Genotyping and strain distribution of *Mycobacterium avium* subspecies *hominissuis* isolated from humans and pigs in Belgium, 2011–2013

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Mycobacterium avium represents a health concern for both humans and pigs. The characterisation of its subspecies is an important step improving the understanding of the epidemiology and the control of this pathogen. Ninety-two human *M. avium* strains were selected for a retrospective study. Subspecies determination by *rpoB* sequencing and IS*1245*/IS*901* analysis showed that 98.9% of Belgian human *M. avium* strains belong to the subspecies *hominissuis* (MAH). Some of these MAH strains present particular IS*1245*/IS*901* profiles (absence of IS*1245* and false IS*901* detection provoked by the presence of IS*Mav6*). In addition, 54 MAH strains isolated from submandibular lymph nodes of Belgian pigs with lymphadenitis were included in this study. Genotyping of human and porcine isolates was performed using multispacer sequence typing (MST). In total, 49 different MST types were identified among pig (n = 11) and human (n = 43) MA isolates, with only five shared by both hosts. Among these MST types, 34 were newly identified. Our findings demonstrate the extensive genetic diversity among MAH isolates. Some genotypes were more prevalent in human or pigs but no correlation was observed between MST type and place of residence or the farm of origin for human and porcine isolates respectively, suggesting an environmental source of infection.
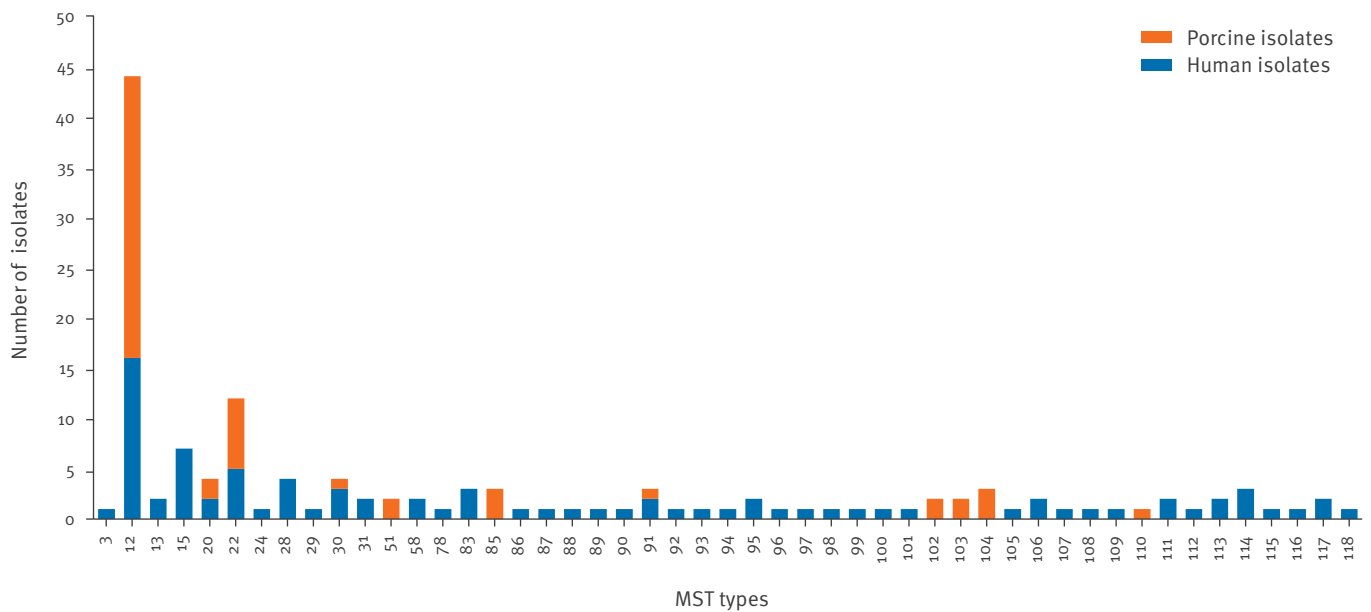
Introduction

