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Full Length Article

Genotyping of *Mycobacterium avium* field isolates based on repetitive elements

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Abstract The economic and zoonotic importance of infections caused by *Mycobacterium avium* complex (MAC) strains in human, animals and birds are increasing. At present, few data are available about the genetic diversity of field isolates of *M. avium* subsp. *hominissuis* (MAH) and subspecies *avium* (MAA). The close relationship between human and swine isolates indicates a possible zoonotic role for such strains. In the present work 73 *M. avium* field strains isolated from feces and lymph nodes of diseased/slaughtered animals in Hesse State, Germany were investigated. Forty eight primers were used for the confirmation, differentiation and finally the genotyping of the isolates based on the presence of polymorphism of different repetitive loci. These include the Large Sequence Polymorphism (LSP), the Mycobacterial Interspersed Repetitive Units (MIRU) and Variable Number Tandem Repeats (VNTR). The genotyping of MAA ($n = 27$) and MAH ($n = 16$) isolates revealed 33 different genotypes (18 MAA, 14 MAH and 1 shared profile). The described methods show great potential for epidemiological mapping of *M. avium* subspecies.

1. Introduction

The members of *Mycobacterium avium* complex (MAC), are a heterogeneous group of slowly-growing mycobacteria [1]. Two genetically distinct species within MAC are characterized, *M. avium* and *Mycobacterium intracellulare*. The *M. avium* was originally separated into three subspecies; *M. avium* subsp. *avium* (MAA); *M. avium* subsp. *paratuberculosis* (MAP) and *M. avium* subsp. *silvaticum* [2]. The importance of infections caused by MAC in both animals and humans could be extracted from its ability to induce economic loses and public

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health problems [3]. The close relationship between human and swine isolates indicates that, either pigs may be an important vehicle for *M. avium* infections in humans or both pigs and humans share common sources of infection [4]. However, the presence of *M. avium* in water and soil and its transmission by insects and rodents may be the source for further spread among animals and their keepers [5–8].

Previous researchers have presented that all strains isolated from humans or pigs were avirulent for poultry even *IS901* positive strains. It was suggested that the passage of bird strains in humans leads to a loss of their virulence for poultry due to the loss of the mobile genetic elements responsible for such virulence [9]. For clarity in *M. avium*-related disease, Mijs et al. [10] grouped the isolates from humans and pigs together as a fourth subspecies called *M. avium* subsp. *hominissuis* (MAH). This classification was confirmed by the sequencing of 16S rRNA and internal transcribed spacer 16S–23S ribosomal DNA. Similar results were also obtained by the use of restriction fragment length polymorphisms (RFLP) [9,11–15]. Later on, the restriction fragment length polymorphism of *IS901*, *IS1245* and *IS1311* was used to detect the genetic diversity among different *M. avium* strains [16,17]. The presence or absence of *IS901* was used also as another molecular approach for the characterization of *M. avium* strains. It was detected in 97.8% of strains from birds, in 74.1% from strains of animal origin and in 38% from the pig isolates. About 97.8% from those 38% were virulent for poultry. On the other hand, only 3.4% from the *IS901* negative strains isolated from pigs were virulent for poultry [9].

In the last decade, the prevalence of MAH in slaughtered pigs increased dramatically from both diseased [18] and healthy pigs [18,19]. Therefore, it is believed that the real threat for swine industry is clearly underestimated which may lead to serious economic and public health problems [20]. Although Genotyping of *M. hominissuis* was carried out by different teams [17,20], the correlation between the genotypes and their capability of disease induction still debated. While some reports state that there is no association between the *MAH* genotype and disease induction [20] and that human and pigs share the same genotypes [4,21], others reported that animal strains differ from human isolates [17,22]. However, the difference in immune response according to the type of the invading MAH genotype is reported [23,24].

The present study aims to investigate the strain diversity among *M. avium* field isolates using Mycobacterial Interspersed Repetitive Units (MIRU) and Variable Number Tandem Repeats (VNTR) which were applied for the genotyping of MAP strains [25,26] and the Large Sequence Polymorphism (LSP) described by Semret et al. [27]. The aim of the present study is (1) to test the possibility of applying the previously described MAP genotyping methods for the closely related MAH and MAA, and (2) to provide useful epidemiological information about MAH and MAA distribution and their genotypes in the field.

