Comparative examination of *Rhodococcus equi* strains isolated from pathological- and environmental samples in Hungary

Theses
of the PhD work

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1. INTRODUCTION

Rhodococcus genus belongs to the group of nocardioform *Actinomycetes* and a subgroup of mycolic acid containing bacteria, at present there are 12 species in it. All these species are saprophytic, with the exception of *Rhodococcus equi*, which is facultative pathogenic and is important both in human and veterinary medicine (Bell et al., 1998).

The bacterium was isolated for the first time by Magnusson from suppurative bronchopneumonia of foals in 1923 and he named it *Corynebacterium equi*. Some years later the species was reclassified and placed to the genus *Rhodococcus* on the basis of cell wall composition, biochemical and genetic examinations (Magnusson, 1923; Bell et al., 1998).

Diseases caused by *R. equi* occur worldwide. They are characterised by suppurative pneumonia with abscess formation, ulcerative enteritis and lesions in intestinal lymph nodes, arthritis in 1-4 month old foals and sometimes abortion in mares. In some studs this microorganism is responsible for 45% of pneumonia cases and 10% of death among foals (Zink et al., 1986). The diseases are also widespread in Hungary (Szeredi et al., 1996; Varga et al., 1999).

In addition to horses *R. equi* bacteria can also be isolated from lymph nodes and abscesses of pigs, cattle, sheep and goats. Some pigs carry this microorganism in their submaxillar lymph nodes without any clinical symptom (Katsumi et al., 1991; Barton and Hughes, 1980; Rao et al., 1982; Takai et al., 1996).

During the past 15 years several *R. equi* caused cases were observed in humans, too, mostly in persons receiving immunosuppressive treatment after organ transplantation or suffering from HIV infection (Prescott, 1991; Takai et al., 1995).

2. AIMS

1./ Frequency of occurrence of *R. equi* in pathological samples from horses, other species and soil,

2./ Comparison and evaluation of selective media for isolation of *R. equi* described in the literature and development of a new selective plating medium with better selective capacity,

3./ Comparison of cultural, morphological and biochemical characteristics of *R. equi* strains isolated from different sources throughout Hungary,

4./ Examination of metabolic fingerprint (carbon source utilization, enzyme activity) and antibiotic susceptibility of *R. equi* strains,

5./ Serological classification of *R. equi* strains,

6./ Characterisation of plasmid profile and a comparative examination of 16S ribosomal RNA gene of strains of different origin,

7./ Development of an immunohistochemical method for diagnosis of *R. equi* infection and

8./ Preparation and testing of different *R. equi* vaccines in field experiments.

3. MATERIALS AND METHODS

1./ *R. equi* strains were isolated from different parts of Hungary from pathological materials (lung, intestinal- and mediastinal lymph nodes, nasal- and rectal swabs from horses, swine submaxillar lymph nodes, humans) and from soil samples using different selective and non selective plating media.

2./ Efficacy of four selective media (NANAT, TINSDALE, M3T and CAZ-NB) described in the literature and that of four other ones (PNP, NC, TVP and TCP) prepared by us was compared by examination of the recovery rate, the colony morphology of the *R. equi* strains and inhibition of contaminant bacteria. The efficacy of the two best selective media were compared by quantitative recovery of *R. equi* from horse faeces and soil samples.

3./ Examination of the morphological, cultural and biochemical characteristics of all *R. equi* isolates were carried out by standard methods. Strains were identified on the basis of species specific DNA sequences using polymerase chain reaction, too.

4./ Enzyme activity (19 constitutive enzymes) and carbon source utilization (95 carbon sources) of representative strains were characterized by using API-ZYM (bioMerieux, France) strips and BIOLOG (Biolog Inc., Hayward, Canada) plates. Antibiotic susceptibility (Minimum Inhibition Concentration, MIC) against ten antibiotics (amikacin, gentamicin, neomycin, rifampicin, vancomycin, erythromycin, amoxicillin, penicillin G, minocycline, oxytetracycline) was examined using broth dilution method.