Among non-tuberculous mycobacteria (NTM), *Mycobacterium avium* complex (MAC), composed of two major species *M. avium* and *M. intracellulare*, is the most frequently isolated from patients [1,2] and the most common NTM identified as responsible of pulmonary disease [3]. In humans, lymphadenitis due to NTM primarily affects children and is caused by a variety of species, with predominance of *M. avium* [4]. In industrialised countries, in the absence of antiretroviral therapy, MAC is also recognised as the most common opportunistic bacterial infection in patients with advanced AIDS [1,3], often with disseminated disease, rather than the typical lung involvement seen in immunocompetent patients [1].

The species *M. avium*, is divided into four subspecies; *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *silvaticum* (MAS), *M. avium* subsp. *paratuberculosis* (MAP) and *M. avium* subsp. *hominissuis* (MAH) [5-7]. Recently, a phylogenetic study showed that MAH represents diverse groups of organisms from which two distinct groups, MAP and MAA/MAS, have evolved independently [8]. These subspecies of *M. avium* are genetically close, yet differ widely in their host range and pathogenicity. MAP is responsible for an intestinal illness in ruminants known as Johne's disease [9] and could be implicated in human Crohn's disease [10]. MAA and MAS mainly infect birds causing a tuberculosis-like disease, whereas MAH is a frequent agent of human and pig mycobacterioses [6,11]. Although MAH can infect a wide variety of animals, pigs are its primary animal host species, causing granulomatous lesions mainly in the lymph nodes of the digestive tract, which can reduce the value of carcasses [12]. Moreover, an association between MAH and human lymphadenitis has been described [13]. MAH represents an increasing

Distribution of multispacer sequence typing types among porcine (n = 52) and human (n = 87) isolates, Belgium, 2011–2013

Materials and methods

Bacterial isolates and study design

A total of 146 Belgian *M. avium* isolates from human patients (n = 92) and pigs (n = 54) were characterised in this study.

public health concern given its pathogenicity for both humans and pigs, and so precise characterisation of MA subspecies and genotypes is essential for epidemiological studies, identification of the source of infection, and to establish or exclude its possible zoonotic role [14-16].

In the period from 2010 to 2013, 2,964 NTM cultures were sent for identification to the Belgian National Reference Centre for Tuberculosis and Mycobacteria (WIV-ISP), of which 1,104 belonged to the *M. avium* complex, with the following distribution between the two species: 574 (19.4% of the total) *M. avium* and 530 (17.9% of the total) *M. intracellulare*.

According to these data, *M. avium* is the predominant NTM isolated from Belgian patients. As the identification of *M. avium* isolates in Belgium is usually carried out by sequencing of the gene coding for 16SrRNA or by line probe assay (based on the analysis of the 23SrRNA gene), the distribution of the four *M. avium* subspecies remains largely unknown. In order to better characterise the *M. avium* strains circulating in Belgium, we selected clinical *M. avium* isolates from the above mentioned *M. avium* isolates and performed subspecies identification by sequencing of the *rpoB* gene and analysis of the IS*1245*/IS*901* elements. We also performed genotyping of the MAH isolates by using multispacer sequence typing (MST). As mycobacteriosis in pigs is mainly caused by MAH [16-18], we also genotyped previously identified MAH isolates from submandibular lymph nodes of Belgian pigs with lymphadenitis. The MST types of strains of human and porcine origin were compared.