2. Materials and methods

2.1. Bacterial isolates

Seventy three *M. avium* field strains were genotyped in the present study. The strains were isolated from feces of 34 diseased cattle and lymph nodes of 39 slaughtered pigs during the routine examination of slaughtered pigs in Hesse State. The strains were isolated in Landesbetrieb Hessisches Landeslabor, Giessen, Germany where they were identified by different PCR; namely: IS*900*, IS*901*, DT1, IS*1311*, IS*1245*, 16S rRNA, *f57*, IS MAv-2, LSP^A8 and LSP^A17 specific PCRs to differentiate among the subspecies MAP, MAH and MAA. In addition to the field isolates, another four MAA reference strains (ATCC15769, ATCC 25291, ATCC 35712 and ATCC 19075) were also involved in the present study. For PCR control, the MAH control positive strain ATCC 700898 was used. The reference strains were obtained from the strain collection of the LHL.

For bacteriological culture, three tubes of HEYM agar (Harold's Egg Yolk Medium contain Mycobactin J and ANV, Becton Dickinson, Heidelberg, Germany) were used for each sample and were then incubated at 37 °C for 16 weeks, after which bacterial growth was tested once weekly. After 4 weeks of the incubation, those bacterial colonies apparently appeared like the mycobacterium colonies (diameter 1–2 mm, entire and white color) were stained with Ziehl–Neelsen (ZN) according to the manufacturer's instruction (Merck, Darmstadt, Germany). The colonies were examined microscopically after being stained with acid fast ZN stain.

A total of 73 growing isolates illustrated ZN positive, a single colony was subcultured on a new HEYM agar tubes and incubated at 37 °C for 4–12 weeks for PCR identification.

2.2. Preparation of genomic DNA

The isolates were processed for DNA extraction by suspending 3–5 colonies in 180 µl TE lysis buffer (lysozome 20 mg/1 ml Roche Diagnostics, Mannheim, Germany in 20 mmol/l Tris–HCl, 2 mmol/l EDTA, Triton 100 1.2% pH 8.0) incubated for 1 h at 37 °C. Subsequently, 35 µl of proteinase K (Qiagen, Hilden, Germany) and 200 µl AL lysis buffer (Qiagen) was added, vortex shortly and incubated 2h at 56 °C. The suspension was heated for 10 min at 100 °C and then cooled to 4 °C. From the last mixture, the DNA was extracted using DNeasy® Tissue Kit according to the manufacturer's instruction (Qiagen).

2.3. Confirmation and differentiation of the isolates

For molecular characterization of the isolates, 48 different PCR primers (WMG, Germany) were used. The isolates were examined by different species specific PCRs. Through the use of the multiplex of Shin et al. [28], amplification of gene fragments of the 16S rRNA, *f57*, IS MAv-2, *IS900* and *IS901* the MAP isolates could be separated from the other two closely related MAH and MAA. All non-MAP strains were then subjected to LSP^A8 and LSP^A17 specific PCRs to differentiate between MAH and MAA colonies.

2.4. Genotyping of the bacterial isolates using MIRU and VNTR analysis techniques

The isolates were amplified using 15 different primer pairs. The PCR mixture was performed in final volume 30 µl containing primers, dNTP-mix, GeneAmp Gold Buffer, $MgCl_2$ and AmpliTaq Gold® polymerase as described in SSR analysis methods

as well as 1.5 µl dimethylsulfoxide (DMSO) (Roche) and 18.4 µl sterile distilled aqua. As a template 2.5 µl DNA was added finally to PCR mixture. The PCR amplicons (10 µl) were separated in 2% agarose gel using a 100 bp DNA ladder marker and the number of repeats was calculated from the size of the amplicon.

For both identification and genotyping of the isolates, different PCR reactions were required. The applied reactions included the amplification of the following DNA fragments: IS*900,* IS*901,* DT1, IS1311, and 16S rRNA (according to Shin et al. [28]), IS1245 [29], LSPA 8 and LSPA 17 [27], MIRU2, MIRU3 and MIRU4 [25], MIRU5, MIRU6, MIRU7 and VNTR8 [30], VNTR1658 [26], VNTR3, VNTRX3, VNTR7, VNTR25, VNTR47 and VNTR292 [31] and finally the RDI130 [32].

