JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Kyselková et al. 2013 : 3 (1) 76-80

ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY TESTING OF RAPIDLY-GROWING MYCOBACTERIA FROM GRASSLAND SOILS

Martina Kyselková[1], Alica Chroňáková[1], Jan Němec[1], Lucie Kotrbová[1,2], Dana Elhottová[1]*

Address(es): Martina Kyselková, PhD,
[1]Biology Centre AS CR, v.v.i., Institute of Soil Biology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic, phone number: +420387310134.
[2]University of South Bohemia, Faculty of Science, Branišovská 3, CZ-37005 České Budějovice, Czech Republic.

*Corresponding author: martinak@upb.cas.cz

ARTICLE INFO

Received 15. 3. 2013
Revised 15. 7. 2013
Accepted 19. 7. 2013
Published 1. 8. 2013

Short communication

OPEN ACCESS

ABSTRACT

Rapidly growing mycobacteria (RGM) are common soil saprophytes, but certain strains cause infections in human and animals. The infections due to RGM have been increasing in past decades and are often difficult to treat. The susceptibility to antibiotics is regularly evaluated in clinical isolates of RGM, but the data on soil RGM are missing. The objectives of this study was to isolate RGM from four grassland soils with different impact of manuring, and assess their resistance to antibiotics and the ability to grow at 37°C and 42°C. Since isolation of RGM from soil is a challenge, a conventional decontamination method (NaOH/malachite green/cycloheximide) and a recent method based on olive oil/SDS demulsification were compared. The olive oil/SDS method was less efficient, mainly because of the emulsion instability and plate overgrowing with other bacteria. Altogether, 44 isolates were obtained and 23 representatives of different RGM genotypes were screened. The number of isolates per soil decreased with increasing soil pH, consistently with previous findings that mycobacteria were more abundant in low pH soils. Most of the isolates belonged to the *Mycobacterium fortuitum* group. The majority of isolates was resistant to 2-4 antibiotics. Multiresistant strains occurred also in a control soil that has a long history without the exposure to antibiotic-containing manure. Seven isolates grew at 37°C, including the species *M. septicum* and *M. fortuitum* known for infections in humans. This study shows that multiresistant RGM close to known human pathogens occur in grassland soils regardless the soil history of manuring.

Keywords: Rapidly-growing soil *Mycobacterium;* mycobacterium isolation and cultivation; grassland soil; antibiotic resistance

INTRODUCTION

Rapidly growing mycobacteria (RGM) form a separate clade within *Mycobacterium* genus that comprises species growing saprophytically in soil and water, and causing opportunistic or accidental infections in human and animals. Living RGM could be recovered from various environments such as soil, farm animal beddings, dust, rocks, aerosol and water (**De Groote and Huitt, 2006; Hartmans *et al.*, 2006; Pakarinen *et al.*, 2007**). RGM are characterized by their rapid growth under optimal laboratory conditions (*i.e.* within one week) and can be distinguished from slow growing mycobacteria based on their 16S rRNA sequences (**Hartmans *et al.*, 2006**). So far, 148 species of RGM have been described.

The infections due to RGM have been increasing in past years, and include pulmonary and other infections in immunocompromised patients, as well as infections related to injuries in healthy individuals (**Tortoli, 2003).** The treatment of RGM infections may be difficult because of their high resistance to antibiotics. This resistance is due to their intrinsic resistance, which is ensured by the composition of the cell wall and presence of several multidrug efflux pumps (**Fajardo *et al.*, 2008**), and due to the acquired resistance (**Pang *et al.*, 1994).** While the intrinsic resistance must have already existed in the pre-antibiotic era, the acquired resistance is thought to be a result of the increased selection pressure caused by human use of antibiotics (**Martinez, 2009**). The antibiotics clarithromycin, amikacin, cefoxitin, imipenem, tobramycin, doxycycline, ciprofloxacin, sulphonamides, linezolid and fluoroquinolones are recommended to treat RGM infections (**Brown-Elliott and Wallace, 2005**) and may represent the antibiotic selection pressure in the clinical environment.

