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RESTRICTION FRAGMENT LENGTH POLYMORPHISM WITH THE PROBE IS900 IN *MYCOBACTERIUM PARATUBERCULOSIS*

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Les ADN de 452 souches de Mycobacterium paratuberculosis isolées à partir d'animaux, d'échantillons de l'environnement de l'étable, et de patients atteints de la maladie de Crohn dans 17 laboratoires de 14 pays et 3 continents ont été digérés par la technique de restriction endonucléique PstI et BstEII et croisés avec l'amorce IS900. L'amorce préparée à partir du plasmide pMB22 a d'abord été marquée radioactivement avec alfa-32PdATP, puis l'amorce préparée par amplification PCR a été utilisée et le marquage a été réalisé non-radioactivement par ECL (Amersham). Les résultats RFLP ont été scannés avec une caméra CCD et analysés en utilisant le logiciel Gel manager (Biosystematika, Travistock, UK). Après digestion par restriction endonucléique PstI, 11 types de RFLP ont été détectés et identifiés comme étant A - K faisant référence à Pavlik et coll., 1995 (Veterinary Microbiology; 45: 311-318). Après digestion par restriction endonucléique BstEII, 15 types de RFLP indiqués comme étant C1-3, C5, C7-15, S1, et I1 ont été détectés faisant référence à Collins et coll. 1990 (J.Clin.Microbiol.; 28: 1591-1596). Après une digestion partielle de l'ADN à partir des souches individuelles, l'ensemble des types de 21 RFLP différents ont été détectés, indiquant par ex. B-C1. Parmi les souches bovines, 17 types de RFLP on été détectés, 9 chez les ovins, 5 chez les caprins, et toujours un type a été détecté chez le rhinocéros, le capricorne, le cerf et le chevreuil. Parmi 6 souches en provenance de patients atteints de la maladie de Crohn, 3 types de RFLP ont été détectés. Aucune relation n'a été trouvée entre la croissance de la souches et l'hôte, l'origine de la souche (pays, continent) et les pays. Les résultats ont été utilisés pour l'étude de l'épizootologie moléculaire de la paratuberculose.

The significance of paratuberculosis (a serious bacterial disease of ruminants caused by *Mycobacterium paratuberculosis*) is rising steadily (3, 13). In the Czech Republic, like in other countries, due to certain alterations of conditions in agriculture, the former favourable epizootological situation, is now exacerbated (12, 13, 14, 15).
Practically it's not possible to differentiate individual strains of *M.paratuberculosis* by bacteriological or biochemical methods. As a result, since the early 80s, methods of molecular biology (e.g. DNA fingerprinting) have been applied (4, 10, 11, 16, 18). DNA hybridization with various probes such as 5SrRNA, has been particularly beneficial (2). However, the use of specific fragment IS900 (8), is recognized to be the best contributor. Several laboratories apply it in the method RFLP - Restriction Fragment Length Polymorphism (1,5,6,7,17).
Therefore, our study is aimed at the standardization of RFLP. Special emphasis was laid on the universal preparation of probes, non-radioactive labels and final results. A suitable software was used in our laboratory for an additional assessment of the RFLP results of all examined strains.

MATERIALS AND METHODS

Strains of *M.paratuberculosis*

DNA of 452 strains of *M.paratuberculosis* were isolated from animals (95.3%),environmental samples (3.3%), and from patients with Crohn's disease (1.4%). Strains from animals include cattle (84.3%), sheep (6.2%), goats (3.8%) and others (5.7%). 91.6% of the examined strains are from 11 European countries (Czech Republic, Denmark, Estonia, France, Germany, Holland, Hungary, Norway, Slovakia, Sweden, Great Britain), 5.3% from New Zealand and Australia, and 3.1% from the USA.

Growth-rate of the strains

442 strains after 12 weeks of incubation period, showed distinct colonies and were identified as **"relatively fast-growing strains"**. While the rest ten strains with longer incubation period (up to 8 months) were designated as **"relatively slow-growing strains"**.

Method RFLP

The isolation of DNA, its digestion by both restriction endonucleases *PstI* and *BstEII*, electrophoresis, vacuum-blot, preparation and radioactive labelling on probes alfa-^{32}PdATP from the plasmid pMB22 with a specific probe IS900 (obtained from Dr. McFadden in 1989), were previously described (14).

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Preparation of probes by PCR amplification
The probe, introduced in the sequence IS900, was prepared by the amplification of DNA of *M.paratuberculosis* ATCC 19698 (9). The resulting amplified product 453kbp (controlled by electrophoresis in 2% agarose) was purified using Wizard PCR purification kit (Promega) and labelled as ECL by the Direct Labelling Kit (Amersham). Similar procedure was used during the evaluation of RFLP results after the digestion by restriction endonuclease PstI. A dendrogram of all RFLP types was done by the software Gel Manager.

Nomenclature of RFLP types
After the digestion by **restriction endonuclease *PstI***, four RFLP types designated A, B, C, D were distinguished according to the study (13, 14). Later on, new RFLP types designated E, F, G, H, I, J, K were identified. After the digestion by **restriction endonuclease *BstEII***, RFLP types were divided into three groups: C, S, I, based on the study (5). In group C, types C1-7, in group S, RFLP types S1-2 and in group I, RFLP types I1-2 were described. In the study (7) was described another RFLP type S3, which is also included in the data-base. Newly identified RFLP types in group C were labelled by new numerical series (C8-15).

Labelling of RFLP types of the *M.paratuberculosis* strains after parallel digestion of DNA by restriction endonucleases *PstI* and *BstEII*
After the differentiation of RFLP types of each strain, we used the label: RFLP type after digestion by restriction endonucleases *PstI-BstII* (e.g. A-C10).

