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Full Length Article

Molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* field isolates recovered from dairy cattle in Germany

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Abstract In the present work a total of 143 *Mycobacterium avium* subsp. *paratuberculosis* (Map) field isolates recovered from faeces of dairy cattle in Hessen State, Germany were investigated. Different Short Sequence Repeats (SSR), Mycobacterial Interspersed Repetitive Units (MIRU) and Variable Number Tandem Repeats (VNTR) were applied to classify these cattle-group field isolates into subgroups. When combining the results obtained from SSR sequencing together with the data obtained from other genotyping PCR techniques applied in the current study, 78 different Map profiles were detected. One profile dominates over the investigated field isolates. This profile was detected in 24 isolates (16.8%) with 11 G (SSR); amplicon size of 280 bp, 300 bp, 200 bp, 200 bp, 210 bp, 350 bp, 210 bp, 300 bp and 300 bp for the primer MIRU 2, MIRU 3, X3, Primer 3, Primer 7, Primer 25, Primer 47, 292 and VNTR 1658, respectively. One profile was represented by eight isolates. Two profiles were represented by six members each. The remaining isolates were

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subdivided into smaller groups (2 profiles each 4 isolates, 3 profiles each 3 isolates, 13 profiles as 2 isolates each and finally 56 profiles were represented by a single strain for each profile).

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1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is one of the main bacterial agents involved in the induction of serious digestive disorders in ruminants (Johne's disease). Although Johne's disease was first described in Germany [1], yet little data are available on the prevalence of Johne's disease in German cattle. Assessments based on field surveys in neighbouring areas estimate that flock level prevalence of German cattle herds ranged between 5% and 15% [2].

Worldwide, efficient control programs of *M. avium paratuberculosis* faced difficulties due to many factors such as (a) the long incubation period of the disease, (b) the presence of undetected shedders in the herds, (c) the possible re-introduction of infection to the herds by free ranging birds, wild animals and even insects, (d) the absence of rapid *M. avium* subsp. *paratuberculosis*-specific diagnostic tools and efficacious vaccines, (e) the lack of knowledge of disease prevalence and the genetic variation among local Map isolates in some countries, specially those who are not engaged in active control programs [1,3–5].

In order to study the strain diversity among Map isolates, different molecular typing techniques such as multiplex polymerase chain reaction (PCR) for IS*900* integration loci (MPIL), Pulse Field Gel Electrophoresis (PFGE) Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) were applied [1,5–9]. Recently, new techniques were developed depending mainly on the identification, characterization and comparison of certain sequence repeats such as the Short Sequence Repeats (SSR, [1,10,11]), the Mycobacterial Interspersed Repetitive Units (MIRU; [12]) and the Variable Number Tandem Repeats (VNTR; [13]). Genotyping of the panel of Map field isolates in this study might provide useful molecular epidemiological information about the dominant Map field genotypes, distribution and -prevalence in Hessen and may detect the origin of widely distributed isolates to permit adequate intervention strategies. The previously described SSR, MIRU and VNTR genotyping methods were applied to investigate the genetic diversity among Map field isolates originated from dairy herds in Hessen [14,15].

2. Material and methods

2.1. Bacterial isolates

M. avium subsp. *paratuberculosis* isolates were included from 143 traditional culture positive dairy cows reared in Hessen State, Germany. HEYM agar (Herold's Egg Yolk Medium containing Mycobactin J and Amphotericin B, Nalidixic acid and Vancomycin (ANV), Becton Dickinson, Heidelberg, Germany) was used as media for cultivation of fecal samples following a protocol described previously by Salem et al. [16]. Tubes were incubated at 37 °C for 16 weeks, and monitored for bacterial growth each week.

Presumptively, Map colonies appeared with a diameter of 1–2 mm and white color. Colonies were stained with Ziehl–Neelsen (ZN) according to the manufacturer's instruction (Merck, Darmstadt, Germany). Map appeared in clumps of acid fast, rod-shaped bacilli. Subcultures were made on new tubes of HEYM agar and incubated at 37 °C for 4–12 weeks for final PCR identification step [1]. Different control isolates including the *M. avium* subsp. *avium* 7376/04, *M. avium* subsp. *avium* 6890/04 (kindly provided from the National Reference Center for Mycobacteria NRC – Borstel, Germany), *M. avium* subsp. *silvaticum* DSM 44175 and *M. intracellulare* DSM43223 (kindly provided from Friedrich-Loeffler-Institute, Jena, Germany) were also included.