In soil, there can also be a substantial selection pressure on antibiotic resistance, due to the entry of antibiotic residues in the form of manure from ATB–treated animals. Antibiotics such as sulfonamides and tetracyclines have been massively used in agriculture, in order to increase the animal growth (now prohibited in the EU), and to treat or prevent diseases. Animal feces are therefore a source of ATB residues and ATB-resistant fecal bacteria, which may transfer their resistance genes to the indigenous soil microflora. Indeed, the same resistance genes were detected in various human, animal and soil isolates (**Pasquali *et al.*, 2004; Kobashi *et al.*, 2007**), and they are likely to circulate between the different environments. Persistent opportunistic pathogens such as RGM, surviving in dust, drinking water or amoebas (**Thomas *et al.*, 2008**), are likely to contribute to the transfer of ATB-resistance between soil, water and clinical environment. Antibiotic resistance in environmental RGM is therefore of interest from both ecological and clinical point of view. The objectives of this study was to isolate RGM from four grassland soils with different impact of manuring, and assess their resistance to antibiotics and the ability to grow at 37°C and 42°C.

MATERIAL AND METHODS

Soil sampling and composition

Soil sampling and soil physicochemical characteristics were previously described by **Kyselková *et al.* (2012)**. Briefly, RGM were isolated from four grassland soils in three farms with different management located in South Bohemia, Czech Republic in years 2007-2010. Soil PM (pig-manured) was from a conventional farm engaged in intensive pig fattening, where animals are commonly treated with antibiotics (chlortetracycline, doxycycline, amoxicillin, florfenicol). Soil PM has been periodically manured (2-3 times per year) with pig slurry for past 30 years. Soil NM (non-manured) was from a small family farm in the neighborhood, which never received pig slurry, the soil was fertilized with cow manure sporadically. Soils CE (cow-excrement) and CO (control) were both from an eco-farm specialized in cattle husbandry. The cattle stay on pastures all over the year and receive no antibiotics. Soil CE was sampled in a part of pasture where cattle stay during the winter. The CE soil is highly enriched with cow excrements and vegetation cover is damaged. In contrast, soil CO was from a part of pasture with a low impact of the cattle and preserved vegetation. In each site, soil from the depth of about 10-30 cm (under the plant roots) was sampled with a sterile spade from three points 5-20 m apart. The soil from the three points was mixed and sieved. Soil was kept at 4°C during the transport to the laboratory and prior the accompanying physicochemical and microbial analyses. Soil samples

for RGM isolation with the NaOH/malachite green/cycloheximide decontamination method were frozen at minus 20°C (**Iivanainen, 1995**). The soils PM, NM, CE and CO had pH respectively 5.7±0.1, 5.9±0.0, 7.4±0.3 and 4.9±0.1, the organic carbon 89±10.2 $g.kg^{-1}$, 63±8.9 $g.kg^{-1}$, 155.0±16.5 $g.kg^{-1}$ and 59.4±8.5 $g.kg^{-1}$, and the total nitrogen 2.4±0.2 $g.kg^{-1}$, 1.4±0.0 $g.kg^{-1}$ 6.5±0.2 $g.kg^{-1}$ 1.8±0.1 $g.kg^{-1}$ (mean±SD, n = 6; **Kyselková *et al.*, 2012**).

