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Evaluation of Three Methods for DNA Fingerprinting of *Corynebacterium pseudotuberculosis* Strains Isolated from Goats in Poland

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Abstract

Phenotypic approaches based on metabolic and biological characteristics of *Corynebacterium pseudotuberculosis* have been limited due to insufficient discrimination between closely related isolates. In this paper we present performance and convenience of three molecular typing methods: BOX-PCR, random amplification of polymorphic DNA (RAPD) and amplification of DNA fragments surrounding rare restriction site (ADSRRS-fingerprinting) in genome analysis of these bacteria. Among examined 61 strains there were distinguished four, eight and 10 different genotypes by BOX-PCR, RAPD and ADSRRS-fingerprinting, respectively. The value of discrimination index was the lowest for BOX-PCR (D = 0.265), much bigger for RAPD (D = 0.539) and the highest for ADSRRS-fingerprinting (D = 0.604). The good discriminatory ability and reproducibility of RAPD and ADSRRS-fingerprinting indicates that those techniques may be particularly applied for epidemiological studies of *C. pseudotuberculosis* isolates. We found that ADSRRS-fingerprinting is a rapid method offering good discrimination power, excellent reproducibility and may be applied for epidemiological studies of intraspecific genetic relatedness of *C. pseudotuberculosis* strains.

Key words: *Corynebacterium pseudotuberculosis*, BOX-PCR, RAPD, ADSRRS-fingerprinting

Introduction

Corynebacterium pseudotuberculosis is an important animal pathogen belonging to the family of *Corynebacteriaceae*. It is a small, pleomorphic, Gram-positive, nonmotile and nonsporeforming rod. In sheep and goats *C. pseudotuberculosis* causes the caseous lymphadenitis (CLA), a common, chronic, economically important disease characterized by caseous abscessation of lymph nodes and internal organs (Brown and Olander, 1987; Williamson, 2001).

This organism has been classified into two biotypes based on host's preferences and nitrate-reducing activity (Biberstein *et al.*, 1971). Isolates from small ruminants (biotype *ovis*) are the most often unable to reduce nitrate to nitrite whereas isolates from horses and cattle (biotype *equi*) are usually nitrate-positive. There is not any described case of natural transmission *C. pseudotuberculosis* between small ruminants and horses or cattle.

Up to now extensive phenotypic diversity has been reported within these bacteria, from cell shape to biochemical properties. In spite of this several molecular typing methods based on direct analysis of genomic polymorphism were used to characterize strains of *C. pseudotuberculosis*, *e.g.* restriction endonuclease analysis (REA) of chromosomal DNA (Songer *et al*., 1988; Sutherland *et al*., 1996; Literák *et al*., 1999), rybotyping (Sutherland *et al*., 1996; Costa *et al*., 1998; Literák *et al*., 1999), pulsed field gel electrophoresis (PFGE) (Connor *et al*., 2000) and random amplification of polymorphic DNA (RAPD) (Foley *et al*., 2004).

This paper describes studies on the genotype analysis of isolates *C. pseudotuberculosis* using three PCR-based techniques, BOX-PCR, RAPD and ADSRRS-fingerprinting (amplification of DNA fragments surrounding rare restriction site). The major advantage of all these methods is that no knowledge of DNA sequence of the subject microbes is necessary. We evaluate these molecular typing methods for its differentiation power and potential use as an epidemiological tool for infection of *C. pseudotuberculosis* in goats in Poland. Results of the conducted study are particularly important to understand epidemiology and to control the *C. pseudotuberculosis* infection.

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Experimental

Materials and Methods

***Corynebacterium pseudotuberculosis* strains:** 61 isolates of *C. pseudotuberculosis* were studied. All strains were isolated from goats with the symptoms of CLA. Animals were derived from 20 herds from different regions of Poland. The swabs taken from abscesses were aseptically inoculated on blood agar supplemented with 5% sheep blood. The plates were incubated at 37°C under microaerophilic conditions (candle jar system) for 24–48 h. All suspected colonies: small, circular, convex, whitish to cream, dry, opaque and surrounded with a narrow zone of hemolysis were picked up for further analysis. Pure cultures of isolates were prepared and then identified biochemically by oxidase, catalase reactions and Api Coryne tests (bioMérieux, France). Isolates were stored at –20°C in trypticase soy broth (TSB; bioMérieux, France) with 20% glycerol. As a type strain the reference strain *C. pseudotuberculosis* ATCC 49327 was used (LGC Promochem, England).

