**Frequency of Mycobacterium chimaera among Belgian patients, 2015**

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*Mycobacterium chimaera* arouses an increasing public health concern, as this non-tuberculous mycobacterium (NTM) has recently been associated with life-threatening cardiac infections. *M. chimaera* and *Mycobacterium intracellulare* are genetically very close but recently appeared to present different epidemiological and clinical significance. Therefore, it has become important for laboratories to use adequate techniques allowing precise species identification. To date, most commercially available laboratory assays cannot distinguish them and erroneously identify *M. chimaera* as *M. intracellulare*. We performed a re-analysis of the 149 *M. intracellulare* strains received by the Belgian National Reference Laboratory using 16S rRNA gene sequencing, representing 25% of all NTM collected in 2015. We found that *M. chimaera* represents the majority (*n*=94, 63 %) of the previous *M. intracellulare*. This study reports the large presence of *M. intracellulare/chimaera* among Belgian patients infected by an NTM and the predominance of the species *M. chimaera* among this group. This study also stresses the public health importance of *M. chimaera* and demonstrates the inability of commonly used laboratory techniques to correctly diagnose these infections.

**INTRODUCTION**

The *Mycobacterium avium* complex (MAC), originally composed of two main species (namely, *M. avium* and *Mycobacterium intracellulare*), is responsible for most non-tuberculous mycobacteria (NTM) infections in many areas (Falkinham, 1996; Prevots & Marras, 2015). *Mycobacterium chimaera*, a novel species of this complex, was first characterized in 2004 (Tortoli et al., 2004), and it later became clear that up to 86% of the strains originally identified as *M. intracellulare* had to be re-classified as *M. chimaera* (Schweickert et al., 2008). From a clinical point of view, although the treatment recommendations are similar across the MAC, this novel species has been associated with tuberculosis-like pulmonary infections (Alhanna et al., 2012; Cohen-Bacrie et al., 2011; Bills et al., 2009; Schweickert et al., 2008; Cayrou et al., 2010). Since 2011, Switzerland (Sax et al., 2015; Achermann et al., 2013), Germany, The Netherlands (Kohler et al., 2015) and the United Kingdom (Public Health England, 2015) have reported severe cardiovascular and systemic infections due to *M. chimaera* following cardiac surgery. The origin of these infections is probably linked to the spread of the bacteria in the operating theatres through ‘heater–cooler’ devices which have been contaminated during the manufacturing process (Sommerstein et al., 2016; Haller et al., 2016; European Centre for Disease Prevention and Control, 2015).

No cardiac infection caused by *M. chimaera* or other NTM has been reported in Belgium to date. Nevertheless, an important number of pulmonary infections provoked by *M. intracellulare/chimaera* are diagnosed each year. Based on this observation and the epidemiological context, we performed a re-analysis of *M. intracellulare/chimaera* strains in order to determine the relative frequency and the possible differences in regard to the clinical significance of these two species.

**METHODS**

*Reporting of NTM infections in Belgium.* Notification of NTM infections is not mandatory in Belgium. Therefore, peripheral laboratories do not systematically report or send the clinical isolates to the National Reference Laboratory (NRL). Nevertheless, the vast majority of clinical laboratories do send their strains on a voluntary basis for NTM identification and drug susceptibility testing.
Clinical data. Clinical data (site of infection, previous mycobacterial infections and ambulatory/hospitalized status) and patient information are routinely collected for all requests received by the NRL. Clinical data recorded in the context of the present study had not been collected for research purposes but as part of the routine data collection for diagnosis. Anonymity of data was ensured prior to analysis. Comparison of the sex ratio between patients infected by M. chimaera and M. intracellulare was assessed using the chi-squared test ($\chi^2$).

Identification of M. chimaera. While most commercially available techniques allow a correct differentiation between M. avium and M. intracellulare, they are generally not able to distinguish M. intracellulare and M. chimaera, as these species are genetically very close. Until recently, the NRL used the GenoType CM line probe assay (LPA) (Hain Lifescience) and an in-house PCR as a first-line approach for the identification of NTM.

For the purpose of this study, all strains identified as M. intracellulare by the LPA or the in-house PCR assays were re-tested by 16S rRNA gene sequencing (Kirschner et al., 1993), a method allowing distinction between both species based on one-nucleotide discrimination (Tortoli et al., 2004). Briefly, PCR products were analysed by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide and UV illumination. The remaining PCR products were purified using a QIAquick 96 PCR Purification kit (Qiagen) and sequenced with primer 285F (Kirschner et al., 1993). Sequencing was performed by using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and analysed on an ABI 3130xl Sequencer (Applied Biosystems). The obtained sequences were submitted to BLAST (Basic Local Alignment Search Tool) analysis against the National Center for Biotechnology Information and Ribosomal Database Project databases. As there is only one nucleotide mismatch, at nucleotide position 403, between the 16S rDNA sequences of M. intracellulare and M. chimaera, the BLAST analysis was specifically inspected in this region using the sequence of M. tuberculosis H37Rv as reference. Quality of the obtained sequence/electropherogram was checked during the analysis step. The Belgian NRL is accredited under the international standard ISO 15189 to perform 16S rRNA gene sequencing.