2.2. Polymerase chain reaction for identification and genotyping of the bacterial isolates

For DNA extraction 3–5 colonies were suspended in 180 µl TE lysis buffer (lysozyme 20 mg/1ml, Roche Diagnostics, Mannheim, Germany in 20 mmol/l Tris–HCl, 2 mmol/l EDTA, Triton 100 1.2% pH 8.0) and were 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, vortexed shortly and incubated for 2 h at 56 °C. The suspension was heated for 10 min at 100 °C and than cooled to 4 °C. From the last mixture, the DNA was extracted using DNeasy® Tissue Kit (Qiagen) according to the manufacturer's instruction. For bacterial identification the Map specific PCR primer pairs to detect the IS*900* (primer TJ1–TJ2) and the f57 gene (primer F57–R57) were used according to Bull et al. [12] and Vansnick et al. [17], respectively (Table 1). The PCR amplification for IS*900*- and f57 gene was performed in 30 µl reaction mixtures in 0.2 ml reaction tubes containing the following reaction mixtures: 1.0 µl of each primer (10 pmol/µl), 1.5 µl dNTP-mix (10 mmol) (MBI Fermentas, St. Leon-Rot, Germany), 3 µl GeneAmp 10× PCR Gold Buffer (150 mM Tris–HCl, 500 mM KCL, pH 8.0, Applied Biosystem, Darmstadt, Germany), 1.8 µl MgCl2 (25 mM, Applied Biosystem), 0.2 µl AmpliTaq Gold® polymerase (5 U/µl, Applied Biosystem), 19.9 µl sterile aqua dest. and 2.5 µl DNA template. Finally, 12 µl of each PCR reaction was mixed with 2 µl loading dye solution (MBI Fermentas) and separated by 2% agarose gel electrophoresis (Biozym, Hessisch-Oldendorf, Germany) at 120 V in 1× TBE buffer (0.04 mol/l Tris, 0.001 mol/l EDTA, pH 7.8) and a 100 bp DNA ladder (Roche, Mannheim, Germany), followed by gel staining for 5 min with 5 µl/ml ethidium bromide solution (Sigma, Taufkirchen, Germany). The amplicons were visualized under an UV trans-illuminator (Biorad, Munich, Germany). For genotyping of the bacteria all 143 MAP isolates were investigated using 24 different genotyping primers. The primer sequence, thermocycler and reference programs are also listed in Table 1.*2.3. Sequencing and sequence analysis*

After performing the SSR targeting PCRs using the primer pair SSR 5-SSR 6, the purified DNA (30 µl) was sequenced from both directions by SEQLAB Sequence Laboratories

Table 1 The primers used to genotype the 143 MAP isolates involved in the present work. All primers were synthesized by MWG Biotech (Ebersberg, Germany). The PCR programs included an initial denaturation step at 94 °C for 10 min for *Taq* polymerase activation, and a final extension step at 72 °C for 7 min.

