Research Article

Multilocus Sequencing of Corynebacterium pseudotuberculosis Biotype Ovis Strains

Boglárka Selleyei,1 Krisztián Bányai,1 Dániel Bartha,1 István Hajtós,2 László Fodor,3 and László Makrai3

1CAR, HAS, Institute for Veterinary Medical Research, P.O. Box 18, Budapest 1581, Hungary
2Government Office for Borsod-Abaúj-Zemplén County, Vologda U. 1, Miskolc 3525, Hungary
3Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, P.O. Box 22, Budapest 1581, Hungary

Correspondence should be addressed to László Makrai; makrai.laszlo@univet.hu

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Thirteen Corynebacterium pseudotuberculosis biotype ovis strains isolated from clinical cases of caseous lymphadenitis in Hungary were characterised using multilocus sequencing and their phylogenetic comparison was carried out on the basis of four housekeeping genes (groEL1, infB, dnaK, and leuA). The in silico analysis of the 16 frequently studied housekeeping genes showed that C. pseudotuberculosis strains could be readily distinguished from C. diphtheriae and C. ulcerans strains; however, sequences of the same genes in the two biotypes of the C. pseudotuberculosis were highly similar; the heterogeneity values were low. Genes dnaK, infB, groEL1, and leuA showed marked genetic variation within C. pseudotuberculosis, and strains of the two biotypes of C. pseudotuberculosis could be differentiated. Analysis of the individual genes showed a fairly conservative nature of C. pseudotuberculosis biotype ovis strains. The greatest genetic differentiation was seen in the dnaK and infB genes and concatenations of these two genes were very useful in the genetic separation of the studied strains.

1. Introduction

Corynebacterium pseudotuberculosis was isolated from a sheep and described by Preisz [1] as a Gram-positive, facultative intracellular pathogen. Two major biotypes can be distinguished based on the ability of reduction of nitrate; the nitrate negative biotype (C. pseudotuberculosis biotype ovis) causes caseous lymphadenitis (CLA) in small ruminants [2–4], whereas the nitrate positive biotype (C. pseudotuberculosis biotype equi) is the causative agent of ulcerative lymphangitis in horses, cows, camels, buffaloes, and occasionally humans [5, 6]. CLA is a chronic, contagious disease that is characterised by abscess formation in or near major peripheral lymph nodes (external form) or within the internal organs and lymph nodes (internal form). CLA occurs worldwide; however, it is more frequent in the tropical areas and causes important economic losses in ovine and caprine herds by reducing wool, meat, and milk production [4, 7–10].

Classification of C. pseudotuberculosis was originally based on cultural, morphological, and biochemical characteristics [11]. In addition to nitrate reduction various molecular methods, restriction fragment length polymorphism (RFLP) of chromosomal DNA, ribotyping, and whole genome sequence analysis were used for the differentiation of C. pseudotuberculosis biotypes [12–14]. C. pseudotuberculosis biotype equi strains showed greater genetic divergence than C. pseudotuberculosis biotype ovis strains when examined with pulsed field gel electrophoresis (PFGE), BOX-PCR, random amplified polymorphic DNA (RAPD), and amplification of DNA surrounding rare restriction sites (ADSRRS) [3, 15, 16].

Multilocus sequence typing has become popular in bacterial genotyping technique over the past decade, including genotyping Corynebacterium spp.; it allows the identification of potential molecular genetic marker(s) in conservative
2. Materials and Methods

2.1. Bacterial Strains and Culture Condition. Thirteen C. pseudotuberculosis biotype ovis field strains isolated between 1994 and 2014 were randomly selected from the strain collection of the Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary. After identification using standard methods [17, 18], they had been stored at −80 °C until use. Fragments of the selected housekeeping genes were amplified by PCR in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Carlsbad, CA, USA). The 25 μl amplification reaction mixture contained 1x Dream Taq Buffer, 200 nM dNTP-mix, 1 μM of each primer, 1U Dream Taq (Thermo Fisher Scientific, Carlsbad, CA, USA), template DNA, and water. The PCR program included 3 min of initial denaturation at 95 °C and 20 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C.