Isolation of DNA: The genomic DNA was extracted from the *C. pseudotuberculosis* cells using a Genomic Mini AX BACTERIA kit (A&A Biotechnology, Poland). Bacterial pellet from 3 ml of TBS culture was resuspended in 100 µl of Tris-HCl buffer (pH 8.5) supplemented with 15 µl lysozyme solution (100 mg/ml). The samples were incubated for 16 h at 37°C. Next isolation steps were performed according to a protocol recommended by the kit producer instruction. The DNA from each strain was dissolved in distilled, DNase/RNase free water and stored at –20°C until use. The quantity of DNA was determined visually, after electrophoresis in 1% agarose gel. The intensities of obtained bands were compared with the DNA bands given by molecular mass marker – MassRuler™ DNA Ladder, Mix (MBI Fermentas, Lithuania).

Optimization of PCR conditions: Optimizations of BOX-PCR and RAPD were performed according to an original protocol of Taguchi and Wu modified by Cobb and Clarkson (1994). The effects and interactions of four components ($MgCl_2$, dNTPs, primer and DNA template) were investigated.

BOX-PCR: The BOX-PCR fingerprinting was performed using the BOXA1R primer (5'-CTACGGC AAGGCGACGCTGACG-3') (Invitrogen, USA). The 25 µl of reaction mixture after optimization contained: 2.5 µl 10×PCR buffer (MBI Fermentas), 4 mM $MgCl_2$, 0.4 mM of each dNTP (MBI Fermentas), 50 pmol of primer, 30 ng DNA template, 1 µl BSA (20 mg/ml) and 1.25 U of Taq polymerase (MBI Fermentas). Three separate reactions for each isolate were conducted. Thermocycling conditions were as follow: initial denaturation at 95°C for 4 min, then 35 cycles of denaturation at 94°C for 3 s and 92°C for 30 s, annealing at 50°C for 1 min and extension at 65°C for 8 min. After the last cycle the samples were incubated at 65°C for 8 min.

RAPD: All *C. pseudotuberculosis* strains were fingerprinted by RAPD using the 10-nt primer OPA03 (5'-AGTCAGCCAC-3') (Invitrogen, USA). The selection of primer was based on the number and size of obtained amplification products. The temperature profile was the same as the one described by Foley *et al.* (2004). Optimal reaction mixture was determined as 2.5 µl 10 × PCR buffer, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP (MBI Fermentas), 20 pmol of primer OPA03, 30 ng DNA template, 1 µl BSA (20 mg/ml), 1 U of Taq polymerase (MBI Fermentas) and water to 25 µl.

Gel electrophoresis of PCR products: Amplification products (20 µl) were separated in a 1.5% agarose gels in 1 × TBE buffer at a constant voltage (2 V/cm). MassRuler™ DNA Ladder, Mix (MBI Fermentas) was used for estimating the molecular size weight of obtained bands. After electrophoresis gels were stained with ethidium bromide solution (0.5 µg/ml) for 30 min at room temperature and visualized, photographed and analysed by the VersaDoc Imaging System (model 1000) and Quantity One software (version 4.4.0) (Bio-Rad, USA).