Mycobacteria isolation and plating

RGM from soils PM and NM were isolated either directly on Tryptic-Soy agar plates (TSA; BBL™ Trypticase™ Soy Broth, 2% agar) with 25 $mg.L^{-1}$ chlortetracycline (two isolates) or using the decontamination method (**Iivanainen, 1995**) as follows. Frozen soil was thawed at 4˚C overnight and 4 g of thawed soil were mixed with 40 mL of sterile tryptic-soy broth (BBL™ Trypticase™ Soy Broth; Becton Dickinson and Company; Sparks, USA) using vortex (Vortex-Genie2; Mo Bio Laboratories, Inc., Carlsbad, California; maximum speed for 2 min). The homogenate was left to stand for 2 min, and upper 25 mL were pipetted into a sterile centrifuge tube. Remaining soil particles were settled by centrifugation (500 x g, 4°C, 5 min; Sigma 3K30 centrifuge, Sartorius Stedim Biotech, Goettingen, Germany). Fifteen mL of the supernatant were separated into three sterile centrifuge tubes and incubated at 28°C, 180 RPM, for 5 h to allow the microbial spores to germinate. Since the temperature 37°C may inhibit the growth of soil microorganisms, we used 28°C instead of 37°C recommended by **Iivanainen (1995)**. Afterwards, NaOH (2.5 mL, 1.5 M, Lachema, Brno, The Czech Republic), malachite green (2.5 mL of 0.3% w/v solution, Carl Roth, Karlsruhe, Germany) and cycloheximide (2.5 mL of 2.5 $mg.mL^{-1}$ solution, Sigma-Aldrich, St. Louis, Missouri) were added to 5 mL of the soil suspension. The mixture was incubated 30 min at room temperature and mixed by inverting the tube three times each five minutes of incubation. The suspension was than neutralized with 2.5 mL of 1.5 M HCl and centrifuged at 8,600 x g, at 4°C, 15 min. The supernatant was discarded and the sediment was washed with 30 mL of sterile distilled water, centrifuged again, and resuspended in 3 mL of sterile water. Fifty µL of the suspension were plated in three replicates on solid M2 medium (**Shirling and Gottlieb, 1966**), MYM medium (source: The Czech National Collection of Type Cultures, Brno; formula for 1 liter: yeast extract 2 g, proteose peptone No.3 2 g, casitone 2 g, $Na_2HPO_4.12H_2O$ 2.5 g, KH_2PO_4 1 g, sodium citrate 1.5 g, $MgSO_4.7H_2O$ 0.6 g, Tween 80 0.5 g, glycerol 50 mL, agar 20 g, pH 7.0) and Ogawa egg medium (**Tsukamura *et al.*, 1986**), and incubated at 28°C for up to two weeks. Obtained colonies were subcultured and checked for acid fastness by Ziehl-Neelsen staining.

RGM from soils CE and CO were isolated using the decontamination method as described above, with two modifications in plating. First, the neutralized soil suspension was diluted with sterile distilled water to 10^{-1} and 50 µL of both diluted and non-diluted suspension were plated. Second, the media used for plating were as follows: M2 agar, TSA and Humic acid-Vitamin (HV) agar (**Hayakawa and Nonomura, 1987**).

In parallel to the decontamination method, olive oil/SDS method described by **Yamamura and Harayma (2007)** was used for RGM isolation from CE and CO soils, as follows. Samples were allowed to dry at room temperature for one week, and 0.5 g of dry soil was suspended in 5 mL of sterile olive oil (Carl Roth GmbH+Co.KG, Karlsruhe, Germany), followed by vortex mixing for 5 minutes at maximum speed. To remove large soil particles, the suspension was centrifuged at 1,600 x g for 5 min, and the supernatant (100 µL) was plated on a HV agar, M2 agar, and TSA together with 50 µL of 20% (w/v) SDS (Sigma-Aldrich). The plates were incubated at 28°C for 2 weeks and checked regularly for appearance of colonies. Colonies surrounded with clear zones of demulsification were subcultured and checked for acid fastness by Ziehl-Neelsen staining. The Ziehl-Neelsen positive isolates were further identified with 16S rRNA gene sequencing, as described in **Kyselková *et al.*, 2012**).

Susceptibility to antibiotics and growth at 37°C and 42°C

The antibiotic susceptibility of isolates to doxycycline (DOX), amikacin (AMI), rifampicin (RIF), sulfonamides (SSS, contains sulfadiazine), clarithromycin (CLA), vancomycin (VAN), ofloxacin (OFL) and amoxicillin/clavulanate (AMC) was assessed using the agar disk diffusion method (**Hindler and Munro, 2010**). The isolates were first grown on M2 medium agar at 28°C for 5 to7 days. A homogenous bacterial suspension was prepared by vortexing (Vortex-Genie2; Mo Bio Laboratories, Inc., Carlsbad, California) and ultrasonication (Ultrasonic Compact Cleaner UC 006DM1, Tesla, Rožnov pod Radhoštěm, Czech Republic) of several colonies in 4 mL of sterile 0.9% NaCl. The turbidity of the suspension was adjusted with sterile 0.9% NaCl to match the McFarland standard 0.5 (densitometer DEN-1, Biosan, Riga, Latvia). The suspension was spread onto Mueller-Hinton agar medium (Bio-Rad, Hercules, California) supplied with antibiotic disks (Bio-Rad; AMI, 30 µg; RIF, 5 µg; SSS, 300 µg; CLA, 15 µg; VAN, 30 µg; OFL, 5 µg; AMC, 20/10 µg; DOX, 30 µg). Inhibition zone diameters were recorded after five days of incubation at 28°C. Light growth within an obvious zone around SSS disk was always disregarded and the diameter of the outer zone was measured (**Hindler and Munro, 2010**).