2.2. In Silico Sequence Analysis and Primer Design. In order to distinguish C. pseudotuberculosis biotypes from the closely related species and to find potential markers, the nucleotide sequences of 16 housekeeping genes of 15 C. pseudotuberculosis, 13 C. diphtheriae strains, and one C. ulcerans strain (Table 2) were compared. The nucleotide sequences of 7 genes including the ATP synthase alpha chain (atpA), the DNA polymerase III alpha subunit (dnaE), the chaperone Hsp70 (dnaK), the elongation factor G (fusA), the 2-isopropylmalate synthase (leuA), the 2-oxoglutarate dehydrogenase E1 and E2 components (odhi), and the DNA-directed RNA polymerase beta chain (rpoB) were taken from [22, 23], while sequences of 9 genes involving ATP synthase beta chain (atpD), the heat shock proteins GroEL and GroES (groES, groEL1, and groEL2), the translation initiation factor IF2 (infB), the DNA recombinational repair system (recA, recN), the alpha subunit of DNA dependent RNA polymerase (rpoA), and the manganese-dependent superoxide dismutase (sodA) were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/genbank/). In silico analysis of the selected genes was performed with the ClustalW multiple alignment algorithm [24] in MegAlign software of the Lasergene 7 program suite (DNASTar, Madison, WI, USA) to find potentially useful markers for genetic differentiation and molecular epidemiological investigations.

Four genes (groEL1, infB, dnaK, and leuA) were selected for primer design; they are located at different genomic regions of bacteria (Figure 1). Primer sets were designed to amplify 580 bp, 654 bp, 641 bp, and 647 bp long fragments from the respective genomic regions; pending on the genes, the number of SNPs varied from 7 to 31 within the amplified fragments (Table 3). PCR primers were designed using Oligo Primer Analysis software 7 [25].

2.3. PCR and Sequencing. A loopful of 48 h culture of C. pseudotuberculosis was suspended in 6% Chelex-solution (Bio-Rad Laboratories, Hercules, CA, USA) and then heated to 65 °C for 30 min and to 100 °C for 8 min; afterwards, it was centrifuged. The supernatant was collected in a new tube and stored at −20 °C until use. Fragments of the selected housekeeping genes were amplified by PCR in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Carlsbad, CA, USA). The 25 μl amplification reaction mixture contained 1x Dream Taq Buffer, 200 nM dNTP-mix, 1 μM of each primer, 1U Dream Taq (Thermo Fisher Scientific, Carlsbad, CA, USA), template DNA, and water. The PCR program included 3 min of initial denaturation at 95 °C and 20 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C.

The objective of this study was characterisation of Hungarian field strains of C. pseudotuberculosis biotype ovis using multilocus sequencing and their phylogenetic comparison on the basis of selected housekeeping genes.

genes for the differentiation of closely related bacterial strains [17, 18].

Reference strains of C. pseudotuberculosis biotype ovis (DSM-7180) and C. pseudotuberculosis biotype equi (DSM-7177) from the Leibniz Institute, German Collection of Microorganisms, and Cell Cultures (Braunschweig, Germany) were also included in the examinations.

The strains were cultured on Columbia blood agar (LabM, Lancashire, UK) with the addition of 5% (vol/vol) sterile defibrinated sheep blood and incubated at 37 °C for 48 h.