ADSRRS-fingerprinting: ADSRRS fingerprinting was performed essentially as described by Krawczyk *et al.* (2003). Total genomic DNA of *C. pseudotuberculosis* was digested with two restriction endonucleases generating cohesive ends (rare cutter/frequent cutter restrictases): NotI/BglII, EcoRI/BglII, XbaI/BglII, XbaI/BamHI or BcuI/BamHI (10 U/µl; MBI Fermentas). Digestion reaction was performed in a 20 µl reaction mixture containing 100–150 ng of template DNA; 2 or 4 µl 10×buffer recommended for the used pair of enzymes (2 µl buffer O+ for NotI/BglII and EcoRI/BglII, 4 µl buffer Y+/Tango for XbaI/BglII and 2 µl buffer G+ for other combinations of enzymes); 5 U of each enzyme and sterile distilled water up to 20 µl. After 16 h incubation at 37°C to each of the digested samples 180 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.5) and 200 µl isopropanol were added. The samples were gently mixed by several inversions, incubated at room temperature for 15 min and then centrifuged at 12 000 × rpm for 10 min. The pellet was washed with 400 µl of 70% ethanol and centrifuged at 12 000 × rpm for 5 min. The pellet was air dried for 15 min. Cohesive ends of DNA were ligated with earlier prepared adapters (Table I). Adapters were made by diluting two appropriate oligonucleotydes in water to the concentration 20 pmol/µl of each and heating mixture at 90°C for 2 min. Afterwards, the tubes were cooled to an ambient temperature for 10 min.

Dry pellet of digested DNA was dissolved in solution: 2 µl of 10 × ligation buffer (MBI Fermentas),

Table I
Characterization of adapters and primers used in ADSRRS-fingerprinting for *C. pseudotuberculosis*

Adapters and primers		Nucleotides sequences
Adapters for rare cutter enzymes:	NotI	5'-**GGCC**GTCGACGTT-3' 3'-CAGCTGCAACCACCTACTTCC-5'
	BcuI / XbaI	5'- **CTAG**GTCGACGTT - 3' 3'-CAGCTGCAACCACCTACTTCC-5'
Adapter for frequent cutter enzymes:	BglII /BamHI	5'-**GATC**CGTCGACAACGGCGTTCCT TCGTCTACCATCC-3' 3'- GCAGCTGTTGCCGCAAGGAAGCAGATGGTAGG-5'
Primers for:	NotI /BcuI /XbaI	5' CCTTCATCCACCAACGTCGAC 3'
	BglII /BamHI	5' GGATGGTAGACGAAGGAACGC 3'

Cohesive ends compatible with restriction site are bolded.

1 µl of each appropriate double-stranded adapters, 1U T4 DNA ligase (MBI Fermentas) and water to 20 µl. Ligation mixture was vortexed and incubated overnight at 16°C. After ligation, 180 µl TE buffer was added and DNA was precipitaded with isopropanol. The DNA pellet was dissolved in 20 µl TE buffer. The amplification was performed using 2.5 µl of 10×buffer for Pwo polymerase (DNA Gdańsk II; Poland), 2.5 µl of 2 mM (each) dNTP Mix (MBI Fermentas), 1.5 µl of 50 mM $MgCl_2$ (DNA Gdańsk II), 25 pmol of each primer, 3 µl solution of ligation products, 1 U of Pwo polymerase (DNA Gdańsk II) and sterile distilled water to bring total volume to 25 µl. The temperature profile contained in turn: initial denaturation at 94°C for 5 min, filling the ends of the DNA fragments at 72°C for 5 min and 24 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min. It was completed by a final extension at 72°C for 7 min. PCR products were electrophoresed in 8% polyacrylamide gels in 1×TAE buffer. 20 µl of each sample was loaded on the gel and electrophoresis was performed at 20°C at constant power 110 V. MassRuler™ DNA Ladder, Mix was used as a size standard. After electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml), visualized, photographed and analysed by VersaDoc Imaging System (model 1000) and Quantity One software (version 4.4.0) (Bio-Rad, USA). All dendrograms were generated using program STATGRAPHICS Centurion XV.

Index of discrimination: In order to compare all typing methods and select the most discriminatory system for genome analysis of *C. pseudotuberculosis* we used a single numerical index of discrimination (D) described by Hunter and Gaston (1988). The index is based on the probability that two independent isolates, sampled randomly from the test population, will be classified into different types.

Results

The physiological analysis of 61 isolates showed that all of them except one did not reduce nitrate into nitrite and all produced phospholipase D, the main virulence factor of *C. pseudotuberculosis* (data not shown). From this result 60 strains were classified as biotype *ovis* and one strain as biotype *equi*. All isolates were oxidase-negative and catalase-positive. In stained smears they show a characteristic angular and palisade arrangement. The species identification of all isolates was confirmed biochemically by Api Coryne tests.

