Kafkas Univ Vet Fak Derg
21 (4): 507-511, 2015
DOI: 10.9775/kvfd.2014.12769

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Spoligotyping of *M. tuberculosis* Strains from Cattle in Turkey

Nevin TUZCU [1] Begüm KAYAR [2] Elif Bilge UYSAL [3]
Yasin GÜLCÜ [4] Mehdi MARZİ [2] Fatih KÖKSAL [2]

[1] Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Cumhuriyet University , TR-58100 Sivas - TURKEY
[2] Department of Medical Microbiology, Faculty of Medicine, Çukurova University , TR-01100 Adana - TURKEY
[3] Department of Medical Microbiology, Faculty of Medicine, Cumhuriyet University , TR-58100 Sivas - TURKEY
[4] Veterinary Control Institute, TR-42100 Konya – TURKEY

KVFD-2014-12769 Received: 07.12.2014 Accepted: 19.03.2015 Published Online: 19.03.2015

Abstract

Although it is generally accepted that *M.bovis* leads to tuberculosis in cattle, there are statements given from the different regions of the world, referring to the fact that *M. tuberculosis*, which is known as the human tuberculosis agent, causes tuberculosis in cattle as well. The material of the study consisted of 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle with the culture methods; these organ pieces had been taken from the cattle with granulomatous lesion detection after the slaughtering in slaughterhouses located in Çukurova region and brought to the laboratory under sterile conditions. It was determined in the genotyping conducted by using the Spoligotyping method that 13 of the 55 isolates were *M. tuberculosis* and they belonged to the T1 family (SIT53) by becoming dense in one cluster (100%). Consequently, it was shown with this study that *M. tuberculosis*, which leads to tuberculosis in humans, could be transmitted from humans to animals and from animals to humans again, and researching the human and epidemiological tuberculosis cases by using molecular epidemiology-based methods such as spoligotyping might provide useful information about explaining the ways of transmission of tuberculosis.

Keywords: *Cattle, Mycobacterium tuberculosis, Spoligotyping*

Türkiye'de Sığırlardan İzole Edilen *M. tuberculosis* Suşlarının Spoligotiplendirmesi

Özet

Tüberkülozise sığırlarda, *M. bovis*'in sebep olduğu genel olarak kabul edilmesine rağmen insan tüberkülozis etkeni olarak bilinen *M. tuberculosis*'in de sığırlarda tüberkülozise neden olduğuna dair dünyanın farklı bölgelerinden yapılmış bildirimler bulunmaktadır. Çalışmanın materyalini Çukurova bölgesinde bulunan mezbahalarda kesim sonrası granülamatöz lezyon tespit edilen sığırlardan alınan ve steril şartlarda laboratuvara ulaştırılan 95 adet sığıra ait organ parçalarından kültür yöntemleri ile izole ve identifiye edilen 13 adet *M. tuberculosis* izolatı oluşturdu. Spoligotyping yöntemi ile yapılan genotiplendirmede 55 izolattan 13 tanesinin *M. tuberculosis* olduğu ve bunların tek bir küme içerisinde yoğunlaşarak (%100) T1 ailesine (SIT53) ait olduğu belirlendi. Sonuç olarak bu çalışma ile insanlarda tüberkülozise neden olan *M. tuberculosis*'in insanlardan hayvanlara, hayvanlardan da tekrar insanlara bulaşabileceği, insan ve hayvan kaynaklı tüberkülozis vakalarının spoligotyping gibi moleküler epidemiyolojik temelli yöntemlerle araştırılmasının tüberkülozisin bulaş yollarının açıklanması konusunda faydalı bilgiler verebileceği gösterilmiştir.

Anahtar sözcükler: *Sığır, Mycobacterium tuberculosis, Spoligotyping*

INTRODUCTION

Tuberculosis is a chronic disease which is comprised of *M. tuberculosis*, *M. bovis, M. canettii*, *M. microti*, *M. africanum, M. caprae* and *M. pinnipedii* included in the *Mycobacterium tuberculosis* complex (MTC) and has a wide host range including humans, fish, reptiles, birds, wild animals and domestic mammals. *Mycobacteria* are the acid-resistant bacteria known with their abilities to settle in almost all the tissues of the body like lungs, kidneys, bones, liver, skin and brain and the lymph nodules belonging to these tissues, and with their formation of typical granulomas in the tissues they settle in [1,2].