	Target region	Forward and reverse primers sequence (5′–3′)	Amplification conditions (X = cycles)	Reference
	IS*900*	P90: GAA GGG TGT TCG GGG CCG TCG CTT AGG P91: GGC GTT GAG GTC GAT CGC CCA CGT GAC	40X: 95 °C – 60 s; 58 °C – 60 s; 72 °C – 150 s	[22]
	F57	F57: CCT GTC TAA TTC GAT CAC GGA CTA GA R57: TCA GCT ATT GGT GTA CCG AAT GT	40X: 95 °C – 60 s; 58 °C – 60 s; 72 °C – 150 s	[17]
SSR	G repeat	SSR5: GTG ACC AGT GTT TCC GTG TG SSR6: TGC ACT TGC ACG ACT CTA GG	35X: 94 °C – 45 s; 60 °C – 60 s; 72 °C – 150 s	[5]
MIRU	MIRU2	M2.f: GAACGAAGATCCTGGGACTG M2.r: CGACGACGAACACCTCAAC	35X: 94 °C – 30 s; 62 °C – 30 s, 72 °C 60 s	[12]
	MIRU3	M3.f: ACATTCACCCTGTCCATTCC M3.r: CCTCCTTACGGAGCAGGAA	35X: 94 °C – 30 s; 62 °C – 30 s, 72 °C 60 s	
	VNTR1658	1658 Fw: CCC AAC CGT TCC CAA CGA GA 1658 Rv: CCC GGG GAG CAT CAG GTC	30X: 94 °C – 30s; 50 °C – 30s; 72 °C – 30s	[13]
	292[a]	292-F: CTT GAG CAG CTC GTA AAG CGT 292-R: GCT GTA TGA GGA AGT CTA TTC ATG G	40X: 95 °C – 30 s;58 °C – 30 s; 72 °C – 30 s	
	X3	X3F: AAC GAG AGG AAG AAC TAA GCC G X3R: TTA CGG AGC AGG AAG GCC AGC GGG	40X: 95 °C – 30 s; 58 °C – 30 s; 72 °C – 30 s	
	25[a]	25-F: GTC AAG GGA TCG GCG AGG 25-R: TGG ACT TGA GCA CGG TCA T	40X: 95 °C – 30 s; 58 °C – 30 s; 72 °C – 30 s	
VNTR	3[a]	3-F: CAT ATC TGG CAT GGC TCC AG 3-R: ATC GTG TTG ACC CCA AAG AAA T	40X: 95 °C – 30 s; 60 °C – 30 s; 72 °C – 30 s	[14]
	7[a]	7-F: GAC AAC GAA ACC TAC CTC GTC 7-R: GTG AGC TGG CGG CCT AAC	40X: 95 °C – 30 s; 60 °C – 30 s; 72 °C – 30 s	
	47[a]	47-F: CGT TGC GAT TTC TGC GTA GC 47-R: GGT GAT GGT CGT GGT CAT CC	40X: 95 °C – 30 s; 64 °C – 30 s; 72 °C – 30 s	
	10[b]	10-F: GAC GAG CAG CTG TCC GAG 10-R: GAG AGC GTG GCC ATC GAG	40X: 95 °C – 30 s; 60 °C – 30 s; 72 °C – 30 s	
	32[a,b]	32-F: CCA CAG GGT TTT TGG TGA AG 32-R: GGA AAT CCA ACA GCA AGG AC	40X: 95 °C – 30 s; 55 °C – 30 s; 72 °C – 30 s	

[a] DMSO was added to the reaction.

[b] Betaine was added to the reaction. The results obtained by the VNTR 10 and 32 were excluded from the evaluation of the data as they gave multiple bands in many isolates and missing bands in other isolates.

(Germany) in an ABI DNA Sequencer System (Applied Biosystems, Germany). SSR sequences were aligned against sequences in the GenBank database and analyzed using the MegAlign program (DNASTAR, Inc., Madison, Wisconsin, USA) and CLUSTALW (http://clustalw.genome.ad.jp).

2.4. Statistic analysis

Simpson's index was used to detect the genetic diversity of the current panel of Map isolates. Simpson's index measures the probability of two randomly selected isolates to belong to the same subgroup [1,18].

3. Results

Isolates were practically identified as Map using methods based on conventional microscopic examination and Map specific PCRs based on the detection of IS*900* and *f57* genes [1]. Genotyping of 143 Map field isolates was carried out using different SSR, MIRU and VNTR methods. While the use of primers VNTR10 and VNTR32 did not reveal significant information due to the presence of multiple bands in many isolates or the absence of an amplicon in other isolates, the use of other primers showed different SRR or MIRU–VNTR genotypes. By combining all these genotyping methods the isolates could be divided into 78 subgroups under the major cattle group. One particular genotype dominated. Such particular genotype was present in 24 isolates (16.8%) with 11 G (SSR) and amplicon sizes of 280 bp, 300 bp, 200 bp, 200 bp, 210 bp, 350 bp, 210 bp, 300 bp and 300 bp for the primers MIRU 2, MIRU 3, X3, Primer 3, Primer 7, Primer 25, Primer 47, 292 and VNTR 1658, respectively. Remaining isolates were subgrouped as follows: one profile was detected in eight isolates; two profiles were represented by six isolates. Two further profiles by four isolates each, three profiles by three isolates each, 13 profiles by two isolates each and 56 profiles by only one isolate.