One hundred clinical *M. avium* isolates were selected from the collection maintained at WIV-ISP, Belgium. The isolates came from patients who had been diagnosed with *M. avium* in the last trimester of 2011 and in 2012, and for whom drug susceptibility testing (DST) had been requested after clinical, radiological and microbiological evaluation. Isolates were considered clinically relevant based on the criteria specified by the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) statement [19]. We aimed to exclude isolates representing environmental contamination of the samples. The selection represents 63% (100/159) of all *M. avium* isolates received at WIV-ISP for identification during the study period. WIV-ISP is the only Belgian laboratory performing DST for NTM. The *M. avium* species identification, carried out during routine activities at the reference laboratory, was performed by sequencing of the gene coding for the 16SrRNA, as previously described [20]. Eight isolates were eliminated from the study because of poor conservation quality, leaving a final sample of 92 human isolates.

The pig isolates were obtained from another ongoing study analysing submandibular lymph nodes from pigs with lymphadenitis sampled after veterinary inspection by the UREAR-ULg in all Belgian slaughterhouses. Isolates within the present study were sampled in 2012 and 2013. The first 54 *M. avium* isolates obtained were

Table 1

Mycobacterium avium subspecies identification of clinical isolates using IS1245/IS901 and rpoB sequencing, Belgium, 2011–2012, (n=92)

Interpretation based on IS1245/IS901 detection			Interpretation based on rpoB sequencing	
No. of isolates	IS1245/IS901 profile	Subspecies conclusion	No. of isolates	Perfect match with rpoB sequence of
87	IS1245 + / IS901-	*M. avium* subsp. *hominissuis*	72 1 1 4 9	*M. avium* subsp. *hominissuis* *Mycobcterium* sp. 09–7368 *Mycobacterium* sp. 11–0068 (KP098592) *Mycobacterium* sp. 11–006596, 10–5763 or 09–4604 rpoB PCR amplification impossible
3	IS1245 + / IS901 +	*M. avium* subsp. *avium* / *silvaticum*	1 2	*Mycobacterium avium* subsp. *avium* *Mycobacterium avium* subsp. *hominissuis*
2	IS1245 - / IS901 +	Not *M. avium*	2	*M. avium* subsp. *hominissuis* strain TH135

IS: insertion sequence; *M. avium: Mycobacterium avium*; subsp.: subspecies; - : absence of the fragment; + : presence of the fragment.

included. They were isolated by the Belgian veterinary centre CODA-CERVA which supplied them for the study. *rpoB* gene sequencing was used to confirm the subspecies identification that had been made by the CODA-CERVA using the IS1245/IS901 detection method. MST genotyping of these porcine isolates was performed as described above.

Clinical data and patient information

All clinical data recorded in the context of the present study had been collected not for research purposes but as part of the routine data collection for diagnosis. We collected the following data: origin of specimens (site of infection), date of collection, sex and age of patients and their city of residence (or postal code). Anonymity of data was ensured before analysis. The study was approved by the ethical committee of Hôpital Erasme (ULB) (reference P2014/028).

IS901/IS1245 detection

M. avium species identification of the selected strains was confirmed by PCR detection of the insertion sequence (IS) 1245 (present in *M. avium* strains), and the *M. avium* subspecies determined by analysing the presence/absence of IS901. Avian MAA and MAS strains contain IS901, whereas mammalian MAH and MAP strains lack it. The following primers were used for the analysis of IS1245 and IS901 respectively: IS1245F (5' AGGTGGCGTCGAGGAAGAC 3') and IS1245R (5' GCCGCCGAAACGATCTAC 3'), IS901F (5' GCAACGGTTGTTGCTTGAAAGGAAT 3') and IS901R (5' GCGCACGCATGATGAGTGGACTTAC 3'). Both PCR assays were performed as previously described [21,22]. PCR products were analysed by electrophoresis on a 2% agarose gel and visualised by staining with ethidium bromide and UV illumination. Sequencing of some IS901 amplicons was performed using the primers used for the PCR amplification.