RESULTS
After the digestion by restriction endonucleases *PstI* and *BstEII*, we detected a total of 21 various RFLP types: A-C8, A-C10, A-C12, B-C1, B-C2, B-C3, B-C5, B-C7, B-C9, B-C12, B-C14, B-C15, C-S1, D-C12, E-C1, F-I1, G-I1, H-C1, I-C13, J-C1, K-C11.

Stability of RFLP type during subcultivations

The strain *M.paratuberculosis* N^{o} CAMP 6381 (Collection of Pathogenic Animal Microorganisms, Veterinary Research Institute, Brno, Czech Republic) has been regularly preserved and subcultivated since 1988 with the interval of 5-7 months. Until 1996, the multiplied material in 18 consecutive subcultures, prior to final examination, was supplied with the original lyofilized strain from 1988. All 19 subcultivations were of the same RFLP type A-C10.

RFLP types of the *M.paratuberculosis* strains in relation with the host organism
After the parallel digestion of DNA of *M.paratuberculosis* strains by both restriction endonucleases, 17 RFLP types were identified in **cattle** designated as A-C10 (n=92), A-C12 (n=2), B-C1 (n=159), B-C3 (n=6), B-C5 (n=2), B-C7 (n=1), B-C9 (n=11), B-C12 (n=4), B-C14 (n=1), B-C15 (n=2), C-S1 (n=1), D-C12 (n=20), E-C1 (n=67), H-C1 (n=2), I-C13 (n=8), J-C1 (n=1), K-C11 (n=2). In **sheep** 9 different RFLP types designated as A-C8 (n=2), A-C10 (n=10), B-C1 (n=6), B-C2 (n=2), B-C3 (n=1), C-S1 (n=2), E-C1 (n=3), F-I1 (n=1), and G-I1 (n=1). In **goats** 5 different RFLP types designated as B-C1 (n=8), B-C3 (n=2), B-C9 (n=1), C-S1 (n=1), and H-C1 (n=5). In **rhinoceros, capricorns, deer** and two **collection mycobactin-independent strains** *M.paratuberculosis* E2 and ATCC 19698, RFLP type B-C1(n=7)were identified. The strain 316F, belonged to RFLP type B-C7. Strains isolated from six **patients with Crohn's disease** were classified to 3 RFLP types. In Czech Republic the strain **Fryba,** belonged to RFLP type A-C10. Another strain **(VIAS)** from Australia and strains from Holland **(Jeltema, Vanveen** and **Houken),** were classified to RFLP type B-C1. The strain **Linda** (ATCC 43015) from the USA, belonged to the RFLP type B-C5.

Representative RFLP types of different countries or continents
From a total of 21 RFLP types, in **Europe** (11 countries) 14 RFLP types were identified (66.7%), in **Australia** and **New Zealand** 6 RFLP types (42.9%), in the **USA,** 7 RFLP types (33.3%). **The most common RFLP types in the 3 continents** was RFLP type **B-C1**. It was also found in 11 European countries. **The second** was RFLP type **A-C10,** identified in four European countries. **The third** was RFLP type **E-C1**, found only in three European countries. In **Australia** and **USA,** RFLP type **B-C3,** in Europe and USA type **B-C5** and **H-C1**, and in Europe and Australia type **C-S1** were identified. **Only in Europe**, RFLP type A-C8, A-C10, A-C12, B-C2, B-C7, B-C9, B-C14, D-C12, and I-C13 were identified. RFLP type B-C15, F-I1, and G-I1 were identified **only in Australia and New Zealand.** Whereas in **USA**, RFLP type B-C12, J-C1 and K-C11 were classified.

RFLP types of the strains *M.paratuberculosis* in relation with their growth-rate
In the group **"relatively fast-growing 442 strains"**, all 21 types described in the previous study, are identified. During the examination of the **"relatively slow strains"**, 5 RFLP types were identified. **In sheep** (n=7) three RFLP types A-C10, B-C2 and E-C1 were found. **In goats** (n=1) RFLP type C-S1 and in **capricorn** (*Capra cylindricornis*) two strains of RFLP type B-C1 were identified. In cattle no "relatively slow growing strain" was identified. In the group 107 strains RFLP type A-C10, 4.7% of slow growing strains were identified, in the group 185 strains RFLP type B-C1, only 1.1% and in the group 74 strains RFLP type E-C1 1.4% of slow growing strains were identified. One of the two strains RFLP type B-C2 and one of the four strains RFLP type C-S1 were slow growing strains.

Dendrogram of each RFLP type

Dendrograms were made by means of software Gel Manager. All RFLP types after the digestion by restriction endonuclease ***BstEII***, were divided into three clusters strains C, S, I. **The first cluster** RFLP **type C** contained 446 strains, **the second cluster** RFLP **type S** contained four strains and **the third cluster** RFLP **type I** contained two strains. All these three groups were phylogenetically different. Within the clusters we can observe the formation of 7 subclusters. The first subcluster (RFLP types C1, C5, C9), the second subcluster (RFLP types C12, C7), the third subcluster (RFLP types C13, C6, C2), the fourth subcluster (RFLP types C10, C8), the fifth subcluster (RFLP types C3, C4), the sixth subcluster (RFLP types C15, C11), and the seventh subcluster (RFLP type C14). After the digestion by restriction endonuclease ***PstI***, **three main clusters** were formed. **The first** cluster contained RFLP types A, B, C, D, E, H, I, J, K with 446 strains. **The second** cluster contained RFLP type C with four strains and **the third** cluster contained two RFLP types F and G with two strains.

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