BOX-PCR: The concentration of tested reaction components used in optimization procedure had an impact not only on the number of generated products but also on their size. High concentration of Mg^{2+} (above 3.5 mM), dNTPs (above 0.3 mM of each) and primer (above 30 pmol) promoted the amplification of products with molecular weights greater than 1.5 kbp but reduced the number of bands with molecular weights less than 1.5 kbp. We have chosen the 4 mM of Mg^{2+}, 0.4 mM of each dNTP and 50 pmol primer as the optimal level for the total number of amplification products (with molecular weights less and greater than 1.5 kbp). DNA template concentration was estimated as 30 ng.

Genotype analysis of totally 62 strains *C. pseudotuberculosis* by BOX-PCR showed quite complex patterns consisting of numerous bands (from 17 to 21) with distinct intensity and size, from approx. 350 bp to 4500 bp (Figure 1A). The fragment with approx. 800 bp was the most prominent. The profiles show high similarity and all isolates were divided into four genotype groups consisting of 53, five, three and one strain respectively. The generated dendrogram represents the degree of similarity between the four distinguished genotyp groups (Figure 1B). In order to compare the discriminatory power of all examined methods and their usefulness in genome analysis of *C. pseudotuberculosis* the numerical index of the discrimination (D) was established. The value of discrimination index for BOX-PCR was very low and was determined as 0.265.

RAPD: The OPA03 primer was chosen for amplification study of all *C. pseudotuberculosis* isolates. The amplification yields gave a good distribution of fragments (relatively large number of bands in variable

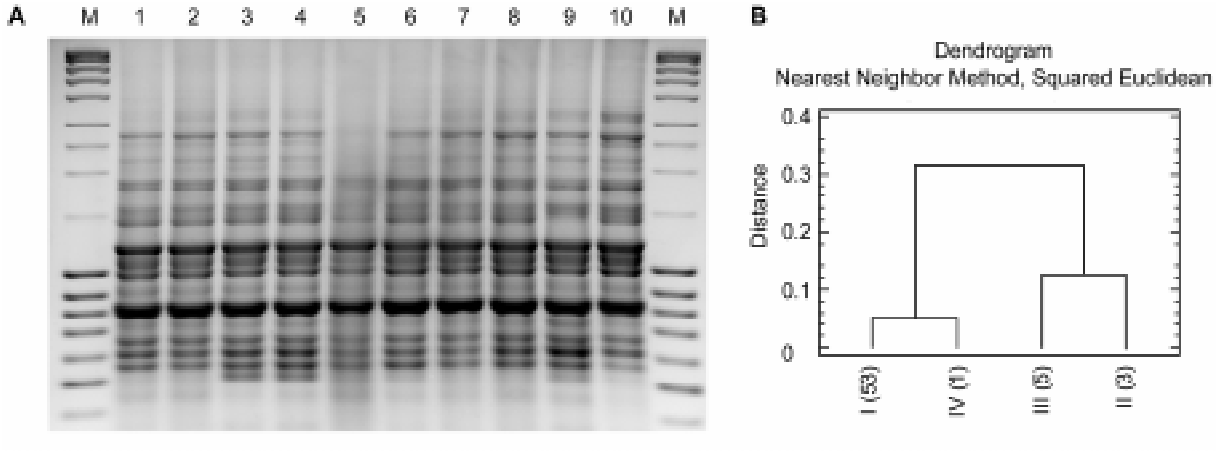

Fig. 1. BOX-PCR analysis of *C. pseudotuberculosis* strains.
A: BOX patterns for ten isolates (lanes 1–10). Lanes M, MassRuler^{TM} DNA Ladder, Mix; MBI Fermentas (10 000–400 bp).
B: Dendrogram showing degree of similarity among tested isolates. Roman numerals denote the genotype groups and arabic numerals – number of strain classified into a given genotype group.

size) enabling a comparatively simple interpretation of the results.