5./ All *R. equi* strains were serotyped according to the system described by Prescott (1981) with agargel immunodiffusion (AGID). Hyperimmune sera were produced in rabbits against reference strains representing each existing serotype and against 30 further strains selected out of 90 non typeable field strains. Antigen extracts were prepared from all strains and examined first with sera produced against reference strains and if no precipitation was seen also with the sera produced against the field isolates.

6./ Plasmid isolation was carried out using alkaline lysis method, virulence genes (vapA, vapB) were detected with polymerase chain reaction and isolated plasmids were typed using restriction enzyme (EcoRI, EcoT22I) polymorphism.

16S ribosomal RNA gene of 32 strains of different origin was amplified using universal bacterial primers (63f, 1387r). The products (1338 bp) of amplification were digested with different restriction enzymes (Tru9I, Hin6I) and characterised after electroforesis. In the case of eleven strains the first 440 bp of the 16 S ribosomal RNA gene were sequenced.

7./ For immunohistochemical purposes hyperimmune sera were produced in rabbits against a *R. equi* strain serotype 1, as strain serotype 1 can frequently be isolated from diseased foals. IgG was prepared by ammonium-sulphate precipitation and it was purified by affinity chromatography. The antigen-antibody binding on paraffin-embedded sections, cryostat sections and impression smears was visualized by using horseradish-proxidase-labelled streptavidin-biotin kit.

8./ Vaccination field trials were carried out in five studs throughout Hungary. For vaccine preparation *R. equi* strain (serotype 1) was cultured on agar plates for 48 hours, colonies were harvested, washed three times in PBS (pH 7.2) inactivated with formalin and then absorbed to aluminum phosphate gel or mixed with incomplete Freund's adjuvant.

In three studs vaccines contained only inactivated *R. equi* bacteria, in two out of them only the foals and in one of them pregnant mares and also their foals were vaccinated. One of the vaccines contained *R. equi* adsorbed to aluminum salt while the other adjuvated with oil.

In two studs vaccination was performed using vaccines containing inactivated *R. equi* bacteria and EHV-2 (equine herpes virus 2) virus. In these studs pregnant mares and also their foals were vaccinated and colostral immunity was also examined. The serological response was measured with virus neutralization test (VN) and indirect haemagglutination test (IHA) after using combined vaccines, while after monovalent vaccines ELISA was used.

4. RESULTS

1./ Based on examination of 379 *R. equi* strains from 43 locations throughout Hungary, it was established that this bacterium species is very widespread in horses in Hungary. *R. equi* was isolated from all (100%) succumbed foals showing typical signs of *R. equi* pneumonia, it was cultured from 14,4% of nasal swab samples and 28,4% of rectal swabs taken from horses in studs where *R. equi* infection occured, from 68,6% of soil samples taken from paddocks and from 14,9% of swine submaxillar lymph nodes.

2./ The new selective plating medium (TCP), containing trimethoprim, cefoperazone, polymyxin B, cycloheximide and potassium-tellurite as selective components, proved to be the best for the isolation of *R. equi* from highly contaminated samples. This medium allowed the growth of at least 62-91 % of *R. equi* present in the samples and the inhibition of the contaminant bacteria and fungi was satisfactory. The colony morphology of *R. equi* on the new medium was typical.

3./ There were no significant differences in morphological and cultural characteristics of R. equi strains isolated in Hungary compared to reference strains. All strains were Gram-positive cocci, obligate aerobic, catalase positive, oxidase positive, no acid was producted from sugars, pigment was producted, urease was producted, nitrate was reducted to nitrit. Although occasionally there were some minor differences in biochemical features (hemolysis on rabbit blood agar, halotolerance, H_2S-production) according to the site of isolation of the strains, but there was no correlation with their origin.

In all strains examined, which were identified as *R. equi* on the basis of cultural, morphological and biochemical characteristics, the species specific 450 bp DNA fragment could be detected using PCR.

The classical cultural, morphological and biochemical examination methods are suitable for identification of *R. equi* on species level, but the identification can also be carried out by the detection of a species specific 450 bp DNA sequence of 16S ribosomal RNA gene.

