic separations of DNA fragments, obtained by the same method for the isolates under investigation. Evaluation of the molecular techniques used for diversification of *H. parasuis* isolates included the following parameters:

– Simpson's diversity index (D), calculated according to the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1)$$

N – the number of isolates used for typing, n_j – the number of type j isolates, S – the number of types obtained.

– The lowest and the greatest number of bands obtained by a given method and the number of profiles. It was assumed in the coefficients above that the isolates showing $\geq 90\%$ affiliation belonged to the same type (clone group) (Hunter and Gaston 1988).

– The procedure in terms of difficulty, availability, length of time and cost was evaluated using the following scale: +++ high; ++ medium; + low.

Results

The results were evaluated taking into account two different aspects of the techniques under investigation. One of them was suitability of the tests for isolating the highest possible number of clone groups (diversifying coefficient, number of profiles), ERIC PCR product yield and diagnostic PCR product digested with restriction endonucleases (Table 1). The other one was subjective evaluation of the method judged with respect to the following criteria: difficulty, availability of equipment and reagents as well as time and cost of the study (Table 1).

The analysis of the dendrographs allowed us to isolate identical or very similar groups of strains, which may account for their molecular affiliation. However, such a conclusion is by no means unanimous, due to incomplete data regarding the links between farms. In either method, no correlation between the virulence of the strain and its position in the dendrograph was observed (Fig. 1).

Electrophoretic separation of PCR products digested with endonucleases showed that irrespective of the enzyme used for the study, the molecular profiles of the samples were different. A brief analysis of the affiliation of the isolates showed 100% similarity in a given group when *Bsr* I endonuclease was used. Further analysis with the use of this enzyme was not carried on because it did not allow us to distinguish the strains. In contrast, with the use of *Mbo* II enzyme, it was possible to produce a dendrograph exhibiting genotypic affiliation of *H. parasuis* isolates under investigation (Fig. 1).

Six main groups exhibiting medium genotype affiliation ranging from 68 to 70% were isolated. A dendrograph of 52 isolates (90% affiliation) allowed to isolate 21 clone groups. The diversity coefficient obtained with the use of this enzyme was 0.946.

A dendrograph of electrophoretic pictures obtained with ERIC-PCR method (Fig. 1) allowed to isolate 4 main groups, exhibiting lower genotype affiliation, ranging from 20 to 50%. A dendrograph (90% affiliation) made possible to isolate as much as 45 clone groups. Coefficient D obtained by this method was 0.988, which shows that this technique proved to be better than PCR/RFLP.