The ability of the isolates to grow at 37°C and 42°C was tested at M2 medium agar plates. Bacterial suspensions (see above) were spread on the plates and incubated up to 7 days. A control plate was incubated at 28°C for each isolate.

RESULTS AND DISCUSSION

Isolation of soil RGM

The selective isolation of mycobacteria from soil is usually based on their resistance to acids, bases and disinfectants. Here we used one conventional decontamination method (**Iivanainen, 1995**) and a method based on the ability of mycobacteria to demulsificate an olive oil/SDS emulsion (**Yamamura and Harayma, 2007**). With the decontamination method, we obtained six RGM isolates from soil PM, five from NM, three from CE and eighteen from CO.

The olive oil/SDS method (**Yamamura and Harayma, 2007**) was used only for soils CE and CO, in parallel to the decontamination method. Although we used the concentration of SDS that was optimized by the authors for maximum RGM recovery and minimal contamination (*i.e.* 10 mg SDS per plate), we had problems with the growth of other (non-mycobacterium) actinomycetes on HV agar, as well as with fast-growing slimy bacteria on M2 agar and TSA. In addition, the emulsion itself was rather unstable. Eventually, we obtained one and nine pure RGM isolates from soils CE and CO, respectively. We also isolated the non-mycobacterium bacteria because they are interesting in the point of view they survived in the SDS/olive oil emulsion, and these bacteria are pending further characterization. Concluding, the decontamination method seems to be more suitable for RGM isolation than the olive oil/SDS method.

The remarkable difference in the number of isolates between CE and CO soils (4 vs. 24 with both methods) may be due the increased pH in soil CE because of cattle excrement deposits (*i.e.* pH 7.4 vs. pH 4.9 in the control soil CO). Indeed, several studies indicate that environmental mycobacteria are more likely to be isolated from soil or sediment with low pH (**Brooks *et al.*, 1984; Iivanainen *et al.*, 1999**). Also **Donoghue *et al.* (1997)** recovered as little as 1-2 mycobacteria (on average) per sampled site in permanent pastures with pH range 6.5-8.5 using a decontamination procedure. The pH of soils PM and NM was comparable (*i.e.* respectively 5.7 and 5.9), and similar numbers of isolates were recovered from the two soils using the same method.

In total, 44 pure cultures of soil RGM were obtained from the four soils. All isolates were non-pigmented. These 44 isolates were divided into 14 distinct groups according to their genome relativeness (based on the similarity of their BOX-PCR profiles) in a parallel study (**Kyselková *et al.*, 2012**). Most of the isolates (*i.e.* 61%) as well as distinct genotypes were recovered from the most acidic CO soil. Representatives of each group were identified with 16S rRNA sequencing (**Kyselková *et al.*, 2012**). Most of the soil isolates were identified as *Mycobacterium septicum,* which belongs to the *Mycobacterium fortuitum* group. These bacteria are often recovered from soil (**Donoghue *et al.*, 1997; Chilima *et al.*, 2006; Hartmans *et al.*, 2006; Yamamura and Harayma, 2007**), but are also known as human pathogens (**Schinsky *et al.*, 2000; Tortoli, 2003**).

Resistance to antibiotics and growth at 37°C and 42°C

Twenty-three isolates representing different BOX-PCR groups (as defined in **Kyselková *et al.*, 2012**) were tested for the resistance to amikacin, rifampicin, sulfonamides, clarithromycin, vancomycin, ofloxacin, amoxicillin/clavulanate, and doxycycline (Table 1). Since standard breakpoints of disc diffusion method are not available for RGM, we arbitrarily set them according to the zone size distributions among the strains (examples for doxycycline and sulfonamides in Figure 1). An isolate was considered resistant to an antibiotic if the respective zone was up to 20 mm for doxycycline, 20 mm for amikacin, 10 mm for rifampicin, 10 mm for sulfonamides, 15 mm for clarithromycin, 10 mm for vancomycin, 20 mm for ofloxacin, and 10 mm for amoxicillin/clavulanate. In the case of doxycycline the 20 mm zone can be approximately interpolated to the MIC 8-16 µg/mL (**Wallace *et al.* 1979**), which are the breakpoints usually used for RGM (**Swenson *et al.*, 1985; Brown-Elliott and Wallace, 2002**). Based on the arbitrary breakpoints, 83% tested isolates were resistant to rifampicin, 74% to doxycycline, 52% to sulfonamides, 48% to vancomycin and 22% to amoxicillin/clavulanate. None of the soil isolates tested was resistant to amikacin, ofloxacin or clarithromycin. Most of the isolates were resistant to 2-4 antibiotics (Figure 2), with maximum multiresistance to five antibiotics in one isolate of *M. septicum* from soil CE.