The results obtained by the procedure chosen as optimal indicated that the increase of the Mg^{2+} concentration to the level 3.5 mM altered the discriminatory power of RAPD (demonstrates a substantial increase in amplification). The optimum concentration of dNTP, primer and template were determined as: 0.2 mM (of each), 20 pmol and 30 ng, respectively. We observed a distinct decrease above this level for the number of amplification products in the RAPD profile suggesting that the highest concentration of these components should not be used. Genotyping of all strains by RAPD showed various patterns of bands between approx. 3100 bp and 250 bp. The method was more discriminating and generated eight different profiles. Four genotypes were shared by more than one strain (41, nine, six and two isolates) whereas four isolates had unique RAPD patterns. Each profile comprised from 12 to 16 bands and eight fragments were shared by all strains. Four bands, with approx. 1050, 1100, 1300 and 2500 bp were permanently more intense than the others. The obtained RAPD patterns and dendrogram for representative isolates are presented in Figure 2A and 2B. The discrimination index had miles bigger value, D = 0.539.

ADSRRS-fingerprinting: In preliminary experiments the restriction system NotI/BglII was selected as the most appropriate for genotype analysis of studied strains. Other combinations of enzymes generated too large number of products, especially in a very small size, which made the interpretation not clear and difficult.

Among 62 strains tested by ADSRRS-fingerprinting, 10 different genotypes were identified. Five genotypes included only one isolate, each of them had a profile completely different from other tested strains. The rest of genotypes in 38, eight, five, four and two

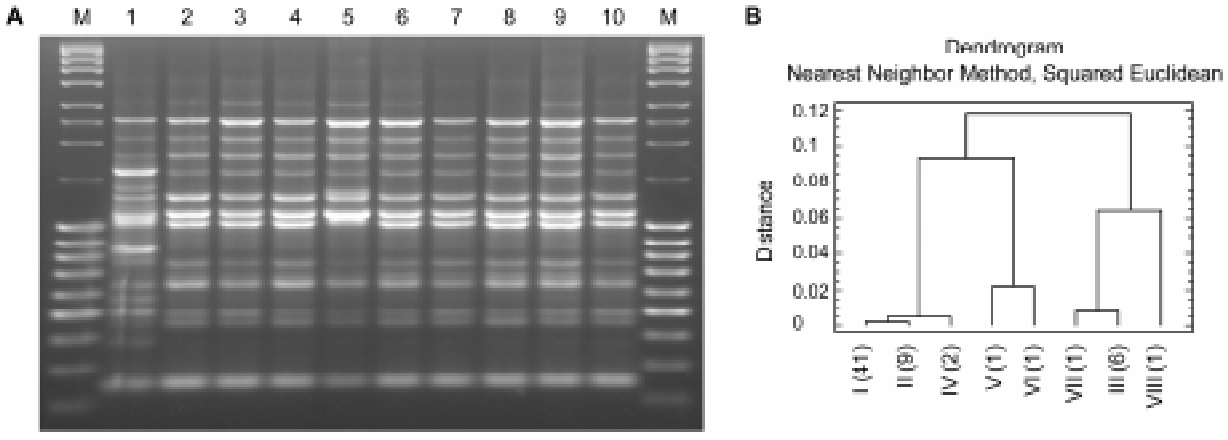

Fig. 2. RAPD analysis of *C. pseudotuberculosis* strains.
A: RAPD patterns of selected isolates (lanes 1–10). Lanes M, MassRuler^{TM} DNA Ladder, Mix (10 000–200 bp).
B: Dendrogram revealing degree of similarity among tested isolates. I–VIII: genotype groups (number of strains).