Although it is generally accepted that *M. bovis* leads to tuberculosis in cattle, there are statements from studies made in different regions, referring to the fact that *M. tuberculosis*, which is known as the human tuberculosis agent, causes tuberculosis in cattle as well. It was reported by many researchers that tuberculosis caused by the *M. tuberculosis* was seen in domestic and wild animals after

İletişim (Correspondence)
+90 346 2191010/3916
ntuzcu@hotmail.com

short and long-term contacts with humans and existed especially in cattle [3-5]. Although there are researches in Africa stating that the incidence rate of the *M. tuberculosis* in cattle is under 1% [3,6,7], it was stated that the *M. tuberculosis* dependent tuberculosis rate in cattle is 7.4% in countries like Sudan where the human tuberculosis incidence is high [8]. It was reported in another study conducted in India that the 30.8% of the tuberculosis occurrences identified in cattle were the tuberculosis cases that develop depending on the *M. tuberculosis* [9].

People with tuberculosis spread the agents with their urine, sputum and stools. There are reports stating that the *M. tuberculosis* infection develops in cattle through eating the feeds that have agents or the inhalation of the contaminated respiration air [5,10]. Direct transmission of the *M. tuberculosis* among cattle is doubtful.

The *M. tuberculosis* complex genome includes 36-base-pair direct repeat (DR) loci at different numbers and there are sequences called *spacer* at the length of 35-41-base pairs between these loci. The genetic relation between the strains can be determined by using the spoligotyping method on basis the number of DR copies and the existence or absence of the spacer sequences. The spoligotyping method has been used frequently over the last years to put forth the molecular epidemiology of the tuberculosis in humans and animals. Spoligotyping is a PCR-based reverse dot blot hybridization method and it is fast, simple and repeatable [11].

Simeon Cadmus et al. stated in the spoligotyping they conducted on human and cattle tuberculosis isolates in Nigeria that 51 of the 60 human MTC-member isolates were *M. tuberculosis*, these isolates had 18 different spoligo-patterns and the most observed pattern was the NH1 pattern, which belongs to LAM 10-CAM family including 35 isolates. Same researchers stated that 15 of the 17 MTC members they obtained from cattle were *M. bovis*, one member was *M. tuberculosis*, another member was *M. africanum* and the *M. tuberculosis* isolate belonged to the NH1 (LAM10-CAM) pattern, which is most frequently seen in humans [4].

It was aimed in this study to perform a molecular characterization of the *M. tuberculosis* strains by using the spoligotyping method, which were isolated from the lesions with the suspicion of tuberculosis obtained from the cattle slaughtered in the slaughterhouses located in Çukurova region.

MATERIAL and METHODS

The material of this study consisted of 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle by using the culture methods; these organ pieces had been taken from the cattle with granulomatous pneumonia detection after the slaughtering in slaughterhouses located in Çukurova region and brought to the laboratory under sterile conditions. These 95 cattle were chosen out of 6.800 cattle slaughtered for meat production.

Culture

The tissue samples, which were taken from the lesions with the suspicion of tuberculosis observed in the lung and lymph nodes in the macroscopic examination carried out after the slaughtering and brought to the laboratory, were decontaminated in accordance with the protocol specified by Petroff [12].

Samples were inoculated onto the LJ medium with (4 gr/l) and without pyruvate and left for incubation at 37ºC [12,13]. EZN-stained preparates were prepared from the bacterial growth. Biochemical tests were applied to the bacterial growth, which were positive in terms of the ARB [14]. The strains which had morphological eugonic growth in the LJ medium, positive niacin accumulation test, nitrate reduction reaction, TCH reaction and negative catalase activity at 68ºC were evaluated as *M. tuberculosis* [5,15] .

Spoligotyping

DNA extraction was made by using the Mickle device from the colonies multiplied in the LJ medium of the 13 isolates, which were evaluated as *M. tuberculosis* as a result of the biochemical tests (Mickle tissue disintegrator). The spoligotyping method was applied after the extraction by using the DRa and DRb primer pairs, array of which is given below [16,17].