Amikacin and quinolones have been used for the initial therapy of serious infections caused by RGM from *M. fortuitum* group because of the low occurrence of resistance (**Brown-Elliott and Wallace, 2002**), and the combination of amikacin and ciprofloxacin was still among the most efficient agents against *M. fortuitum* in a study from 2010 (**Santos *et al.*, 2010**). Likewise, all soil isolates tested in our study were susceptible to amikacin and the quinolone ofloxacin. The majority of isolates from this study were resistant to the antituberculosis drug rifampicin, which is consistent with previous findings on atypical mycobacteria (**Nguyen and Thompson, 2006**). Compared to the proportions of susceptible clinical strains within *M. fortuitum* group summarized by **Brown-Elliott and Wallace (2002),** our environmental strains were more

often susceptible to clarithromycin and vancomycin, and less often to doxycycline and sulfonamides (though sulfadiazine used here instead of sulfamethoxazole). The frequent resistance to doxycycline and sulfonamides in soil isolates might be due to the massive use of tetracycline and sulfonamide groups of antibiotics in agriculture. The comparisons with clinical isolates are nevertheless very rough because of different approaches for susceptibility testing and the lack of defined resistance breakpoints of disk diffusion test for RGM. The disk diffusion method used in this study is considered as a useful screening tool for environmental or taxonomical studies, but not suitable for therapeutic purposes due to some technical problems such as 'partial zones' of inhibition for sulfonamides (**Brown-Elliott and Wallace, 2002**) observed also in this work. The present soil management did not seem to influence the occurrence of resistance since multiresistant strains, including strains resistant to sulfonamides and doxycycline, were recovered also from soil at an eco-farm where antibiotics have not been used for veterinary purposes for past 17 years.

The ability to grow at 37°C was shown in seven isolates that originated from soil NM, CE and CO (Table 1). None of the tested isolates grew at 42°C. The isolates growing at 37°C belonged to the species *M. septicum*, *M. fortuitum* and *M. litorale*, and were resistant to 2-4 antibiotics. *M. septicum* and *M. fortuitum* are known human pathogens (**Tortoli, 2003**), and the growth at 37°C indicates that the isolates may be able to colonize human body. The direct relationship between an environmental source and clinical disease due to mycobacteria is however difficult to evidence (**De Groote *et al*., 2006**). *M. litorale* is a novel species isolated from soil in China (**Zhang *et al*., 2012**) and nothing is known about its possible clinical impact.

Table 1 Growth at 37°C and resistance to antibiotics in rapidly growing mycobacteria from grassland soils

Isolate[a]	Soil[b]	Growth at 37ºC[c]	Antibiotic resistance (zone in mm)[d]							
			AMI	RIF	SSS	CLA	VAN	OFL	AMC	DOX
M. septicum Site1-11A	PM	-	28	**6.5**	**6.5**	32	21	37	15	40
M. septicum Site1-2A	PM	-	50	**6.5**	45	35	22	40	25	43
M. septicum Site1-8A	PM	-	45	**6.5**	50	40	**6.5**	45	16	**12**
M. septicum Site1-IIA/46	PM	-	43	**6.5**	55	38	**6.5**	41	20	**6.5**
M. septicum Site2-2C	NM	-	43	**6.5**	50	45	**6.5**	46	17	**6.5**
M. fortuitum subsp. *acetamidolyticum* Site2-4C	NM	+	29	**6.5**	**6.5**	25	**6.5**	40	20	44
M. aubagnense Site2-IIIC/14	NM	-	39	28	**6.5**	40	12	40	28	**10**
M. septicum Site3-B10	CE	+	45	**6.5**	**6.5**	42	**6.5**	47	**6.5**	**8**
M. litorale Site3-B14	CE	+	47	**6.5**	80	42	**6.5**	46	**6.5**	**8**
M. septicum Site3-B33	CE	-	47	**6.5**	40	45	28	42	22	**9**
Mycobacterium sp. Site3-B34	CE	+	48	**6.5**	**6.5**	37	**6.5**	45	14	**6.5**
M. septicum Site4-B1	CO	+	28	**6.5**	50	17	**6.5**	45	**6.5**	**11**
M. septicum Site4-B15	CO	-	43	**6.5**	40	33	**6.5**	37	23	50
M. septicum Site4-B2	CO	-	45	**6.5**	50	40	14	38	20	**6.5**
M. septicum Site4-B25	CO	-	46	**6.5**	**6.5**	30	18	37	28	42
M. septicum Site4-B29	CO	+	37	11	**6.5**	36	12	36	18	**6.5**
M. septicum Site4-B30	CO	-	43	16	**6.5**	30	26	35	23	**8**
M. septicum Site4-B31	CO	-	44	**6.5**	**6.5**	35	26	35	30	**8**
M. septicum Site4-B36	CO	+	47	**6.5**	47	45	**6.5**	47	**6.5**	**6.5**
M. septicum Site4-B38	CO	-	40	27	**6.5**	42	23	40	32	45
M. alvei Site4-B4	CO	-	47	**6.5**	**6.5**	35	28	38	16	**6.5**
M. septicum Site4-B5	CO	-	45	**6.5**	45	40	**6.5**	45	24	**10**
M. septicum Site4-B6	CO	-	38	**6.5**	**6.5**	42	16	40	**6.5**	**6.5**