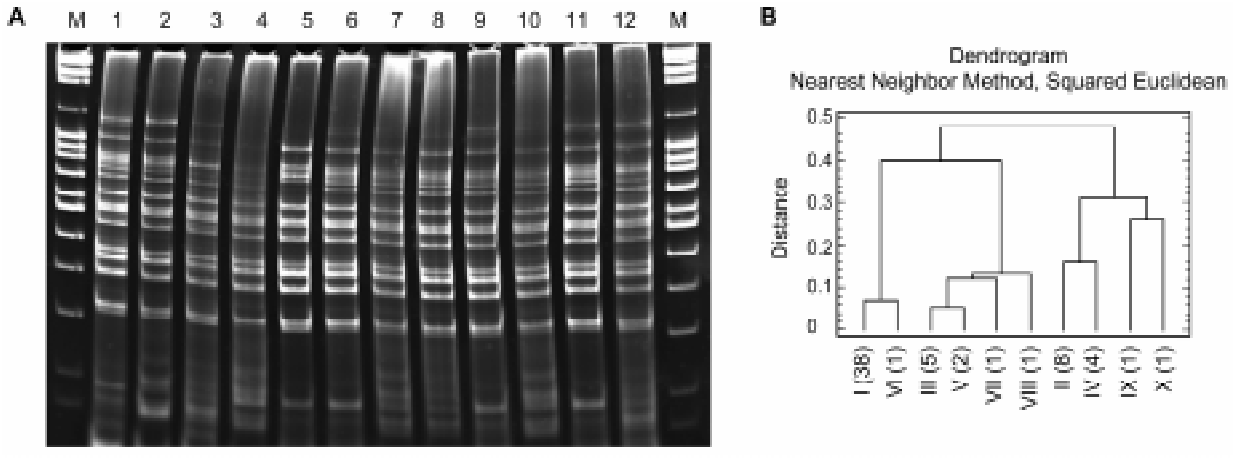

Fig. 3. ADSRRS-fingerprinting analysis of *C. pseudotuberculosis* strains.
A: ADSRRS patterns for 12 isolates (lanes 1–12). Lanes M, MassRuler^{TM} DNA Ladder, Mix (10000–80 bp).
B: Dendrogram illustrating degree of similarity among tested isolates. I–X: genotype groups (number of strains).

strains were shown. Amplification patterns consisted of variable number (from 11 to 20) bands in different size (in the 1400–100 bp range). All strains had the same nine DNA fragments. The fingerprinting results for representative isolates are shown in Fig. 3A. The banding patterns obtained for all strains were compared and dendrogram based on the results of ADSRRS-fingerprinting assay is shown in Fig. 3B. The same genetic patterns were observed in any of the three independent experiments. The discrimination index had the highest value, D = 0.604.

There were some variations between the BOX, RAPD and ADSRRS genotypes. For example, two isolates having the same genotype by one of the method were shown to have distinct profile in another one (see Table II). It could suggest that combination of two the most discriminatory methods – RAPD and ADSRRS-fingerprinting may be the most efficacious in typing of clinical isolates of *C. pseudotuberculosis.*

Finally, a combined dendrogram based on genome patterns obtained by RAPD and ADSRRS-fingerprinting was generated (Figure 4). Among the 62 strains, 19 different genotypes were identified. Eight genotypes were shared by more than one strain: 28 isolates

Table II
The genotyping results for 62 *C. pseudotuberculosis* strains analysed by BOX-PCR, RAPD and ADSRRS-fingerprinting

C. pseudotuberculosis strain	BOX--profile	RAPD--profile	ADSRRS--profile	RAPD--ADSRRS profile*
ATCC 43927, 1, 2, 5, 7, 9, 14, 15, 18, 21, 29, 44, 46, 48, 51, 52, 53, 55, 57, 58, 61, 64, 66, 67, 68, 73, 74, 75	I	I	I	I
6, 13, 23, 24, 69	I	I	II	II
11, 12, 26, 50, 65	I	I	III	III
70	I	I	IV	XIII
17	I	I	VII	XIV
16	I	I	X	XVI
28, 30, 34, 72	I	II	I	IV
22, 59	I	II	II	VI
25, 45	I	II	V	VII
31	I	II	VIII	XV
63, 71	I	III	I	VIII
60	I	VI	I	XI
19	I	VIII	I	XVIII
56	II	V	I	X
20, 32, 62	III	III	IV	V
10	III	III	IX	XIX
4	III	VII	I	XVII
47	IV	IV	II	XII
33	IV	IV	VI	IX

* Genome profiles determined by combining the results of two the most discriminatory methods – RAPD and ADSRRS-fingerprinting.