Table 1: The list and registered data of C. pseudotuberculosis strains included in the examinations.

| ID | Host | Place of isolation | Strains | Year |
|----|------|--------------------|---------|------|
| C1 | Goat | Szentistván | C. pseudotuberculosis biotype ovis | 1994 |
| C3 | Sheep | Vizsoly | C. pseudotuberculosis biotype ovis | 2003 |
| C7 | Goat | Vizsoly | C. pseudotuberculosis biotype ovis | 2003 |
| C11 | Sheep | Boldogkővárálya | C. pseudotuberculosis biotype ovis | 2005 |
| C12 | Goat | Érd | C. pseudotuberculosis biotype ovis | 2005 |
| C14 | Goat | Alsószolca | C. pseudotuberculosis biotype ovis | 2006 |
| C15 | Goat | Rudabánya | C. pseudotuberculosis biotype ovis | 2006 |
| C19 | Goat | (DSM-7180) | C. pseudotuberculosis biotype ovis | 1988 |
| C21 | Horse | (DSM-7177) | C. pseudotuberculosis biotype equi | 1988 |
| C22 | Sheep | Mezőcsát | C. pseudotuberculosis biotype ovis | 2013 |
| C23 | Goat | Csétény | C. pseudotuberculosis biotype ovis | 2013 |
| C24 | Sheep | Pétervasára | C. pseudotuberculosis biotype ovis | 2009 |
| C25 | Goat | Szendrő | C. pseudotuberculosis biotype ovis | 2011 |
| C26 | Goat | Csongrád | C. pseudotuberculosis biotype ovis | 2014 |
| C27 | Goat | Kisvásárhely | C. pseudotuberculosis biotype ovis | 2014 |
Table 2: The source of *Corynebacterium* sp. examined in the in silico sequence analysis.

| *C. pseudotuberculosis* | Biotype | Host | Origin | *C. diphtheriae* | Host | Origin |
|-------------------------|---------|------|--------|-----------------|------|--------|
| FRC41                   | Ovis    | Human | France | NCTC13129       | Human | UK     |
| 3/99-5                 | Ovis    | Sheep | Scotland | BH8 | Human | Rio de Janeiro |
| P54B96                 | Ovis    | Antelope | South Africa | C7 | Human | Atlanta, GA, USA |
| 1002                 | Ovis    | Goat | Brazil | 241 | Human | Rio de Janeiro |
| C231                  | Ovis    | Sheep | Australia | HC01 | Human | Rio de Janeiro |
| I9                    | Ovis    | Cow | Israel | VA01 | Human | Rio de Janeiro |
| PAT10                 | Ovis    | Sheep | Argentina | CDCE 8392 | Human | Bethesda, MD, USA |
| 42/02-A              | Ovis    | Sheep | Australia | 31A | Human | Rio de Janeiro |
| 267                  | Ovis    | Llama | USA | HC03 | Human | Rio de Janeiro |
| 316                  | Equi    | Horse | USA | HC02 | Human | Rio de Janeiro |
| CIP 52.97            | Equi    | Horse | Kenya | INCA 402 | Human | Rio de Janeiro |
| 1/06-A              | Equi    | Horse | USA | PW8 | Human | New York |
| 31                   | Equi    | Buffalo | Egypt | HC04 | Human | Rio de Janeiro |
| 258                  | Equi    | Horse | Belgium | *C. ulcerans* | |
| Cpl62             | Equi    | Camel | UK | BR-AD22 | Dog | |

Sequences were aligned using the ClustalW algorithm in the MEGA 6 software [26] and trimmed manually at the same position before being used for comparison with sequences of other *C. pseudotuberculosis* strains deposited in the GenBank. The same gene set from *C. diphtheriae* HC02 was used as an outgroup.

After the single gene alignments, the sequences were joined to make a concatemer of loci at head-to-tail in-frame. The phylogenetic trees were constructed by using the neighbour joining (NJ) method. The bootstrap technique [27] was employed to evaluate the reliability of tree topologies by resampling the sequence alignment 1000 times.

2.5. Nucleotide Sequence Accession Numbers. The determined partial gene sequences were deposited in GenBank under accession numbers: from MF491615 to MF491629 (*dnaK*); from MF464070 to MF464084 (*infB*); MF476990 and from MF476992 to MF477005 (*groEL1*); MF446654 and from MF446656 to MF446669 (*leuA*).

3. Results

As the use of MLST protocol of *Corynebacterium diphtheriae* (https://pubmlst.org/cdiphtheriae/) proved to be inadequate for analysis of *C. pseudotuberculosis* strains, developing of new scheme has been required.