Legend:

[a]Isolates were identified by 16S rRNA gene sequencing in Kyselková *et al*. (2012).

[b]PM, periodically manured with pig slurry from ATB-treated pigs; NM, non-manured; CE, highly impacted by excrements from bio-cattle that do not receive antibiotics; CO, preserved, with negligible impact of excrements from bio-cattle.

[c]None of the isolates grew at 42ºC.

[d]AMI, amikacin; RIF, rifampicin; SSS, sulfonamides; CLA, claritromycin; VAN, vancomycin; OFL, ofloxacin; AMC, amoxicillin/clavulanate; DOX, doxycycline; TET, tetracycline. For antibiotic doses, see Material and Methods. The inhibition zones smaller than breakpoint values are marked in bold.

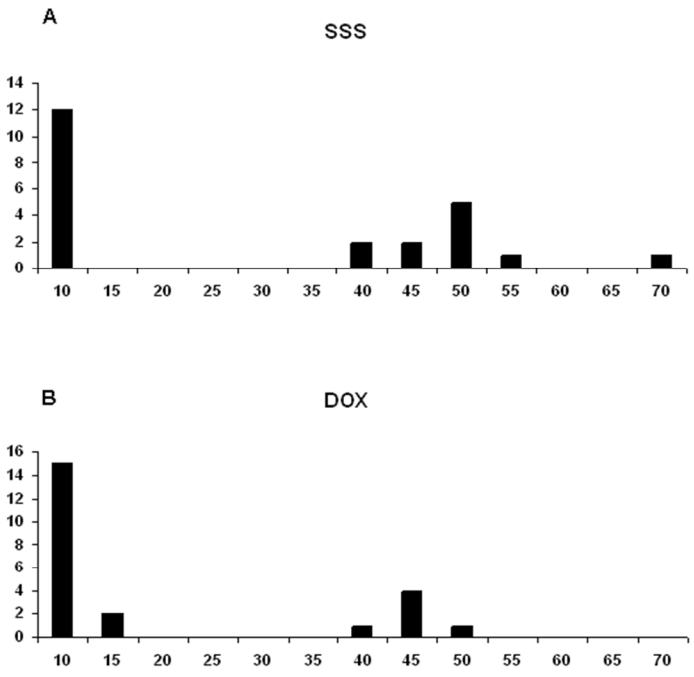

Figure 1 Distribution of resistance to sulfonamides (A) and doxycycline (B) in soil RGM isolates (n = 23). X-axis: categories of zone sizes (mm), **10**, 6.5-10; **15**, 11-15; **20**, 16-20; **25**, 21-25; **30**, 26-30; **35**, 31-35; **40**, 36-40; **45**, 41-45; **50**, 46-50; **55**, 51-55; **60**, 56-60; **65**, 61-65; **70**, >65. Y-axis: number of isolates with the corresponding zone size.