Gene sequencing

Subspecies identification among *M. avium* isolates was also performed by sequencing of the *rpoB* gene as described by Ben Salah et al. [23]. Briefly, the primers Myco-F (5' GGCAAGGTCACCCCGAAGGG 3') and Myco-R (5' AGCGGCTGCTGGGTGATCATC 3') were used to amplify a variable region of the *rpoB* gene [23]. PCR products (10 μl) were visualised on a 2% agarose gel after staining with ethidium bromide. The remaining PCR products were purified using a QIAquick 96 PCR Purification Kit (Qiagen) and sequenced with primers Myco-F and Mycoseq-R (5' GGACATCATCCTGAACACCCACG 3'). Sequencing was performed by using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analysed on an ABI 3130xl Sequencer (Applied Biosystems). The obtained forward and reverse sequences were re-assembled using the bioinformatics software Lasergene SeqMan Pro and used for sequence comparisons by BLAST with the NCBI database (National Centre for Biotechnology Information).

For a few strains, the subspecies identification was also confirmed by sequencing of the *hsp65* gene using the classification proposed by Turenne et al. [24] and the method described by Dirac et al. [25].

Multispacer sequence typing

Genotyping of the *M. avium* isolates was performed by MST. The PCR amplification of the spacers MST2, MST4, MST15 and MST16 was performed as described by Cayrou et al. [26]. Amplicon purification and sequencing were performed as described above, with the same forward and reverse primers as used for the different MST PCRs.

The MST type was determined by consulting the MST database [27].

Statistical analysis

A 95% confidence interval (CI) of prevalence of clinical *M. avium* isolates according to the affected organ was estimated using an exact binomial distribution. The comparison of age between lymphadenitis and non-lymphadenitis patients was assessed using a two-sample t-test with unequal variances, whereas comparison of the sex ratio was assessed using the chi-squared test (Chi 2), and comparison of province of origin by the Fisher exact test [28]. The number of porcine *M. avium* strains according to the MST type and farm was assessed using a Poisson regression. A p value of 0.05 was considered as significant and all analyses were performed in Stata (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

Results

Clinical isolates

The subspecies of *M. avium* isolates is not determined routinely in Belgium. In order to better characterise these *M. avium* isolates, a retrospective study was carried out (n = 92).

Information on the biological origin of the isolate was available for 71 patients. The vast majority of these were isolated from the respiratory tract (n = 51; 95% CI: 59.9–81.9) followed by lymph nodes (n = 13; 95% CI: 10.1–29.3), abscesses (n = 4; 95% CI: 1.6–13.8), blood (n = 2; 95% CI: 0.3–9.8) and peritoneal fluid (n = 1; 95% CI: 0.04–7.6).

The mean age of the selected patients was 58 years (data available for 90 of 92 patients). However, a significant difference in age was observed between lymphadenitis (n = 13; mean = 15.3 years, standard deviation (SD) = 22.8 years) and non-lymphadenitis patients (n = 77; mean = 65.1 years, SD = 18.7 years) (p < 0.0001). The overall sex ratio was 1.04 (47 males and 45 females) but no significant difference was observed between patients with or without lymphadenitis (p value = 0.70). In addition, lymphadenitis and non-lymphadenitis patients did not differ in geographic origin (p value = 0.125).

Subspecies identification

Based on presence of IS*1245* and absence of IS*901* determined by PCR analysis, 87 isolates among the 92 analysed were identified as MAH, and three as MAA/MAS (Table 1). For the two remaining strains, conflicting results were observed between the IS*1245* PCR (absence of the fragment usually present in *M. avium* strains) and sequencing of the 16sRNA used for the selection of the *M. avium* strains included in the study.

Subspecies identification based on the *rpoB* sequence confirmed MAH subspecies for 72 of 87 isolates (Table 1). For the 15 remaining strains, nine were negative in *rpoB* PCR amplification (repeated three times), five strains were identified as *M. species* (M. sp. 11–006596

(JF804804), M. sp. 10–5763 (JF327744) or M. sp. 09–4604 (JF327745) with same blast score, and M. sp. 09–7368 (JF437546)), and one had no perfect match with the *rpoB* sequences currently recorded. The four *M. species* were isolated from a macaque, a pig, a cow and a steer, and characterised as close to *M. avium* subspecies in a study evaluating the usefulness of *rpoB* sequencing for identification of *Mycobacterium* isolates of veterinary origin [29]. The new *rpoB* sequence discovered in this study was deposited in the GenBank database under accession number KP098592. This strain was isolated from a lymph node of a 64-year-old patient with lymphadenitis.