The in silico analysis of the 16 frequently studied housekeeping genes showed that *C. pseudotuberculosis* strains could be readily distinguished by sequence analysis from *C. diphtheriae* and *C. ulcerans* strains; however, sequences of the same genes of the *C. pseudotuberculosis* strains belonging to the two biotypes were highly similar, and the heterogeneity values were low (Table 4). Genes *dnaK*, *infB*, *groEL1*, and *leuA* showed marked genetic variation within *C. pseudotuberculosis* biotypes and they were selected for further genetic analysis.

2.4. Phylogenetic Analysis. Fragments of four protein-coding highly diverse housekeeping genes (*groEL1*, *infB*, *dnaK*, and *leuA*) were sequenced for all strains.

Figure 1: The location of *in silico* studied 16 housekeeping genes at the complete genome map of *C. pseudotuberculosis* FRC41 [19]. The partial regions of rimmed four genes in frames were amplified and sequenced in this study.
**Table 3: Primer sequences used for amplification and sequence analysis.**

| Locus | Putative gene product | Primer name | Sequence | Product size (bp) | Position on NC_0143291 (FRC41 strain) |
|-------|-----------------------|-------------|----------|-------------------|---------------------------------------|
| dnaK  | Chaperone Hsp70       | Cps dnaK F  | 5'-TCCTTACCCAGTGGCCCTATCC-3' | 580  | 2081944–2081964  |
|       |                       | Cps dnaK R  | 5'-GAGTTCCAGCGCCATCACC-3'    |      | 2082507–2082524  |
|       |                       | Cps groEL1 F| 5'-ACCTTCACGGGATCATTG-3'     | 654  | 1978987–1979004  |
|       |                       | Cps groEL1 R| 5'-TTGGGTGATGCTGTAAGGC-3'    |      | 1979623–1979641  |
|       |                       | Cps infB F  | 5'-ATTGC GGGA CTTGGACG-3'    | 641  | 1397768–1397784  |
|       |                       | Cps infB R  | 5'-GCATTATGTGACACAGGC-3'     |      | 1398390–1398409  |
| infB  | Translation initiation factor IF2 | Cps leuA F  | 5'-AGCTCAGTGGCGGCTGACC-3'    | 647  | 161252–161271    |
|       |                       | Cps leuA R  | 5'-ATGCGCCGTGGCGGTCC-3'      |      | 161915–161899    |
| leuA  | 2-isopropylmalate synthase | Cps leuA F  | 5'-AGCTCAGTGGCGGCTGACC-3'    | 647  | 161252–161271    |
|       |                       | Cps leuA R  | 5'-ATGCGCCGTGGCGGTCC-3'      |      | 161915–161899    |
differentiation and molecular epidemiological investigations. The sequence diversity in these regions exceeded 5%.

*"C. pseudotuberculosis" biotype ovis and *"C. pseudotuberculosis" biotype equi field strains were differentiated with sequence analysis of the target gene fragments. Analysis of the individual genes showed a fairly conservative nature of *"C. pseudotuberculosis" biotype ovis strains, although segregation of some Hungarian strains (e.g., C1 and C19, C3, C23, and C27) together with strains from Australia, Israel, USA, South Africa, Scotland, France, and Brazil was also evident but they were clearly located in the cluster of *"C. pseudotuberculosis" biotype ovis. The greatest genetic differentiation was seen in the *dnaK* and *infB* genes and concatenations of these two genes were very useful in the genetic separation of the studied strains (Figure 2).

4. Discussion

*"C. pseudotuberculosis" biotype ovis is the causative agent of caseous lymphadenitis, a contagious, chronic disease of sheep and goats [3].

Several previous studies showed high sequential homology of the *"C. pseudotuberculosis" biovar ovis strains confirming their clonal origin; however, diversity of the clinical signs in sheep and goats and different efficacy of the vaccines suggest that more than one pathogen clone can exist [14].