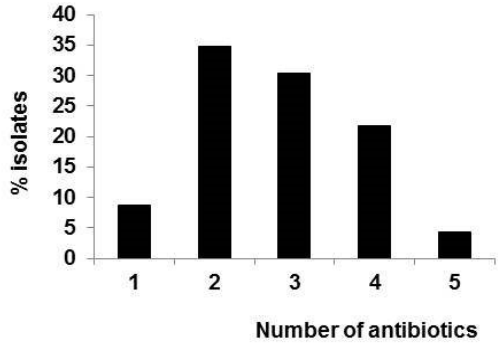

Figure 2 Percents of RGM isolates resistant to 1-5 antibiotics.

CONCLUSION

The NaOH/malachite green/cycloheximide decontamination method was more efficient than the olive oil/SDS-based method for RGM recovery from grassland soils. Multiresistant RGM close to known human pathogens occurred in grassland soils regardless the history of manuring and exposition to antibiotics. No isolate was, however, resistant to amikacin or ofloxacin, which are important agents in RGM infection treatment.

Acknowledgments: This work was supported by the Grant Academy of the Czech Republic, project P504/10/2077, Ministry of Education, Youth and Sport of the Czech Republic, project LC06066 and by project Postdok_BIOGLOBE - CZ.1.07/2.3.00/30.0032 co-financed by the European Social Fund and the state budget of the Czech Republic. The South Bohemia University in České Budějovice is acknowledged for PhD- student support to L. Kotrbová (GAJU 04-142/2010/P). We thank to Zuzana Stehlíková for her excelent technical assistance and MUDr. J. Scharfen, National Reference Laboratory for Pathogenic Actinomycetes, Dept. Medical Microbiology and Immunology, Regional Hospital Trutnov, Inc., Trutnov, Czech Republic, for helpful discussion.

REFERENCES

BROOKS, R., W., PARKER, B., C., GRUFT, H., FALKINHAM, J., O., 3rd. 1984. Epidemiology of infection by nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. *American Review of Respiratory Disease*, 130, 630-633.

BROWN-ELLIOTT, B., A., WALLACE, R., J., JR. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clinical Microbiology Reviews*, 15, 716-746.

BROWN-ELLIOTT, B., A., WALLACE, R., J., JR. 2005. Infections caused by nontuberculous mycobacteria. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition* (Mandell, G., L., Bennett, J., E., Dolin, R., eds), Elsevier Church Livingstone, Philadelphia, 4016 p. ISBN 0-443-06643-4.

CHILIMA, B., Z., CLARK, I., M., FLOYD, S., FINE, P., E., M., HIRSCH, P., R. 2006. Distribution of environmental mycobacteria in Karonga district, northern Malawi. *Applied and Environmental Microbiology*, 72, 2343-2350.

DE GROOTE, M., A., HUITT, G. 2006. Infections due to rapidly growing mycobacteria. *Clinical Infectious Diseases*, 42,1756–1763.

DONOGHUE, H., D., OVEREND, E., STANFORD, J., L. 1997. A longitudinal study of environmental mycobacteria on a farm in south-west England. *Journal of Applied Microbiology*, 82, 57-67.

FAJARDO, A., MARTINEZ-MARTIN, N., MERCADILLO, M., GALAN, J., C., GHYSELS, B., MATTHIJS, S., CORNELIS, P., WIEHLMANN, L., TUMMLER, B., BAQUERO, F., MARTINEZ, J., L. 2008. The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE*, 3, e1619.

HARTMANS, S., DE BONT, J., A., M., STACKEBRANDT, E. 2006. The genus *Mycobacterium* – nonmedical. *The Prokaryotes Vol. 3* (Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., Dworkin, M., eds), Springer-Verlag, New York, 1143 p. ISBN 0-387-25493-5.

HAYAKAWA, M., NONOMURA, H. 1987. Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *Journal of Fermentation Technology*, 65, 501-509.

HINDLER, J., F., MUNRO, S. 2010. Disk diffusion test. *Clinical microbiology procedures handbook, Vol. 2* (Garcia, L., S., Isenberg, H., D., eds) ASM Press, Washington, DC, 2540 p. ISBN 1-55581-527-8.

IIVANAINEN, E. 1995. Isolation of mycobacteria from acidic forest soil samples: comparison of culture methods. *Journal of Applied Microbiology*, 78, 663-668.

IIVANAINEN, E., MARTIKAINEN, P., J., VAANANEN, P., KATILA, M.-L. 1999. Environmental factors affecting the occurrence of mycobacteria in brook sediments. *Journal of Applied Microbiology*, 86, 637-681.