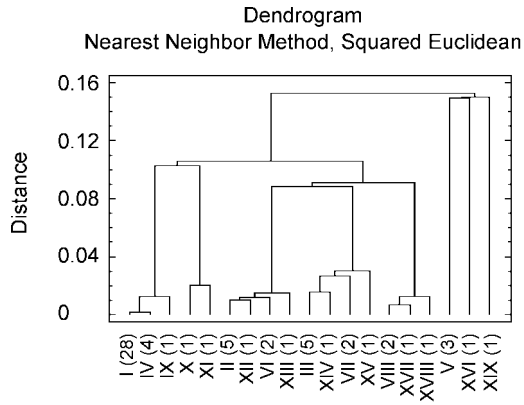

Fig. 4. Combined dendrogram based on the RAPD and ADSRRS patterns showing degree of similarity among tested isolates of *C. pseudotuberculosis.*
I–XIX: genotype groups (number of strains).

(genotype I), five (genotype II and III), four (genotype IV), three (genotype V) and two (genotype VI, VII, VIII) whereas eleven isolates had unique profile (genotype IX–XIX). The value of discrimination index was higher than discrimination index determined by each of this method individually (D = 0.784).

Discussion

In recent years, many reports have shown the great value of molecular techniques in identification and genotype differentiation of infectious disease agents. Genotyping methods are used with increasing frequency for epidemiological investigations of many pathogenic bacteria. They allow the detection of polymorphisms at the level of nucleic acids and thus can provide more precise and reliable information on variability among tested strains than phenotypic techniques. They are rapid, more discriminatory and less affected by variations due to growth phase or conditions. They enable the discrimination of genera and species and they can also determine relationship and differences among individual strains. However, the efficiencies of various molecular methods can differ considerably.

In the presented study there were used three different fingerprinting techniques based on PCR to assess the genome diversity among *C. pseudotuberculosis* strains which cause infections in goats in Poland. We have evaluated the usefulness of each examined method in molecular typing of these bacteria on the basis of the number and size ranges of obtained amplification products and analysis of the numerical index of the discrimination (D). The first essential step was the choice of reaction conditions and appropriate primer/restriction enzymes combination to maximal improvement of the discriminatory power of fingerprintings. The number of generated amplification products has to be sufficient so as to allow the differentiation among related but distinct bacterial strains and a reliable demonstration of the relationship between isolates and easy interpretation of results. Too complex patterns make the analysis difficult and time-consuming. The amplification profiles generated by all techniques showed a moderate complexity. The number of obtained in every test, different in size (in the 4500–100 bp range), DNA fragments were relatively large (above 10 bands) and interpretation of data was not problematic. However, all methods characterized different level of discrimination and, by extension, usefulness for the molecular typing of *C. pseudotuberculosis* isolates. Among the 62 examined strains of *C. pseudotuberculosis* (61 isolates and one reference strain) it was possible to distinguish four, eight or 10 different banding patterns depending on using method.

The strains were first genotype by BOX-PCR and we found that this technique showed a low discriminatory power (D = 0.265). To our knowledge the BOX-PCR fingerprinting has never been tested on *C. pseudotuberculosis*. Anyhow, this method was proved to be a great tool for molecular typing of many species of bacterial pathogens, for example plant-pathogenic bacteria (Louws *et al*., 1994), pneumococcal strains (van Belkum *et al*., 1996) and *Aeromonas* spp. (Tacão *et al*., 2005). According to our data BOX-like sequences are also present in many copies within the genome of *C. pseudotuberculosis* species. However, so small diversity obtained in our studies may indicate that although BOX motifs are prevalent in strains of *C. pseudotuberculosis*, there is a high degree of similarity between number and distribution of these repetitive sequences throughout the genome of analysed strains. All isolates have shown very similar bands patterns and only four genotypes were revealed.