Of the three strains identified as MAA/MAS based on the presence of both IS*1245* and IS*901*, one was confirmed by *rpoB* analysis as MAA, and the other two were identified as MAH. We therefore sequenced the IS*901* amplicons obtained for these three strains. Blast analysis of 173 nt sequenced confirmed the presence of the IS*901* in the isolate identified as MAA but revealed that one of the two MAH actually had the insertion sequence IS*Mav6*, presenting 95% similarity with IS*901* [30].

Finally, for the two strains presenting negative results for the IS*1245* amplification, the *rpoB* sequence was identical to the MAH strain TH135 of the NCBI bank. *hsp65* sequencing yielded codes 1 and 2 according to the classification of Turenne et al. [24]. These *hsp65* codes have been described in MAH strains. It should be noted that these strains were also positive for the IS*901* element, which is unusual for MAH strains. Sequencing of IS*901* revealed the presence of insertion sequence IS*Mav6* instead of the standard IS*901*.

Molecular typing

The 92 human *M. avium* isolates included in this study were genotyped by MST in order to establish their genetic diversity (unrelated clinical isolates) or relatedness (clustered clinical isolates). MST types were interpretable for 87 strains. The distribution of the obtained genotypes is presented in the Figure. Overall, 43 different MST types were identified. Some genotypes were more prevalent such as MST type 12 found in 16 isolates, MST15 in seven isolates, MST22 in five isolates and MST28 in four isolates. Among the human isolates, twenty-nine MST types (86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 105, 106, 107, 108, 109, 111, 112, 113, 114, 115, 116, 117, 118) were identified for the first time and recorded in the MST database developed by the Université de la Méditerranée. The MAA strain belonged to MST type 3, which was previously described as specific for MAA [26]. No association was observed between the MST types and sites of infection. Moreover, analysis of the geographical repartition of the patients revealed no correlation between the MST types and the patient's places/cities of residence.

Repartition of the porcine *Mycobacterium avium* subspecies hominissuis isolates by multispacer sequencing type and farm, Belgium, 2012–2013

Farm	No. of porcine isolates							
	MST 12	MST 51	MST20	MST 103	MST 22	MST 104	MST 102	MST 51
1	6			1				
2	2	1	1					
3	1							
4	2							
5	1							
6	6							
7	10							
8		1						
9		1						
10			1					
11				1				
12					1			
13					6			
14						1		
15						1	1	
16						1	1	
17								1
18								1
Total 18	28	3	2	2	7	3	2	2

MST: multispacer sequencing type.
This table includes only MST types found in two or more isolates/pigs.

Porcine isolates

Within the ongoing Belgian project begun in 2012, the inspection of 125,000 randomly selected pig carcasses was performed by the UREAR-ULg. All submandibular lymph nodes from pigs with lymphadenitis were sampled and transmitted to CODA-CERVA. In the laboratory, a smear of all lymph nodes were stained by the Ziehl-Neelsen method and observed microscopically. The samples with acid-fast resistant stain, as well as a randomised selection of 266 samples selected to be able to detect an expected prevalence of gross lesions of 0.5% with an acceptable error of 0.06% and a confidence interval of 95%, were cultivated on Coletsos (Biorad) and Löwenstein-Jensen (Biomérieux) media. The first 54 porcine MAH isolates were transmitted to the WIV-ISP, where 52 could be MST-typed, yielding 11 different MST types, of which five were also detected in human isolates. The most frequent porcine genotypes, MST type 12 (28 isolates) and MST type 22 (seven isolates), were also among the most frequently detected in humans. On the other hand, the second most prevalent human type (MST 15) was not detected in pigs. As for the clinical isolates, new MST types (n = 6) were identified (85, 91, 102, 103, 104, 110) and recorded in the MST database. The Figure shows the distribution of the MST types among porcine and human strains.