RESULTS

Frequency and distribution of NTM

In 2015, a total of 896 NTM strains, originating from 774 different patients, were characterized at the Belgian NRL. Among the 595 clinically relevant species, 158 (26.5%) were identified as M. avium, and 149 (25%) were identified as M. intracellulare/chimaera.

Differentiation between M. intracellulare and M. chimaera

All strains primarily identified as M. intracellulare were re-identified using 16S rRNA gene sequencing. This identified 94 strains of M. chimaera (63%), while it appeared that only 55 (36.9%) strains had been correctly identified by the lower performing LPA and in-house PCR.

Characteristics of patients infected by M. chimaera and M. intracellulare

Characteristics of the patients infected by M. intracellulare or M. chimaera are reported in Table 1. For both species, the vast majority of the strains were isolated from the respiratory samples (n=135, 97.1%). Only 3 of 88 M. chimaera strains were found from extrapulmonary specimens, and none was cardiovascular.

Mean age of patients infected by M. intracellulare or M. chimaera was similar (about 68 years). M. chimaera was more frequently isolated from women (55.4%) than from men (44.6%), while M. intracellulare infections were more common among men (53.7% vs 46.3%) (statistically not significant).

DISCUSSION

There is increasing evidence that different NTM species and subspecies cause profoundly different clinical and epidemiological presentations. The Belgian NRL performs routine identification of NTM for cultured strains received from clinical laboratories using the Genotype Mycobacterium CM commercial assay as the first-line intention and the 16S rRNA gene sequencing as a second-line test. In 2015, after re-classification of previously undifferentiated M. chimaera and M. intracellulare species, the most common NTM clinically relevant species isolated were M. avium, M. chimaera, Mycobacterium xenopi and M. intracellulare.

In Belgium, reporting of NTM infections is not mandatory and is on a voluntary basis. Therefore, the actual incidence of these infections may still be underestimated. Nevertheless, we report each year an important number of pulmonary infections provoked by M. intracellulare and M. chimaera. Our results show that M. chimaera accounts for 63% of the strains formerly reported as M. intracellulare. This observation was also described in a retrospective study performed in Germany (Schweickert et al., 2008).

Belgium has not notified to date any cardiovascular infection due to M. chimaera, but it should be noted that no active screening strategy has yet been implemented.

We classified 38.3% of the M. chimaera isolates as ‘clinically relevant’ based on IDSA/ATS classification (Griffith et al., 2007). This proportion is intermediate between the original report from Tortoli et al. (2004) where 7 of 12 patients

Table 1. Characteristics of patients infected with M. intracellulare and M. chimaera, Belgium, 2015

<table>
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<tr>
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<th>M. chimaera</th>
<th>M. intracellulare</th>
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<tbody>
<tr>
<td>No. of strains</td>
<td>94</td>
<td>55</td>
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<tr>
<td>Gender (male/female)</td>
<td>41/92 (44.6%)</td>
<td>29/54 (53.7%)</td>
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<tr>
<td>Mean age (years)</td>
<td>68</td>
<td>67</td>
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<tr>
<td>Origin of specimens</td>
<td></td>
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<tr>
<td>Pulmonary</td>
<td>85/88 (96.6%)</td>
<td>50/51 (98.0%)</td>
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<tr>
<td>Extrapulmonary</td>
<td>3/88 (3.4%)</td>
<td>1/51 (2.0%)</td>
</tr>
<tr>
<td>Clinical significance</td>
<td>38/94 (38.3%)</td>
<td>38/55 (69.1%)</td>
</tr>
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(58.3 %) were considered as infected with the bacterium and the 3.3 % clinical significance reported from Germany. The treatment recommendations for all members of the MAC are the same, and therefore, we consider that this imprecise identification did not impact the care to patients. Other authors recommend the sequencing of rpoB or hsp65 genes for the identification of mycobacteria (Andre et al., 2015; Van Ingen et al., 2012; De Zwaan et al., 2014). Although these techniques allow an easier discrimination between many mycobacterial species and subspecies, 16S rRNA gene sequencing is still a valuable technique for the differentiation between M. intracellulare and M. chimaera when the quality of the method is controlled. Commercial assays are relatively imprecise for the identification of NTM, as documented in this study and elsewhere (Ramis et al., 2015). Recently, the GenoType NTM-DR (Hain Life-science) was released and is supposedly able to differentiate the members of the MAC, including the specific detection of M. chimaera. We could not find independent scientific evidence in regard to the performance of this new commercial assay.

One of the main limitations of the present report is linked to the fact that notification of NTM infections is not mandatory in Belgium, although these pathogens have become an increasing public health concern (Cassidy et al., 2009; Winthrop et al., 2010; Hoefsloot et al., 2013). Therefore, the frequency of mycobacterial infections is probably underestimated by the current figures.

In conclusion, we report the large presence of M. intracellulare/chimaera among Belgian patients infected by an NTM and the predominance of the species M. chimaera among this group of genetically close species, usually/previously identified and reported as M. intracellulare. This study shows the importance of using a laboratory technique able to discriminate the two species. Laboratories performing NTM species identification by using some common laboratory techniques can miss the M. chimaera identification. As M. intracellulare and M. chimaera represent important public health concerns and could be associated with severe medical conditions, correct species identification is relevant to progress in the understanding of associated pathologies.

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REFERENCES


