Isolation of non-tuberculous mycobacteria among tuberculosis patients during a five-year period in Croatia

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ABSTRACT

The aim of this study was to evaluate the frequency and to assess the main NTM species isolated among all bacteriologically confirmed TB patients during a five years period in Croatia. From all bacteriologically confirmed TB patients, 4.3% (n=140) had also NTM isolated from respiratory specimens. The most frequently isolated NTM were M. gordonae, M. fortuitum and M. xenopi. The majority of patients (n=127) had only one NTM isolate, and 13 patients (9.3%) had two or more isolates of the same NTM species. One should take into account the possible significance of multiple NTM isolation in TB patients. It is clear that every case should be evaluated individually and considered with caution.

INTRODUCTION

M. tuberculosis, the causative agent of tuberculosis (TB) is a wide known pathogen for many centuries now, while on the other hand, non-tuberculous mycobacteria (NTM) have been identified as human pathogens only several decades ago [1,2]. Given the prolonged time engaged in fighting TB, many aspects of the disease have been identified and extensively studied. Moreover, M. tuberculosis as an obligate pathogen presents a clear picture regarding the clinical relevance of its isolation in patient’s samples and starting treatment. However, many NTMs are still quite a mystery regarding the importance of specific isolates identified from human samples and the decision on if and which therapy to start [2]. Over the years many studies pointed out the importance of NTM in causing true disease and emphasized the need for therapy [3-5]. In order to elucidate the clinical importance of NTM isolation, guidelines on how to diagnose mycobacteriosis were issued [1,2]. Main criterion in the NTM pulmonary disease diagnosis is the exclusion of other illnesses or cause of pathological changes [1]. Considering this criterion, NTM isolates in patients with active tuberculosis are per se not relevant and do not need specific treatment. However, there are very few studies regarding NTM isolated in TB patients and the relevance of co-isolation is not fully understood [6,7]. It is obviously very hard to distinguish changes caused by M. tuberculosis from changes possibly caused by co-infection with NTM in TB patients. Are NTM in TB patients really not relevant or do we have to take the NTM disease criteria “cum grano salis” in specific circumstances?

In this study, we wanted to evaluate the frequency and to assess the main NTM species isolated among all bacteriologically confirmed TB patients during a five years period in Croatia.

Moreover, we wanted to examine whether specific epidemiological conditions or infections caused by specific TB strains influenced the NTM-TB co-infection frequency.

MATERIAL AND METHODS

We conducted a nationwide retrospective study of NTM isolated from all bacteriologically confirmed TB patients in Croatia during a five year-period, 2007-2011. Usual follow-up of two years was included for TB patients first identified in 2011. The data were collected from the Croatian National Reference Laboratory for Mycobacteria (NRL). The Croatian NRL has a full national coverage of both bacteriologically confirmed TB cases as well as NTM isolates. TB patients with NTM isolated during the total of seven years of follow up were divided into two groups; co-isolation group encompassed those with NTM isolated in the period from two months prior up to nine months after TB isolation, while after-isolation group included patients with NTM isolated more than 9 months after M. tuberculosis isolation. Data recorded were as follows: patient’s age, sex, county of residence (n=21), rural or urban region of residence, time of NTM isolation in relation to TB, species of NTM isolated, results for drug susceptibility testing (DST) of M. tuberculosis and M. tuberculosis genotype.

All samples were processed according to standard procedures as previously described [8]. NTM species were identified by molecular methods (GenoType© CM/AS; Hain Lifescience, Nehren, Germany) while for NTM species not included in the panel, phenotypic methods were applied according to previously published guidelines [9-11]. DST for first line antituberculotic drugs was performed for all samples using the proportion method on Löwenstein-Jensen (LJ) solid medium at the following final drug concentrations: isoniazid (INH) at 0.2 μg/ml, rifampicin (RMP) at 40 μg/ml, streptomycin (STM) at 4 μg/ml and ethambutol (EMB) at 2 μg/ml [12-14]. MIRU-VNTR genotyping was performed according to the previously described protocol [15-17]. The MIRU-type was assessed combining the results of the 15 loci tested: 580

(MIRU4), 960 (MIRU10), 1644 (MIRU16), 2996 (MIRU26), 3192 (MIRU31), 802 (MIRU40), 424 (Mtub04), 577 (ETRC), 1995 (Mtub21), 2163b (QUB11b), 2165 (ETRA), 2401 (Mtub30), 3690 (Mtub39), 4052 (QUB26), and 4156 (QUB4156).