Significantly greater genome diversity was observed among the isolates studies by RAPD and ADSRRS-fingerprinting and index of discrimination for these techniques were much higher (0.539 and 0.604, respectively). Among the 62 tested strains, eight (by RAPD) and 10 (by ADSRRS-fingerprinting) different genotypes were identified. In previous studies a high degree of genome similarity and relationship among ovine and caprine strains of *C. pseudotuberculosis* were demonstrated by several different genotypic methods. By the restriction analysis of chromosomal DNA of 94 *C. pseudotuberculosis* isolates there were distinguished only two different genotypes. All strains of *ovis* biotype had identical restriction profiles, different from those obtained for strains of *equi* biotype. Moreover, the number of fragments generated by restriction enzymes was large which made the interpretation difficult and time-consuming, especially in case of large numbers of studying isolates (Songer *et al*., 1998). Ribotyping was shown to be more discriminatory technique which allowed the detection of differences also between strains belonging to the same biotype. Two different patterns for five (Sutherland *et al*., 1996) and 29 (Costa *et al*., 1998) isolates of *equi* biotype were achieved. All strains isolated from small ruminants had the same genotype. REA and ribotype patterns of 10 isolates from ovine and caprine cases of CLA in the Slovak and Czech Republics were also identical (Literák *et al*., 1999). The highest level of discrimination was demonstrated by PFGE and RARD and these assays were shown to be the most useful in epidemiological investigation to determine the relationship between clinical isolates of *C. pseudotuberculosis*. Among 47 United-Kingdom isolates of *ovis* biotype five pulsotypes by PFGE were designated. Two strains of equine origin had distinct sixth pulsotype (Connor

et al., 2000). PFGE is considered to be the most discriminatory and reliable typing method ("gold standard"). However, its serious limitations in use as an epidemiological tool could be an expensive, complex, laborious and time-consuming procedure and the necessity to possess specialized equipment. In genotype differentiation RAPD assay is simpler and more rapid. Thanks to this method eight distinct patterns were detected among 48 strains of *C. pseudotuberculosis* isolated from horses (Foley *et al*., 2004).

The major disadvantage of RAPD is a poor reproducibility of obtained results, especially interlaboratory, because of high sensitivity of these methods to reaction conditions. There are many factors affecting reproducibility of techniques based on DNA amplification by arbitrary primer, such as differences in DNA extractions, template quality, concentration of reaction components (especially Mg^{2+} ions), PCR conditions, the model of using thermal cycler (Tyler *et al*., 1997). Furthermore, the annealing temperature is lower than in classical PCR that is suitable for incomplete hybridization between the primer and the target site and can induce nonspecific amplification products and can lead to differentiation in obtained molecular patterns. However, careful choice of primer and precise optimization of the reaction condition in the present study allowed the reduction of this disadvantage and to improve the reproducibility and quality of patterns. Using the strategy based on modified Taguchi method we investigated, using only a few reactions, not only effects but also interactions between the most important components in mixture reaction (Cobb and Clarkson, 1994). The reproducibility of each single method was assessed by carrying out three separate experiments for the majority of isolates. There was a little variation only in results obtained by BOX-PCR. The variability included the presence of bands in high molecular mass (between 3500–4500 bp). However, these bands in all strains were very weak and less intense than the others. No changes in the RAPD and ADSRRS-fingerprinting patterns were observed in three independent reactions for each isolate. In RAPD control reaction (this is a sample without experimental template DNA) some smear was observed. In literature data the presence of smears and/or weak bands in "no template" control assays of RAPD have been reported. These backgrounds were dependent on the source of Taq polymerase or their concentration and did not seem to influence on typing results because they were not detected when template DNA was added to the reaction mixture (Tyler *et al*., 1997; Gzyl and Augustynowicz, 1999).

The present study indicates that *C. pseudotuberculosis* strains causing infections in goats in Poland are genetically diverse. Identical genome patterns were found in strains isolated from animals from herds in different regions and strains which had distinct genome profiles were isolated from goats from the same herd. One genotype was significantly predominant and was represented by 85%, 66% or 61% of isolated depending on using method (BOX-PCR, RAPD and ADSRRS-fingerprinting respectively). The amplification profile of the reference strain of *C. pseudotuberculosis* (ATCC 43927) showed the high similarity to the studied isolates. It had the identical pattern to that of the predominant genotype. One isolate had ability to reduce nitrate to nitrite but its genotype was the same as the one obtained for the majority of nitrate-negative caprine isolated. It was classified also to the most abundant genome profile.

In conclusion, we used three fingerprinting methods, BOX-PCR, RAPD and ADSRRS-fingerprinting as molecular typing tool for strains of *C. pseudotuberculosis*. The most discriminative turned out ADSRRS-fingerprinting and this technique could be very useful alone or in combination with RAPD, for the screening of differentiation and relationship between clinical isolates of these bacteria.

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