KOBASHI, Y., HASEBE, A., NISHIO, M., UCHIYAMA, H. 2007. Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. *Microbes and Environments*, 22, 44-51.

KYSELKOVÁ, M., CHROŇÁKOVÁ, A., VOLNÁ, L., NĚMEC, J., ULMANN, V., SCHARFEN, J., ELHOTTOVÁ, D. 2012. Tetracycline resistance and presence of tetracycline resistance determinants *tet*(V) and *tap* in rapidly growing mycobacteria from agricultural soils and clinical isolates. *Microbes and Environments*, 27, 413-422.

MARTINEZ, J., L. 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society B*, 276, 2521–2530.

NGUYEN, L., THOMPSON, C., J. 2006. Foundations of antibiotic resistance in bacterial physiology: the mycobacterial paradigm. *Trends in Microbiology*, 14, 304-312.

PAKARINEN, J., NIEMINEN, T., TIRKKONEN, T., TSITKO, I., ALI-VEHMAS, T., NEUBAUER, P., SALKINOJA-SALONEN, M., S. 2007. Proliferation of mycobacteria in a piggery environment revealed by mycobacterium-specific real-time quantitative PCR and 16S rRNA sandwich hybridization. *Veterinary Microbiology*, 120, 105–112.

PANG, Y., BROWN, B., A., STEINGRUBE, V., A., WALLACE, R., J., JR, ROBERTS, M., C. 1994. Tetracycline resistance determinants in *Mycobacterium* and *Streptomyces* species. *Antimicrobial Agents and Chemotherapy*, 38, 1408-1412.

PASQUALI, F., DE CESARE, A., RICCI, A., KEHRENBERG, C., SCHWARZ, S., MANFREDA, G. 2004. Phage types, ribotypes and tetracycline resistance genes of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strains isolated from different origins in Italy. *Veterinary Microbiology*, 103, 71-76.

SANTOS, A., CREMADES, R., RODRÍGUEZ, J., GARCIA-PACHON, E., RUIZ, M., ROYO, G. 2010. Activity of various drugs alone or in combination against *Mycobacterium fortuitum*. *Journal of Infection and Chemotherapy*, 16, 64-67.

SCHINSKY, M., F., MCNEIL, M., M., WHITNEY, A., M., STEIGERWALT, A., G., LASKER, B., A., FLOYD, M., M., HOGG, G., G., BRENNER, D., J.,

BROWN, J., M. 2000. *Mycobacterium septicum* sp. nov., a new rapidly growing species associated with catheter-related bacteraemia. *International Journal of Systematic and Evolutionary Microbiology*, 50, 575-581.

SHIRLING, E., B., GOTTLIEB, D. 1966. Methods for characterization of streptomycetes. *International Journal of Systematic Bacteriology*, 16, 313-340.

SWENSON, J., M., WALLACE, R., J., JR., SILCOX, V., A., THORNSBERRY, C. 1985. Antimicrobial susceptibility of five subgroups of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. *Antimicrobial Agents and Chemotherapy*, 28, 807–811.

THOMAS, V., LORET, J.-F., JOUSSET, M., GREUB, G. 2008. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environmental Microbiology*, 10, 2728–2745.

TORTOLI, E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev*, 16, 319–354.

TSUKAMURA, M., YANO, I., IMAEDA, T. 1986. *Mycobacterium moriokaense* sp. nov., a rapidly growing, non photochromogenic mycobacterium. *International Journal of Systematic Bacteriology*, 36, 333-338.

WALLACE, R., J., DALOVISIO, J., R., PANKEY, G., A. 1979. Disk diffusion testing of susceptibility of *Mycobacterium fortuitum* and *Mycobacterium chelonei* to antibacterial agents. *Antimicrobial Agents and Chemotherapy*, 16, 611-614.

YAMAMURA, H., HARAYMA, S. 2007. Method for selective isolation of mycobacteria using olive oil emulsified with SDS. *Bioscience, Biotechnology, and Biochemistry*, 71, 1553-1556.

ZHANG, Y., ZHANG, J., FANG, C., PANG, H., FAN, J. 2012. *Mycobacterium litorale* sp. nov., a new rapidly growing species from soil in China. *International Journal of Systematic and Evolutionary Microbiology*, 62, 1204-1207.