2.5. Statistic

Simpson's index and Simpson's index of diversity were used to detect the genetic diversity among the investigated strains. Simpson's index measures the probability that two isolates randomly selected belong to the same subgroup.

3. Results

In the present work, 73 *M. avium* strains were isolated from bovine feces ($n = 34$) and swine Lymph nodes ($n = 39$). The identity of the strains was confirmed through microscopic examination and by the application of species specific PCRs described previously. The 73 isolates could be subdivided into MAP ($n = 34$), MAH ($n = 16$), and MAA field strains ($n = 23$). These strains, in addition to 4 MAA reference strains were genotyped (Tables 1 and 2) using different set of subspecies specific primers. For MAH and MAA genotyping, amplicons obtained by the use of *30* different primers were used. The genotyping of the isolates revealed 33 different genetic profiles (18 MAA, 14 MAH and 1 shared profile). The described methods show great potential for epidemiological mapping of *M. avium* subspecies (Table 2).

For MAH, with the exception of primer pair MIRU4, VNTR3 and VNTR7, all other primer pairs revealed size polymorphism. Meanwhile, the VNTR3 and VNTR7 were also, in addition to VNTR292, unable to subdivide the examined MAA isolates. The Simpson's index of diversity (*D*) ranged from 0.12 in RD 130 and 1 in VNTR3 and VNTR7. The detailed band sizes and Simpson's index are listed in Tables 3–5.

4. Discussion

Due to the increasing economic and zoonotic importance of infections caused by *M. avium* complex (MAC) strains worldwide, it was necessary to investigate the ability of different genotypes to induce infections and whether one or more subtypes dominate in the field. To achieve this goal, the present work was designed to investigate the application of the previously described MAP genotyping primers for the closely related MAH and MAA, and to compare the discriminative value of these methods in both subspecies.

Here, 73 different *M. avium* field strains were collected from cattle and swine in Germany. The identity of the isolates was confirmed by PCRs. All strains isolated from swine were identified and confirmed to be MAH as the pigs are susceptible for infections caused by both MAH and MAA [33]. The genotyping of MAA ($n = 27$) and MAH ($n = 16$) isolates revealed 33 different genotypes (18 MAA, 14 MAH and 1 shared profile). The described methods show great potential for epidemiological mapping of *M. avium* subspecies.

Out of these 33 groups, one cluster is represented by 4 members, one cluster with 3 members, five clusters with 2 members, while the remaining isolates could not be grouped together into clusters. Among all MAH strains, only two MAH isolates cluster together, showing higher genomic flexibility in MAH in comparison to MAA. In one cluster only mixed members of MAH and MAA could be grouped together. Although the presence of high genomic diversity among MAH field isolates agrees with Despierres et al. [20], it controverts other published data referring to the presence of epidemic isolates which dominate in the field [34]. In agreement with our results, the use of MIRU–VNTR typing is an efficient method for the genotyping in MAH but less efficient for the genotyping of the closely related MAA isolates [35].

When comparing the obtained results with the results obtained by the genotyping of MAP strains isolated from the same geographic area (data submitted for publication), we find that although the used primers are originally designed to genotype MAP strains, yet they showed more differentiation power when applied to MAH and MAA strains. This can be also attributed to the higher conservation of MAP genome when compared to MAA and MAH or can be also an indicator for the earlier evolution of MAH than MAP strains. This opinion is supported by Turenne et al. [36] who achieved similar results by the use of Multilocus Sequence Analysis of MAH isolates. The division of the filed isolates into a large number of subgroups, although all strains were isolated from a limit

Table 1 The subdivisions of the 73 *Mycobacterium avium* field strains based on the different PCR systems.