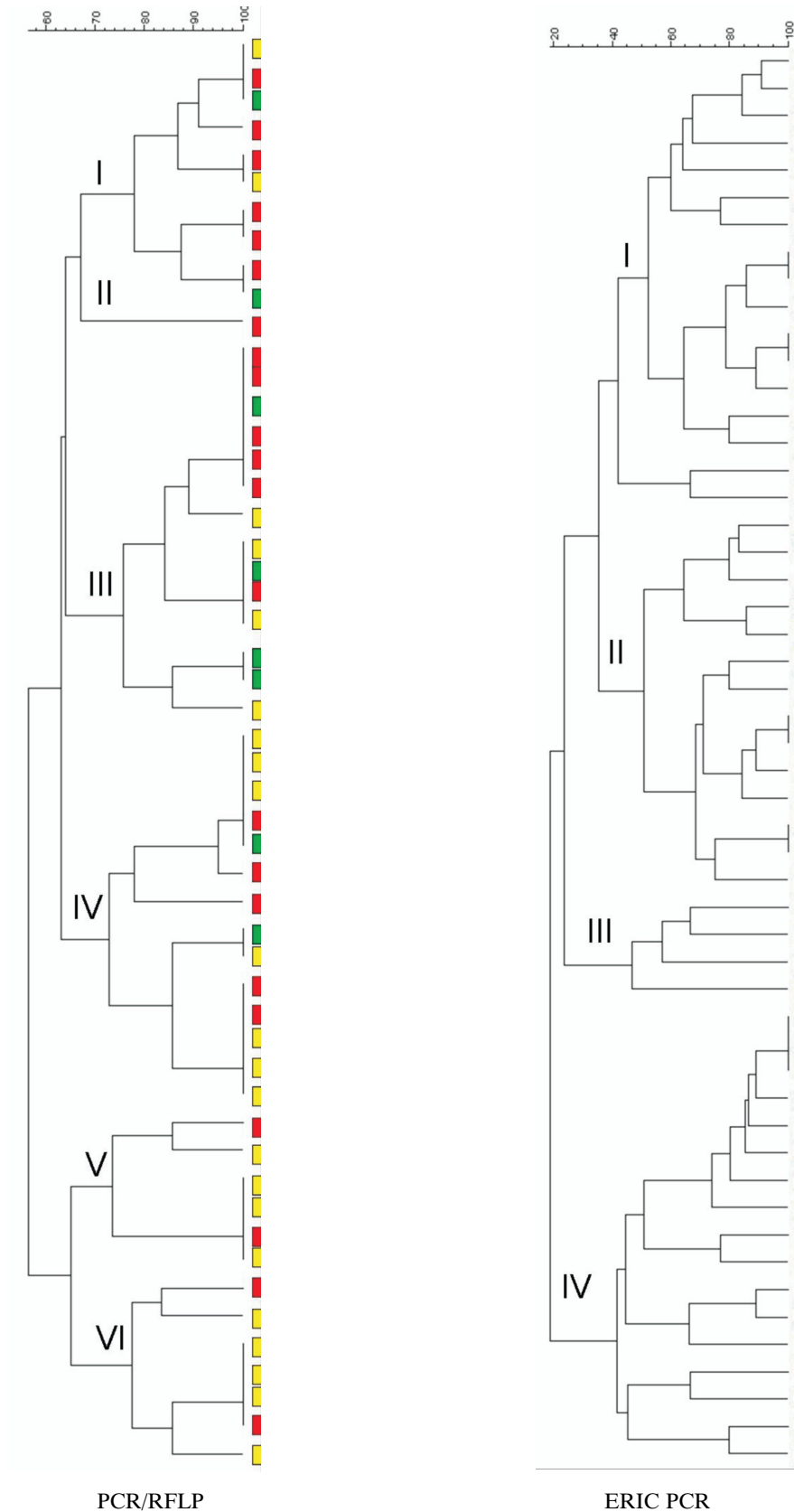


Fig. 1. Dendrographs obtained from computer analysis of electrophoretic pictures of *H. parasuis* isolates analysed by PCR/RFLP (MboII) and ERIC PCR. The affiliation degree (percentage) of the isolates is shown on a scale. Red colour shows strains I – isolated from animals with clinical signs typical of Glasser's disease, yellow colour shows strains II – isolated from animals with diagnosed pneumonia, green colour shows strains III – isolated from nasal smears of healthy animals from herds free of *H. parasuis* infection.

Discussion

Molecular methods are not affected by environmental factors, and for this reason, they are better than phenotypic methods. These methods, based on unique electrophoretic fingerprints, can be used for the assessment of the entire genome of a given microorganism or its fragment (gene, etc.). From them, ERIC-PCR method is frequently used for typing *H. parasuis* strains. High discriminatory power of ERIC-PCR method was found by other authors (Oliveira et al. 2003, Olvera et al. 2006, Olvera et al. 2007b) and it was markedly higher than in ID serotyping. The same authors found high genotypic diversity among the serotypes. ERIC-PCR test allowed them to distinguish several isolates obtained from different farms. This method was used for epidemiological studies by Olvera et al. (2007a), who determined the strains responsible for the outbreak of acute Glässer's disease from non-pathogenic strains present in the upper respiratory tract. They also evaluated the efficiency of the programme of eradication of virulent strains of *H. parasuis*.

However, it is worth to underline that the use of this method by no means ensures high repeatability of molecular patterns (Olvera et al. 2007b). This constraint is associated with the use of variable sequences of oligonucleotide primers, varied DNA concentrations and remarkable impact of amplification conditions on the final result. These disadvantages can be reduced by applying a uniform procedure. Unfortunately, when the investigations are carried out in two or more laboratories, repeatability of the results is difficult to achieve. According to Olvera et al. (2007b), repeatability of the results is better with PCR/RFLP than with the use of ERIC PCR due to the use of higher stringency conditions.

Molecular typing with the use of PCR/RFLP was studied by Lin (2003) who used a species-specific PCR product described by Oliveira et al. (2001), subjected to a digestion with endonuclease *HindIII*. In total, he distinguished 15 clone groups from 85 field strains and 7 reference strains of *H. parasuis*. Other authors used *tbpA* (de la Puente Redondo et al. 2003) and *aroA* genes (del Rio et al. 2006), containing more variable sequences. De la Puente Redondo (2003) analysed *tbpA* gene using a combination of nucleases (*AvaI*, *TaqI* and *RsaI*) and found 0.93 discriminatory power for field isolates of *H. parasuis*. Amplification of *aroA* gene and digestion with *Sau3AI* and *RsaI* endonucleases confirmed genetic diversity within a gene, which enabled typing and distinguishing of *H. parasuis* from some bacteria of *Actinobacillus* genus isolated from pigs (del Rio et al. 2006).

The results obtained in the present study show that the two methods used for typing of *H. parasuis* had high discriminatory power (~ 0.95). Taking into

account this parameter it can be concluded that ERIC PCR is more suitable than PCR/RFLP. This justifies the use of ERIC PCR for routine epidemiological analyses of the mentioned pathogen. Taking into account the complexity of method used, ERIC-PCR based on random amplification of DNA, proved to be comparable to PCR/RFLP.

The last mentioned technique is relatively less expensive and labour-consuming, especially when diagnostic PCR method is used for the epidemiological studies. The main advantage of PCR/RFLP is related with the fact that the method can be performed directly on clinical samples.

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