DRa: 5'-GGT TTT GGG TCT GAC GAC-3' (biotin labelled at the 5' end)

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

DRa and DRb primer pairs targeting DR area is synthesized. As DRa primer is labelled with biotin, it is preserved at +4ºC. DRb primer is splitted into small amounts and preserved at -20ºC. In each process, positive (*M. bovis*, *M. bovis* BCG, *M. tuberculosis* H37Rv, or clinical isolate whose genotype is known) and negative control (dH_2O) are used. For each strain; dH_2O 8.5µl, DMSO 1.0 µl, 2x PCR Master Mix (Fermantas) 12.5 µl, DRa (25 pmol/µl) 0.25 µl, DRb (25 pmol/µl) 0.25 µl, template DNA 2.50 µl are used. Tubes containing PCR reaction mixture are placed in Thermal Cycler device (Applied Biosystem AB) and heat cycle as: 95ºC 5 min, 40 cycle 94ºC 1 min, 55ºC 1 min, 72ºC 45 sec and then 72ºC 10 min 4ºC ∞. By using below mentioned octal coding key, results are converted to "Octal code" made of 15 characters between 0 and 7. By using databases, groups and clades are determined by obtained data (http://www.pasteurguadeloupe.fr:8081/SITVITDemo/outils Consultation.jsp, http://www.mıru-vntrplus.org, http://www.mbovis.org).

□□□ = 0 □□■ = 1 □■□ = 2 □■■ = 3
■□□ = 4 ■□■ = 5 ■■□ = 6 ■■■ = 7
■ = 1 □ = 0 Spacer 43

RESULTS

It was determined that isolated strains belonged to three different farms and the age of cattle were determined to vary between 3 to 6. It drew attention that farms which arrival of cattle isolated *M. tuberculosis* had aproximately 30-50 Holstein or Holstein hybrid breed and small dairy cows.

In the genotyping of the 13 *M. tuberculosis* isolates conducted by using the spoligotyping method, which were isolated and identified by using the culture methods from the organ pieces of 95 cattle with granulomatous pneumonia detection after the slaughtering and brought to the laboratory for bacteriological isolations under sterile conditions, it was determined that all the isolates belonged to the T1 family included in one profile (100%). The spoligotyping patterns of the *M. tuberculosis* isolates are given in *Table 1* and seen in *Fig. 1*.

DISCUSSION

In this study genotyping were carried out 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle were chosen out of 6.800 cattle slaughtered for meat production and had been taken with granulomatous pneumonia detection after the slaughtering by spoligotyping methods.

Tuberculosis, which is thought to cause the death of approximately 2 million people every year, is one the significant zoonotic diseases that has reached so far. It is estimated that 9 million people got sick, 1.5 million people died and 360.000 of those dead people had HIV positive disease in 2013 [18]. Almost all the deaths resulting from tuberculosis are preventable and the application of tuberculosis control programs is necessary to decrease the deaths resulting from tuberculosis. Beside the early and right diagnosis of the disease, determination of the disease origin, infection-carriers and the type of the Mycobacterium leading to the disease is quite important to achieve success in the tuberculosis control programs. There are studies showing that the intra-dermal skin test (IDDT), which is used in most of the control programs to determine the prevalence of the disease, is not a reliable indicator in the determination of the active disease in cattle and IDDT results and clinical findings related to tuberculosis do not match up with the molecular diagnosis results [5]. It is obvious that supporting the fieldwork with laboratory work by using molecular methods like the spoligotyping method, which was used in this study together with the conventional methods applied in the tuberculosis diagnosis, will make important contributions to the eradication efforts of tuberculosis.

Sensitivity period of the IDDT, which is used for the diagnosis of tuberculosis in livestock, is short and reaction may not be observed when the infection source disappears in the herd. While a negative IDDT does not show that the disease does not exist in places where the tuberculosis incidence is high, a positive IDDT does not mean that the disease always exists [19]. Infection caused by the *M. tuberculosis* must be considered if the animal in the herd, which had a negative tuberculin dermal test before, is young in the first positive reaction determined with the tuberculin dermal test and if the farm staff has tuberculosis.