Subspecies	Number	Sample	f57	*ISMav*-2	*LSP*^{A}*8*	*LSP*^{A}*17*
MAP	34 isolates	Feces – cattle	+	+	–	+
MAH	16	L.N. – swine	–	–	+	–
MAA	27[a]	L.N. – swine	–	–	+	+
Subspecies	DT1 (300 bp)	IS900 (400 bp)	16SrRNA (500 bp)	*IS1311* (600 bp)	*IS901* (750 bp)	*IS1245* (430 bp)
MAP	–	+	+	+	–	–
MAH	–	–	+	+	–	+
MAA	+	–	+	+	+	+

[a] Including four MAA reference strains.

Table 2 The results obtained by the application of LSP to the 43 none-MAP investigated strains.

	Presence of LSP^{A}8 (222 bp)		Presence of LSP^{A}17 (202 bp)		Absence of LSP^{A}17 (398 bp)	
	Non-MAP		*M. avium hominissuis*		*M. avium avium*	
	Number of strains	Number of profiles	Number of strains	Number of profiles	Number of strains	Number of profiles
Reference Strain 1	4	1	–	–	4	4
Field strains	39	1	16	15[a]	27	15[a]

[a] One profile is shared in both groups. The total number of detected profiles is 33 profiles.

Table 3 List of the 33 different genotypic profiles detected among the studied strains of MAA (n = 27; 18 profiles) and MAH (n = 16; 14 profiles) in addition to 1 shared profile.

No. of profiles	No. of isolates present per profile	Identify of the isolates
13	1	MAA
3	2	MAA
1	3	MAA
1	4	MAA
13	1	MAH
1	2	MAH
1	2	MAA + MAH

geographic area and one host, and the absence of one or few dominant genotypes as was the case with the closely related MAP may also indicate the absence of correlation between the genotype and the capability of disease induction in opposite to the conclusion of Eisenberg et al. [34]. However, it was proven that the immune system responds in a different manner according to the invading genotype [23,24].

Genotyping of MAH/MAA isolates was performed by many authors previously using both classical methods as RFLP [37] and the MIRU–VNTR-Short Sequence Repeat (SSR) dependable genotyping [35,38]. The obtained results were not satisfactory although a great success of SSR-depending methods was recorded when used for the genotyping of MAP strains [38–40]. On the other hand, and in agreement with our data, satisfactory results could be achieved by Romano et al. [30]. The team used MIRU–VNTR for the genotyping of different Brazilian MAC isolates including some MAH strains of human origin mainly. Others could successfully genotype MAH strains depending on sequence level variation in conserved housekeeping genes or other repetitive elements

Table 4 Different genotypes yield by the application of different genotyping PCRs. The dominant amplicon sizes are written in bold.

MIRU2 (bp*)	MAH (n**)	MAA (n)	MIRU3 (bp)	MAH (n)	MAA (n)	MIRU4 (bp)	MAH (n)	MAA (n)
140	2	0	0	5	8	200	0	1
150	2	0	295	**7**	**12**	370	**16**	**26**
240	**11**	**19**	400	4	7			
250	0	8						
300	1	0						
MIRU5	MAH (n)	MAA (n)	MIRU6	MAH (n)	MAA (n)	MIRU7	MAH (n)	MAA (n)
125	3	1	200	6	6	160	**11**	**24**
180	1	0	250	1	1	210	1	0
240	**12**	**26**	310	**9**	**20**	270	4	3
VNTR8	MAH (n)	MAA (n)	VNTR1658	MAH (n)	MAA (n)	VNTR3	MAH (n)	MAA (n)
0	3	0	310	2	1	180	**16**	**27**
440	1	3	320	**7**	**18**			
510	0	2	420	6	7			
620	**9**	**19**	480	1	1			
820	3	3						
VNTRX3	MAH (n)	MAA (n)	VNTR7	MAH (n)	MAA (n)	VNTR25	MAH (n)	MAA (n)
200	**10**	**16**	190	**16**	**27**	280	1	6
250	0	1				295	**11**	**21**
300	4	10				355	4	0
380	2	0						
VNTR47	MAH (n)	MAA (n)	VNTR292	MAH (n)	MAA (n)	RD 130	MAH (n)	MAA (n)
180	**13**	**15**	140	4	0	0	**15**	**24**
210	3	12	240	**12**	**27**	180	0	3
						450	1	0

(bp*) = the different amplicon sizes obtained in this PCR reaction, while (n**) refers to the number of strains giving the same amplicon size.