4./ The enzyme activity profile of *R. equi* strains of different origin was rather homogeneous. All strains examined had alkaline- and acid phosphatase, leucine-, valine-, cystine arylamidase, α-, β-glucuronidase and naphtol-AS-BI-phosphohydrolase activity. Correlation between enzyme activity and origin of strains was detected only in the case of

esterase (C4). *R. equi* strains of human origin had no esterase (C4) activity, while half of the other strains produced this enzyme.

All *R. equi* strains were able to utilize six carbon sources (Tween 40, Tween 80, α-D-glucose, α-hydroxibutyric acid, L-lactic acid, metyl-pyruvate), but neither was able to utilize 51 carbon sources. Carbon source utilization (BIOLOG) is an appropriate method for a rapid identification of the strains. Dendrogram, which was made on the basis of the results of carbon source utilization can give additional information about the relationship of the strains.

R. equi strains isolated in Hungary were most susceptible to rifampicin (MIC: 0,125-0,25 μg/ml) and erythromycin (MIC: 0,125-0,5 μg/ml).

5./ Apart from a single exception all 379 *R. equi* strains could be serotyped. In addition to the formerly known 7 serotypes (Prescott, 1981), four further serotypes were established by us. Showing some differences in origin (animal species or location) 45,6% of the strains belonged to serotype 1, 28,2% to serotype 2 and some other strains to further serotypes, 27,75% of the strains belonged to the four new serotypes (recommended serotypes "8", "9", "10", "11"). The majority (74,4%) of the strains isolated from horses belonged to serotype 1. Six out of eight strains isolated from human patients and the majority (56,9%) of strains isolated from swine belonged to serotype 2.

6./ Using PCR vapA virulence gene (virulent strains) was detected in 41,5% of the strains and vapB gene (moderately virulent strains) was found in 13,6% of the strains. The rest of the strains possessed no virulence plasmids (avirulent strains). The majority of the horse strains (88,4%) was virulent, but the majority of the human strains was moderately virulent. Avirulent strains were prevailing in swine (71,9%), but 27,5% of strains were moderately virulent. The majority of the virulent strains contained a 85 kbp type I plasmid, a smaller part of these strains possessed 87 kbp type I virulence plasmid. The majority of moderately virulent strains isolated from swine and human patients possessed the same type S5 (95 kbp) virulence plasmid. Some strains isolated from swine contained another virulence plasmid. Six different kind of virulence plasmids were isolated, from which one was a formerly unknown plasmidtype.

Among the 16S ribosomal RNA gene of strains with different origin we did not find any differences using RFLP and partial sequence analysis.

7./ The immunohistochemical procedure developed by us promoted and accelerated the diagnosis of diseases caused by *R. equi*, since it could detect *R. equi* in pathological samples (impression smears, histological sections) within some hours. The sensitivity of this procedure seems to be same or higher than that of the bacteriological examination.

8./ Antibody levels in the plasma and colostrum samples elevated only slightly compared to controls after vaccination of pregnant mares and 4-6 week-old foals (vaccinated twice, intramuscularly) with aluminum salt or oil adjuvated inactivated vaccines. This increase of titres was not able to prevent in all cases the manifestation of clinical signs. These results do not support the wide-range use of these vaccines in the practice.

5. NEW RESULTS

- Data on the occurrence of *R. equi* in foal pneumonia, feces of clinically healthy horses, soil samples and submaxillar lymph nodes of clinically healthy swine were provided proving the wide occurrence of *R. equi* strains in Hungary.

- A unique *R. equi* culture collection was established, with cultural, morphological and biochemical characteristics of the strains.

- A new selective plating medium was developed, which has better selective capacity, than the formerly known media. This medium is useful in diagnostic work and suitable for isolation of *R. equi* from highly contaminated samples.