Microsoft Excel (Microsoft, Redmont, WA, USA) was used to calculate frequencies, percentages and median age. Comparison between groups was performed using Fishers Exact test.

RESULTS

In the 5-year period there have been 3,260 bacteriologically confirmed TB patients in Croatia. The median age of TB patients was 54 years, and 62.1% of all patients were male. Throughout the study period and the two years follow up, we recorded NTM isolation in a total of 140 TB patients, of which 56.7% were male. More than two third (67.9%) of all patients belonged to the co-isolation group with average NTM isolation time of 3.3 months after TB isolation. The average time of NTM isolation in the “after-isolation” group was 19 months after TB isolation. The median age of patients within the co-isolation group and after-isolation group were 60 and 54 years, respectively. When dividing patients according to the area of residence (rural or urban), we found no significant difference between the two groups. However, we found that in the larger Zagreb county there was three times less likelihood of NTM co-isolation and TB in comparison to all other counties (26 vs 19, p=0.0047).

In the co-isolation group, the most frequently isolated NTM were M. gordonae (32.6%), M. fortuitum (17.9%), M. xenopi (11.6%) and M. vaccae (7.4%). In the after-isolation group the

most frequently found isolates included M. gordonae (42.2%), M. fortuitum (15.6%), M. terrae (15.6%) and M. xenopi (11.1%) (Table I).

The only statistically significant difference between the two groups regarding species isolation was that M. terrae was more often isolated in the after-isolation group (15.6% vs 3.2%; p=0.0128). M. vaccae was exclusively isolated in the co-isolation group but the result was not statistically significant due to small number of isolates (7/95 vs 0/45; p=0.097).

The majority of patients (n=127) had only one NTM isolate, while 13 patients (9.3%) had two or more isolates of the same NTM species. Nine patients had two NTM isolates (M. fortuitum and M. gordonae in three cases, and one case of M. terrae, M. xenopi and M. chelonae, respectively), three patients had three isolates (M. abscessus, M. chelonae and M. gordonae, respectively), and only one patient had 6 isolates of M. xenopi (Table II).

During the study period, there were 15 MDR and 50 patients with TB resistant to INH among all bacteriologically confirmed patients in Croatia. In the co-isolation group four patients had resistant TB strains; three were INH resistant and one was MDR TB. In the after-isolation group we also found TB patients infected with resistant strains; two with MDR TB and one with INH resistance. Thus, 20% of all MDR patients and 8% of those with INH resistant strains also had NTM isolated during the study period.

Out of 140 TB strains included in our study, genotyping information was available for 138. Although none of them were of identical genotype, two thirds (n=92; 66.7%) were part of different clusters, while 46 were orphan strains.

DISCUSSION

The incidence of new TB cases in Croatia is steadily decreasing and during our study period, the TB incidence decreased from 22/100,000 in 2007 to 13/100,000 inhabitants in 2011. Clinicians, as well as microbiologists, are starting to get familiar with NTMs and efforts were made to evaluate the importance of NTM as disease causing pathogens in Croatia [18]. Jankovic et al. estimated that the incidence of pulmonary NTM disease is low, namely 0.23/100,000 inhabitants, significantly lower than TB incidence [18]. However, during the last two decades, we have also observed an increasing trend in the number of NTM isolates (NRL data). In our study we showed the frequency of NTM in patients with bacteriologically confirmed TB. From all bacteriologically confirmed patients, 4.3% had also NTM isolated from respiratory specimens. This is, to our knowledge, the lowest NTM isolation frequency in TB patients. Few studies that investigated such co-infections found variable, but unequivocally higher percentage of NTM isolates. Namely, studies from USA and Canada, countries with a low TB and high NTM incidence, isolated NTM in 14% and 11% of TB patients, respectively [6,19]. Studies from Korea and Taiwan, despite higher TB burden, also found higher incidence (7%) in comparison to our study [7,20]. There are several factors that could have contributed to our somewhat low incidence. One of the most prominent causes is the scarce knowledge of NTM significance among professionals. Clinicians do not consider NTM as possible causing agent, while microbiologists do not always use adequate tools. Namely, in Croatia all laboratories don’t use liquid media for TB culture, thus having less chance to identify NTM. Another reason is the financial aspect of NTM identification. Local laboratories are not equipped for NTM identification and have to send out the isolates to the NRL. This could sometimes be discouraged given the cost and general low awareness of pathogenic potential of NTMs.