Table 5 Comparison of application efficiency of the same primers for the genotyping of *MAH*/*MAA* and *MAP* field strains isolated from the same geographical district based on Simpson index of diversity. The value ranges between 0 and 1, where 1 represents infinite diversity and 0, no diversity.

	MAH[a]	*MAA*[a]	*MAP*[b]
MIRU2	0.52	0.43	0.59
MIRU3	**0.7**	**0.67**	**0.72**
MIRU4	No polymorphism	0.07	No polymorphism
MIRU5	0.42	0.07	No polymorphism
MIRU6	0.57	0.58	No polymorphism
MIRU7	0.5	0.22	No polymorphism
VNTR3	No polymorphism	No polymorphism	0.14
VNTR7	No polymorphism	No polymorphism	0.51
VNTR8	0.65	0.49	No polymorphism
VNTR25	0.5	0.36	0.36
VNTR47	0.32	0.51	0.37
VNTR292	0.6	No polymorphism	0.66
VNTR1658	**0.7**	0.51	0.37
VNTRX3	0.56	0.52	0.34
RD 130	0.12	0.21	No polymorphism

[a] Results obtained from the present work [Elsayed et al., 2013 : current study].
[b] Results obtained from previous works [39,40].

[35,36]. Through the use of Multilocus sequence analysis (MLSA), the greatest variability among MAC group was observed within the *M. avium* subsp. *hominissuis* and could divide MAH into two distinct groups and showed promising results. Recently in 2012, *M. hominissuis* genotyping was carried out by Despierres et al. [20] who could detect 15 genotypes in 29 non-lymphadenitis isolates and 11 genotypes in 24 lymphadenitis isolates (human origin isolates). Tirkkonen et al. [4] compared the use of both *IS1245* RFLP pattern and MIRU–VNTR typing of MAH. Both methods were efficient for this purpose; however, MIRU–VNTR typing has the advantage of being much easier, reproducible, and non-subjective.

The use of MIRU3 and VNTR1658 revealed the best differentiation power according to the Simpsons index of diversity when used in MAH /MAA. While VNTR1658 was also superior in the genotyping of MAP isolates from the same locations, the MIRU3 did not show promising results for genotyping of MAP [39,40]. The obtained data also disagrees with the results obtained by Fernández Silva [41] who reported the absence of polymorphism among MAH and MAA isolates when using VNTR1658. Meanwhile, the use of MIRU5, MIRU6, MIRU7, VNTR8 and RD 130 showed size polymorphism when applied with MAH/MAA but not with MAP [39,40] in opposite to the VNTR3 and VNTR7 which yield size polymorphism with MAP but not MAH/MAA. Finally, the VNTR292 could subdivide both MAP and MAH but not MAA into smaller subgroups.

In addition to the MIRU and VNTR, the use of the RDI130 region which is present inside the f57 region of MAP, can help in genotyping process of MAH–MAA. The amplification of the region in MAP isolates revealed no size polymorphism in the present study. However, Dohmann et al. [32] reported the presence of size polymorphisms when applying the RDI130 to MAP according to the source of the isolate, being 631 bp or 642 bp. The application of the same primers for the amplification of MAH and MAA genomes could amplify fragments of 500 bp and 180 bp strain in three MAA and one MAH strains, respectively.

It is of interest to notice that the dominant amplicon size obtained by all genotyping reactions of MAH (written in bold in Table 4) dominates also when used for MAA which may be attributed to the close genetic relationship between the two subspecies.

In conclusion, a higher degree of discrimination among MAH/MAA isolates, even those isolated from one host and from a definite geographical area, can be achieved by combing the use of both MIRU and VNTR. This may provide the foundation for the development of highly discriminatory typing approach for strain differentiation among isolates of MAH/MAA and should place the focus of planed control programs and management strategies which aim to interrupt the transmission of the pathogen to susceptible animals. The absence of a dominant profile among MAH isolates within swine herds and the conservation of MIRU and VNTR loci described previously in MAP also in MAH enable their use in molecular epidemiologic analysis to track transmission pathways.

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