Analysis at fattening farm level (18 farms) was performed for all pigs infected with a MAH strain belonging to a MST type for which at least two strains were detected in this study. This analysis showed in all cases (six different MST types), that strains with the same MST type could be isolated from pigs originating from at least two different Belgian farms (Table 2). For example, the 28 porcine MST12 strains were isolated from pigs fattened at seven different farms. Farms of origin (farms of birth) supplying piglets to the fattening farms were also different in all cases. Moreover, MAH strains with different MST types were found among pigs originating from the same fattening farm. In addition, the number of different MAH strains isolated in a farm was not related to the MST type or the farm origin (Poisson regression; p value > 0.12).

Discussion

MAC strains are the most frequently isolated NTM, and mycobacterial infections caused by these MAC strains are on the rise in both animals and humans. According to data from Belgium's National Reference Laboratory, *M. avium* currently represents about 20% (167/857) of all NTM isolated from patients. In 2000, this percentage was only 12% (63/506 NTM analysed) suggesting an increasing number of *M. avium* infections.

Although a true relative increase in *M. avium* infections is suspected, this observation could also be partially

attributed to the increased awareness of NTM disease in recent years and the increased availability and sensitivity of laboratory techniques [3]. Indeed, the development of rapid liquid culture methods and molecular tests, such as species-specific PCR or reverse hybridisation assay, has improved and facilitated the detection and identification of NTM infections [3].

The present study was undertaken to characterise *M. avium* isolated from patients living in Belgium. First, the subspecies of a collection of clinical *M. avium* isolates was determined by detection of IS*1245* and IS*901*, which is the most widely used technique for the (sub-)species identification of MAC. By using the strict IS*1245*/IS*901* interpretation model, the majority (87/92, 94.6%) of human isolates were identified as MAH. When applying multiple identification strategies, however, conflicting data were obtained. Three of the five remaining isolates were detected as positive for IS*901* and IS*1245*, which is the usual profile for MAA, but the MAA subspecies identification was only confirmed by *rpoB* sequencing for one of these three isolates, whereas the other two yielded a typical MAH *rpoB* profile. Moreover, the two other remaining isolates, showing positive results for IS*901* but absence of amplicon for the IS*1245* (usually used as a marker for *M. avium* strains) gave the identification MAH by *hsp65* sequencing and MAH strain TH135 by *rpoB* sequencing.

Strain TH135 was isolated in Japan from a HIV-negative patient with pulmonary disease. Genome analysis of this strain, carried out by Uchiya et al., revealed the presence of a new [31] insertion sequence, designated IS*Mav6*, which presents 95% sequence identity with the subspecies marker IS*901* [30]. PCR amplification of IS*Mav6* with primers designed for IS*901* was previously reported, explaining the positive IS*901* PCR results obtained for our two TH135 strains [32]. Moreover, *M. avium* isolates lacking IS*1245* were also reported [29,31,32]. The existence of *M. avium* strains lacking IS*1245* and MAH strains harbouring an IS homologous to IS*901* complicates the interpretation of IS*1245*/*901* results. In this study, we show that the presence of strains with this particular IS profile appears to be quite limited in Belgium with 2.2% (2/92) of *M. avium* strains lacking IS*1245* and 4.4% (4/91) of MAH strains giving false IS*901* PCR amplification. A more problematic situation was described in Japan with 16% and 67%, respectively [32].

Taking into account the MAH identification by *rpoB* sequencing for the four isolates presenting an unusual (but already described) IS*1245*/IS*901* profile, 91 (98.9%) of the 92 *M. avium* analysed isolates belong to the *hominissuis* subspecies, leaving only one isolate being identified as MAA. Contrary, subspecies identification based on *rpoB* sequencing alone, would result in a lower percentage of MAH (82.6%; 76/92). The differences between the two techniques are caused by *rpoB* amplification problems [9] and isolates that are identified as *M. species* by *rpoB* sequencing [6].