The most frequently isolated NTM in our study were M. gordonae, M. fortuitum and M. xenopi. This is of no surprise because these mycobacteria are among most commonly isolated species in Croatia. It is hard to explain the observed differences between the two groups regarding M. vaccae and M. terrae isolation without further investigation of specific regional environmental factors which could favor distinct NTM propagation. However, taking into account that these two species are mostly non-pathogenic, we do not think that they have an important impact on patients with both NTM and TB isolation.

Two or more isolates of the same NTM species were isolated in almost 10% of the TB patients in our study. One should take into account the possible significance of multiple NTM isolation in TB patients. It is clear that every case should be evaluated individually and considered with caution. To our knowledge, none of these patients were treated for NTM infection. While the multiple isolation of M. gordonae and M. terrae in patients with TB are not considered important as these species are rarely pathogenic, more attention should be given to isolates of potentially pathogenic species such as M. chelonae, M. abscessus, M. fortuitum and M. xenopi. In our study, we had presumably only one true NTM disease patient with TB co-isolation. Two months after the first isolation of M. tuberculosis, M. xenopi was isolated in a sputum sample, and during the next year, M. xenopi was isolated in five isolation episodes. M. xenopi was found to be the most frequent NTM causing true pulmonary disease in Croatia. Thus, although TB treatment is the first and only option for most patients, it is very important to keep monitoring possible NTM disease progression and evaluate the need for NTM treatment in specific circumstances.

Interestingly, we found that patients with both NTM and M. tuberculosis isolates encompass 20% of all MDR and 8% of all isoniazid resistant strains in the study period. Compared to the

overall NTM isolation frequency of 4.3%, the isolation frequency is fivefold and twofold higher in MDR and isoniazid resistant group, respectively. There is some evidence that resistant strains are less pathogenic than susceptible strains [21]. Thus, a possible explanation of the high percentage of resistant strains found in our study is the likelihood that less pathogenic strains allow easier co-infection with other microorganisms than susceptible strains. Many reports suggested the importance of previous TB disease as predisposing factor for NTM infection [22-25]. There are several possible explanations for such findings including specific lung changes caused by TB, or just specific host factors influencing efficient defense against infectious pathogens. Although there is also the possibility that specific TB strains could trigger NTM infections, we did not find specific clustering of our patients. The percentage of clustering found in our study (66.7%) is similar to previously described in Croatia [26]. Moreover, all our TB strains were of different MIRU-VNTR genotype.

In conclusion, this is the first study describing NTM and M. tuberculosis co-isolation in Croatia and emphasizing the possible importance of multiple NTM isolates in TB patients.
REFERENCES


**Table I.** Non-tuberculous mycobacteria species isolated in TB patients in Croatia from 2007-2011.

<table>
<thead>
<tr>
<th>NTM species</th>
<th>Co-isolation group</th>
<th>After-isolation group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>31</td>
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<tr>
<td>M. fortuitum</td>
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<tr>
<td>M. xenopi</td>
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<tr>
<td>M. terrae</td>
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<td>30</td>
<td>7</td>
</tr>
<tr>
<td>M. vaccae</td>
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<td>0</td>
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<tr>
<td>M. nonchromogenicum</td>
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<td>M. chelonae</td>
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<td>66.67</td>
<td>2</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>5</td>
<td>83.33</td>
<td>1</td>
</tr>
<tr>
<td>M. peregrinum</td>
<td>3</td>
<td>75.00</td>
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</tr>
<tr>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>M. celatum</td>
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</tr>
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<td>M. scrofulaceum</td>
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</tr>
<tr>
<td>Mycobacterium spp.</td>
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<tr>
<td>Total</td>
<td>95</td>
<td>67.86</td>
<td>45</td>
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</table>

**Table II.** Number of TB patients with multiple NTM isolates

<table>
<thead>
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<th>NTM species</th>
<th>2 NTM isolates</th>
<th>3 NTM isolates</th>
<th>6 NTM isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. fortuitum</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. gordonae</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. terrae</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. xenopi</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. cheloneae</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. abscessus</td>
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<td>1</td>
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</tr>
</tbody>
</table>