Ocepec Matjaz et al.[20] reported in the screening, which they made in a facility consisting of 78 animals in Slovenia, that they did not come across granulomatous lesions pointing at tuberculosis in 3 animals after the slaughtering, which they assessed as strongly positive with the IDDT, but reproduction was seen following the 28th day in the inoculations of the LJ medium belonging to a sample from the mediastinal and portal lymph nodes of a 2-year-old cow, however, there was no reproduction in the MGIT, stonebrink medium and Middle Brook 7H10 medium despite the 8-week incubation, and the isolate was *M. tuberculosis* as a result of the biochemical tests conducted with the colonies they defined in the LJ medium. It should not be forgotten that the cattle can be a potential reservoir in terms of not only *M. bovis*, but also

Table 1. *Spoligotyping pattern of 13 M. tuberculosis isolates*
Tablo 1. *13 M. tuberculosis izolatının spoligotyping paterni*

Strain Number(N)	Octal Code	Spoligotyping	Spoligotyping Family	
			SpoIDB4	
13	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	777777777760771	T1	SIT53

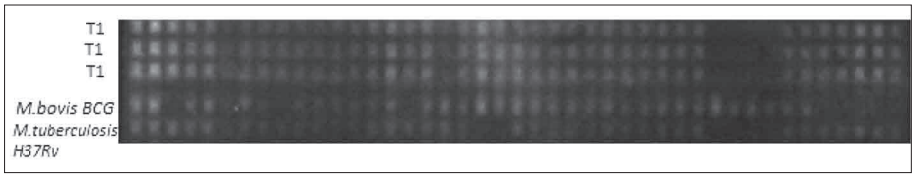

Fig 1. Spoligotyping image of 13 *M. tuberculosis* isolates and control strains

Şekil 1. 13 *M.tuberculosis* izolatının ve kontrol suşların spoligotyping görüntüsü

M. tuberculosis in places where the tuberculosis incidence is high in humans and animals.

In another study including the analysis of 768 samples, which were taken from the cattle with the suspicion of tuberculosis after the slaughtering in Northern India, 54 MTC isolates were determined and it was shown in the biochemical tests conducted on the isolated MTC strains that 14 of the isolates were *M. tuberculosis* [5]. The fact that 13 MTC isolates isolated from 95 cattle with the detection of granulomatous lesions were determined as *M. tuberculosis* in this study resembles the results of Srivastava et al.[5]. Besides, this result reveals that the cattle tuberculosis resulting from the *M. tuberculosis* must also be taken into account in the determination of the tuberculosis eradication strategies, which will be applied in our country.

Researchers stated at the end of the study they conducted between 2005-2007 that the cattle-isolates, which were among the 19 isolates isolated from the animals, were included in the T1 family (SIT53) by reporting in the spoligotyping of the 74 tuberculosis agents isolated from humans, who did cattle business with the animals slaughtered in the southwest of Nigeria and had the diagnosis of tuberculosis, that 32 agents were *M. tuberculosis*. The fact that all the *M. tuberculosis* isolates obtained from the cattle with granulomatous lesion detection after the slaughtering belonged to the T1 family in this study matches up with the results of Jenkins et al.[21].

It is stated in the studies related to the molecular epidemiology of the *M. tuberculosis* belonging to the people in our country that the *M. tuberculosis* Beijing strains have started to be observed at growing rates [17,22,23]. With the spoligotyping method, Zozio et al.[24] stated in their study, where they assessed 245 clinical *M. tuberculosis* isolates from Ankara and Malatya regions, that 206 isolates gathered in 33 clusters and 39 isolates were in specific clusters with single members, the LAM7 TUR family constituted the biggest cluster by 21% and the T1 family followed it with the rate of 16.3% and the Haarlem 3 family with 5.3%. Durmaz et al.[23] reported in the spoligotyping they conducted with 145 human MTC isolates in Malatya that LAM7-TUR was the most common pattern (23.96%) and the T1 family (SIT53) was the second most common pattern (22.5%) in Malatya. The fact that the T1 family (SIT53), which was defined as the second density by the researchers in the molecular typing of the *M. tuberculosis* isolates isolated from the tuberculosis cases, included all the *M. tuberculosis* isolates isolated in this study puts forth the necessity to definitely consider the cattle for presenting the transmission dynamics of the tuberculosis seen in humans.