- It was proved, that all of the *R. equi* strains identified on the basis of conventional (cultural, morphological, biochemical characteristics) methods possessed a 450 bp size DNA fragment as a part of the 16S ribosomal RNA gene. This means that in addition to classical identification methods, detection of this DNA fragment by PCR is suitable for the rapid and specific identification of *R. equi* strains.

- Dendrogram was created on the basis of utilization of 95 carbon sources. This can be a suitable method for comparing of *R. equi* strains of different origin.

- The BIOLOG system was adopted for the first time to the examination of *R. equi* strains of different origin, providing data in this way for the use of this system in veterinary diagnostic work and for the use of metabolic fingerprinting in epidemiological investigations.

- Four new *R. equi* serotypes (serotype „8”; „9”; „10”; „11”) were identified and established using agargel-immunodiffusion test. Data were provided on frequency of serotypes in different animal species and different geographical locations.

- Occurrence of virulence plasmids and plasmidtypes were described in *R. equi* strains isolated in Hungary. Similarity of virulence of *R. equi* strains

isolated from swine in a slaughterhouse and human patients in Hungary was also shown.

- It was proven that variability of 16S ribosomal RNA gene of *R. equi* type strains and strains isolated in Hungary is much smaller, than it could be expected from data in the literature and gene-banks.

- A new, specific immuno-histochemical procedure was developed, which is suitable for detection of *R. equi* bacteria in impression smears, cryostat or paraffin-embedded sections.

6. SCIENTIFIC PUBLICATIONS AND PRESENTATIONS PUBLISHED ON THE SUBJECT OF THE DISSERTATION

SCIENTIFIC PUBLICATIONS

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3.) Szeredi L., **L. Makrai** and B. Dénes (2001): Rapid immunohistochemical detection of *Rhodococcus equi* in impression smears from affected foals on postmortem examination, Journal of Veterinary Medicine B., **48.**, 751-758.

4.) **Makrai L.**, S. Takai, M. Tamura, A. Tsukamoto, R. Sekimoto, Y. Sasaki, T. Kakuda, S. Tsubaki, J. Varga, L. Fodor, N. Solymosi and A. Major (2002): Characterisation of virulence plasmid types in *Rhodococcus equi* isolates from foals, pigs, humans and soil in Hungary, Veterinary Microbiology, **88.**, 377-384.

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PRESENTATIONS

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5.) **Makrai L.**, Varga J., Fodor L., Vendég I., Szigeti G. és Reiczigel J.: *Rhodococcus equi* izolálására használatos szelektív táptalajok összehasonlító vizsgálata, Akadémiai beszámoló, 2001. január 23.

6.) **Makrai L.**, Dénes B., Varga J., Tekes L., Szalay Sz., Szollár I., Sümeghy L., Soós P., Fodor L.: *Rhodococcus equi*-vel szembeni vakcinázási kísérletek tapasztalatai két magyarországi lóállományban, Magyar Mikrobiológiai Társaság 2001. évi Jubileumi Nagygyűlése, Balatonfüred, 2001. október 10-12.

7.) **Makrai L.**, Dénes B., Varga J., Tekes L., Major A., Fodor L.: Magyarországon gyűjtött különböző eredetű *Rhodococcus equi* törzsek szerotipizálása, Magyar Mikrobiológiai Társaság 2001. évi Jubileumi Nagygyűlése, Balatonfüred, 2001. október 10-12.

8.) **Makrai L.**, Takai S., Varga J., Major A., Fodor L.: Különböző eredetű *Rhodococcus equi* törzsek virulenciaplazmidjainak vizsgálata, (poszter) Magyar Mikrobiológiai Társaság 2001. évi Jubileumi Nagygyűlése, Balatonfüred, 2001. október 10-12.

9.) **Makrai L.**, Márialigeti K., Kovács G., Varga J., Fodor L., Senoner Zs., Bognár Cs., Major A.: Különböző eredetű *Rhodococcus equi* törzsek 16S rDNS-ének vizsgálata, (poszter) Magyar Mikrobiológiai Társaság 2001. évi Jubileumi Nagygyűlése, Balatonfüred, 2001. október 10-12.

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