Regarding to the sampling design, different time periods in human (last trimester of 2011 and 2012) and pig (2012–2013) studies were applied for logistic reasons: availability of historical human samples and the opportunity of the ongoing pig study. These non-overlapping time frames may have caused a bias in inter-species strain diversity. To address this issue, human isolates from 2012 to 2013 should be analysed and compared with the current pig set.

Regardless of the technique used, we can conclude that the vast majority of *M. avium* strains isolated in Belgium belong to the subspecies *hominissuis* as described in other countries [13,32]. Our analysis also shows that application of either the IS*1245*/IS*901* or *rpoB* based strategy resulted in the determination of most *M. avium* subspecies, but it was the combination of the two techniques that allowed us to firmly conclude the species and/or subspecies identification for nineteen (20.6%) cases.

Concerning the MAH genotypes, 48 different MST types were identified among pig and human MAH isolates, comprising five types shared by both hosts. Moreover, among these MST types, 34 were newly identified within this study (28 in human, five in porcine and one in both hosts) showing that the genotypes of the Belgian MAH strains are quite different from those isolated in other countries and previously recorded in the MST database. This clearly demonstrates the large genetic diversity of MAH isolates.

There was no correlation between the place of residence of the patients and the MST types of the MAH strains. Indeed, strains with same MST types were isolated from patients living in dispersed places in the country, and for patients living in the same city often various MST types were identified. Also for the porcine strains there was no correlation of MST type with the geographical localisation of the farms. Indeed, analysis of the MST types compared with the pig farms showed that MAH strains with identical MST types could be isolated from pigs originating from different fattening farms, while pigs on the same farms can be infected by MAH strains of various MST types. This genetic diversity of the MAH strains has also been described in other epidemiological studies (using other genotyping tools like IS*1245*-RFLP or MIRU-VNTR), suggesting an environmental source of infection rather than direct transmission between the hosts [14,16,33]. Alternatively, transmission from pigs to humans by consumption of meat could be considered. Indeed, in this situation, no correlation between pig farms and the patients' place of residence is needed. Although this route of transmission cannot be excluded, there is as yet no clear evidence of meat-borne transmission of *M. avium* to humans [34].

While considerable attempts were made to include only *M. avium* human isolates responsible for disease (and

to avoid environmental contamination of the specimens) by selecting isolates of patients for whom DST had been requested by a physician, we consider it likely that the selection of pathogenic isolates studied may be underrepresented as some physicians probably treat their patients after species identification without asking the laboratory to perform DST. Another limitation of our study could be the small sample size, particularly due to the large genetic diversity detected among the MA strains. For instance, our data suggest that some MST types are only detected in *M. avium* isolated from humans or pigs. Analysis of a larger sample is required to formally test this hypothesis.

In conclusion, we report on the predominance of MAH among Belgian patients infected by a *M. avium* strain, and characterisation of Belgian MAH porcine isolates. Genotyping revealed a large genetic diversity of strains (whatever their origin; human or porcine) and the absence of a link between genotypes and the place of residence (human) or the farm of origin (pigs), suggesting an environmental source of infection. Some genotypes are however more frequent in human or in pigs, and some are only found in one of these two hosts. Moreover, the IS*Mav6* element was detected in some human isolates as described in another study [15]. As MAH represents an increasing public health concern, these host-strain particularities deserve more investigations.

Conflict of interest

None declared.

Authors' contributions

VM, KH, DF and CS conceived and designed the experiments; CV and KS performed the experiments; CV, VM and CS analysed the data; KS, LD, JD, FS, DF, CS contributed reagents/materials/analysis tools; CV, LD, MFD, NB, KH, DF, LR, VM and CS wrote the paper.

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