**Figure 2:** Phylogenetic reconstruction based on the concatenated partial sequence of *dnaK* (520 bp), *infB* (577 bp), *groEL1* (588 bp), and *leuA* (597 bp) genes from the Hungarian field isolates and DSM type strains. Analysis was conducted using the neighbour joining (NJ) method. The 1000 bootstrap (BT) values are indicated at branching points. Bar % estimated nucleotide substitutions.
Table 4: Nucleic acid average sequence divergence of housekeeping genes in Corynebacterium species in the in silico study.

| Gene   | Full length (bp) |  C. diptheriae versus C. pseudotuberculosis | Average sequence divergence | C. ulcerans versus C. pseudotuberculosis | C. pseudotuberculosis biotype ovis versus equi |
|--------|-----------------|---------------------------------------------|----------------------------|------------------------------------------|-----------------------------------------------|
| groEL1 | 1641            | 62.9                                        |                            | 27.9                                     | 1.1                                           |
| infB   | 2886            | 13.6                                        |                            | 6.0                                      | 0.8                                           |
| dnaK   | 1836            | 9.1                                         |                            | 5.0                                      | 0.6                                           |
| leuA   | 1818            | 14.0                                        |                            | 8.1                                      | 0.5                                           |
| adh1   | 3687            | 12.5                                        |                            | 49.7                                     | 0.4                                           |
| atpD   | 1446            | 6.2                                         |                            | 3.2                                      | 0.4                                           |
| recN   | 1740            | 27.0                                        |                            | 10.6                                     | 0.4                                           |
| dnaE   | 3561            | 58.2                                        |                            | 7.1                                      | 0.3                                           |
| groEL2 | 1641            | 59.5                                        |                            | 27.6                                     | 0.3                                           |
| recA   | 1110            | 14.1                                        |                            | 7.3                                      | 0.3                                           |
| rpoA   | 1017            | 7.7                                         |                            | 3.5                                      | 0.3                                           |
| fusA   | 2127            | 3.9                                         |                            | 2.6                                      | 0.2                                           |
| groES  | 297             | 6.5                                         |                            | 3.5                                      | 0.2                                           |
| rpoB   | 2974            | 8.0                                         |                            | 3.3                                      | 0.2                                           |
| sodA   | 600             | 10.0                                        |                            | 4.3                                      | 0.2                                           |
| atpA   | 1630            | 8.4                                         |                            | 2.1                                      | 0.0                                           |

The analysis of sequence data of 16 frequently studied housekeeping genes (atpA, dnaE, dnaK, fusA, leuA, adh1, rpoB, atpD, groES, groEL1, groEL2, infB, recA, recN, rpoA, and sodA) of 15 C. pseudotuberculosis, 13 C. diptheriae, and C. ulcerans genomes from the GenBank proved to be related and all studied genes could be used to differentiate C. diptheriae, C. ulcerans, and C. pseudotuberculosis strains. All these genes allow the differentiation of biotype ovis and biotype equi strains of C. pseudotuberculosis but for detailed examination of C. pseudotuberculosis biovar ovis field isolates four genes (dnaK, groEL1, infB, and leuA) seemed to have higher differentiation power. The partial sequence analysis of adequate genes delineated phylogenetic trees with various topology and depth of branches. The highest resolution and topology of the tree were obtained with the dnaK sequences.

The clusters could be explained by movement of breeding animals, but no epidemiological connection was found between the examined C. pseudotuberculosis strains. The distance between the geographical place of isolation and the year of the isolation do not support any epidemiological connection between the strains, so circulation of several clones of C. pseudotuberculosis biotype ovis was verified.

According to our data, multilocus sequence typing can differentiate C. pseudotuberculosis and the most important Corynebacterium species, and examination of selected genes helps to differentiate the two biotypes of C. pseudotuberculosis, and it can be used for epidemiological follow-up of these strains.

Conflicts of Interest
None of the authors have any conflicts of interest to declare.

Authors’ Contributions
All authors have been involved in designing the study, analysing the data, and drafting of the manuscript.

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