This study has a critical and important role in the control programs that will be applied in our region due to the fact that it is the first molecular epidemiological study conducted on the *M. tuberculosis* infection in the cattle of our region.

Consequently, the results obtained through this study showed that *M. tuberculosis*, which leads to tuberculosis in humans, could be transmitted from humans to animals and from animals to humans again, animals may have significant roles in the transmission chain of the *M. tuberculosis*, and researching the human and animal-origin *M. tuberculosis* isolates with molecular epidemiology-based methods such as spoligotyping might give useful information about determining the ways of transmission of tuberculosis in the development of struggle strategies against tuberculosis.

REFERENCES

1. van Soolingen D, Hoogenboezem T, De Haas PEW, Hermans PWM, Koedam MA, Teppema KS, Brennan JB, Besra SG, Janetta top PF, Schouls ML, van Embden JDA: A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: Characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol*, 47, 1236-1245, 1997. DOI: 10.1099/00207713-47-4-1236

2. Beytut E: Kars ili ve yöresinde sığırlarda tüberküloz insidensi ve lezyonların lokalizasyonu üzerine patolojik incelemeler. *Kafkas Univ Vet Fak Derg,* 7, 15-25, 2001.

3. Ocepek M, Pate M, Zolnir-Dovc M, Poljak M: Transmission of *Mycobacterium tuberculosis* from human to cattle. *J Clin Microbiol*, 43, 3555-3557, 2005. DOI: 10.1128/JCM.43.7.3555-3557.2005

4. Cadmus S, Palmer S, Okker M, Dale J, Gover K, Smith N, Jahans K, Hewinson RG, Gordon SV: Molecular analysis of human and bovine tubercle bacilli from a local setting in Nigeria. *J Clin Microbiol,* 44, 29-34, 2006. DOI: 10.1128/JCM.44.1.29-34.2006

5. Srivastava K, Chauhan DS, Gupta P, Singh HB, Sharma VD, Yadav VS, Sreekumaran Thakral SS, Dharamdheeran JS, Nigam P, Prasad HK, Katoch VM: Isolation of *Mycobacterium bovis* and *M. tuberculosis* from cattle of some farms in North India - Possible relevance in human health. *Indian J Med Res*, 128, 26-31, 2008.

6. Yumi U, Tooru M: Tuberculosis as a zoonosis from a veterinary perspective. *Comp Immunol Microbiol Infect Dis,* 30, 415-425, 2007. DOI: 10.1016/j.cimid.2007.05.002

7. Regassa A, Medhin G, Ameni G: Bovine tuberculosis is more prevalent in cattle owned by farmers with active tuberculosis in central Ethiopia. *Vet J* , Aug 28 [E-pub ahead of print], 2007. DOI: 10.1016/j.tvjl.2007.06.019

8. Sulieman MS, Hamid ME: Identification of acid fast bacteria from caseous lesions in cattle in Sudan. *J Vet Med,* B 49, 415-441, 2002. DOI: 10.1046/j.1439-0450.2002.00565.x

9. Prasad HK, Singhal A, Mishra A, Shah NP, Katoch VM, Thakral SS, Singhc DV, Chumberd S, Bald S, Aggarwale, Padmaf MV, Kumarg S, Singh MK, Acharya SK: Bovine tuberculosis in India: Potential basis for zoonosis. *Tuberculosis,* 85, 421-428, 2005. DOI: 10.1016/j.tube.2005.08.005

10. Hutchings MR, Harris S: Effects of farm management practices on cattle grazing behaviour and the potential for transmission of bovine tuberculosis from badgers to cattle. *Vet J, 153,* 142-162, 1997. DOI: 10.1016/S1090-0233(97)80035-4

11. Aranaz A, Liebana E, Mateos A, Dominguez L, Vidal D, Domingo M, Gonzolez O, Rodriguez-Ferri EF, Bunschoten AE, Van Embden JDA, Cousins D: Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: A tool for studying epidemiology of tuberculosis, Australia. *J Clin Microbiol*, 34 (11): 2734-2740, 1996.

12. Ambrosio SR, Márcia de Deus OE, Rodriguez CAR, Neto JSF1, Amaku M: Comparison of three decontamination methods for *Mycobacterium bovis* isolation. *Braz J Microbiol*, 39, 241-244, 2008. DOI: 10.1590/S1517-83822008000200008

13. Munyeme M, Rigouts L, Shamputa IC, Muma JB, Tryland M, Skjerve E, Djønne B: Isolation and characterization of *Mycobacterium bovis* strains from indigenous Zambian cattle using spacer oligonucleotide typing technique. *BMC Microbiol,* 9, 144-152, 2009. DOI: 10.1186/1471-2180-9-144

14. Wayne LG, Kubica GP: Mycobacteria. **In,** Sneath PHA (Ed): Bergeys Manual of Systematic Bacteriology, Vol. 2, 861-867, The Williams&Wilkins Co., Baltimore 1986.

15. Vestal AL: Procedures for isolation and identification of *Mycobacteria*. 15-19, U.S. Department of Health Education and Welfare, CDC Atlanta, Georgia, Publication No CDC-77-8230, 1977.

16. Kamerbeek J, Schouls L. Kolk A, van Agterveld M, van Soolingen D, van Embden J: Simultaneous detection and strain ifferentiation of *Mycobacterium tuberculosis* for diagnosis andepidemiology. *J Clin Microbiol*, 35, 907-914, 1997.

17. Oral Zeytinli Ü: Çukurova bölgesinde akciğer tüberkülozlu hastalardan izole edilen *Mycobacterium tuberculosis* suşlarının Spoligotyping ve MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeat) yöntemiyle tiplendirilmesi. *Uzmanlık Tezi,* Çukurova Üniv. Sağlık Bil. Enst., 2010.

18. WHO: Global Tuberculosis Report 2014. http://www.who.int/tb/publications/global_report/en/2014, *Accssed:* 22.10.2014

19. Solmaz H, İlhan Z, Aksakal A, Gulhan T, Ekin İH: Detection of bovine tuberculosis by tuberculin test and polymerase chain reaction in Van, Turkey. *Turk J Vet Anim Sci*, 33 (3): 229-233, 2009.

20. Ocepek M, Pate M, Zolnir-Dovc M, Poljak M: Transmission of *Mycobacterium tuberculosis* from human to cattle. *J Clin Microbiol,* 43, 3555-3557, 2005. DOI: 10.1128/JCM.43.7.3555-3557.2005

21. Jenkins AO, Cadmus SIB, Venter EH, Pourcel C, Hauk Y, Vergnaud G, Godfroid J: Molecular epidemiology of human and animal tuberculosis in Ibadan, Southwestern Nigeria. *Vet Microbiol,* 151, 139-147, 2011. DOI: 10.1016/j.vetmic.2011.02.037

22. Durmaz R, Ozerol IH, Durmaz B, Gunal S, Senoglu A, Evliyaoglu E: Primary drug resistance and molecular epidemiology of *Mycobacterium tuberculosis* isolates from patients in a population with high tuberculosis incidence in Turkey. *Microbial Drug Resist*, 9, 361-366, 2003. DOI: 10.1089/107662903322762798

23. Durmaz R, Zozio T, Gunal S, Allix C, Fauville-Dufaux M, Rastogi N: Population-based molecular epidemiological study of tuberculosis in Malatya, Turkey. *J Clin Microbiol*, 45, 4027-4035, 2007. DOI: 10.1128/JCM.01308-07

24. Zozio T, Allix C, Gunal S, Saribas Z, Alp A, Durmaz R, Fauville-Dufaux M, Rastogi N, Sola C: Genotyping of *Mycobacterium tuberculosis* clinical isolates in two cities of Turkey: Description of a new family of genotypes that is phylogeographically specific for Asia Minor. *BMC Microbiol,* 44, 1-9, 2005. DOI: 10.1186/1471-2180-5-44