The sequencing results of SSR revealed the presence of G repeats in short repeats of 7, 8, 9, 10, 11, 12, 13, 14 and 15 successive G. The MIRU2 and MIRU3 showed size polymorphisms and divided the isolates into six and seven subgroups, respectively, with a dominating amplicon size of 280 bp in both MIRUs (Table 2). The VNTR typing method applied could also subdivide our isolates into 6, 4, 3, 5, 3, 3 and 3 groups for VNTR 1658, VNTR 292, VNTR X3, VNTR 25, VNTR 3, VNTR 7 and VNTR47, with dominant amplicon sizes of 300 bp, 300 bp, 200 bp, 350 bp, 200 bp, 220 bp and 210 bp, respectively (Table 3). The Simpson's index obtained by the application of different methods ranged from 0.26 to 0.91 (Tables 2 and 3).

4. Discussion

The present work applied recent modern molecular epidemiological techniques in shedding more light into aspects of the regional epidemiology of Map-infection in Germany. 143 Map field isolates were subject to different genotyping methods based on sequence polymorphism for SSR or size polymorphism in case of MIRU and VNTR typing. Through combining the obtained data from different genotyping PCRs and SSR sequencing, a total of 78 alleles could be identified among investigated isolates. The evaluation of the sub-classification of Map and discriminatory power of the used methods was done through the application of Simpson's Index (S.I.). The knowledge of the diversity of isolates, which are shed in different herds within the same region, is important to understand the dynamics of Map transmission.

The obtained results were agreement with some previous works [15,19], the high discriminatory value of using the SSRs for characterization of Map isolates. Methods based on SSR appeared to be more efficient for this purpose than those depending on MIRU–VNTR alone. The applied MIRUs were in turn, more efficient than the VNTR. The use of SSR in a routine work may be limited due to their high costs in comparison to MIRU and VNTR, but the obtained results in the present study based on the S.I. index indicate that differentiation power of MIRU3 (0.28) is comparable to those obtained by SSR typing (0.26). The results obtained revealed the predomi-

Table 2 Summary of the investigated MAP isolates percentage and their distribution in the different subgroups.

Amplicon with sequence polymorphisms and number of isolates (SSR)		Amplicon with size polymorphismus in base pair and number of isolates (MIRU)			
Number of (G) repeat (g)	Number of isolates (%)	MIRU 2		MIRU 3	
Distribution of 143 isolates in subgroups obtained by the use of SSR and MIRU genotyping methods					
7	2 (1.4%)	bp	N (%)	bp	N (%)
8	2 (1.4%)	250	16 (11.2%)	250	4 (2.8%)
9	11 (7.7%)	280	84 (58.7%)	280	64 (44.8%)
10	49 (34.3%)	300	1 (0.7%)	300	32 (22.4%)
11	48 (33.6%)	330	37 (25.9%)	330	20 (14%)
12	22 (15.4%)	350	4 (2.8%)	350	9 (6.3%)
13	5 (3.5%)	NR	1 (0.7%)	400	1 (0.7%)
14	3 (2.1%)			NR	13 (9.1%)
15	1 (0.7%)				
S.I.	0.26	S.I.	0.42	S.I.	0.28

N, number of isolates, (%), percentage of each group in correlation to the total number (143 isolates), S.I., Simpson's index.

Table 3 Summary of the numbers and percentages of MAP isolates of the different subgroups characterized in the present work depending on size polymorphisms by the application of VNTR genotyping methods. The dominant types are typed in bold.

VNTR 1658		VNTR 292		VNTR X3		VNTR 25		VNTR 3		VNTR 7		VNTR 47	
bp	N (%)	bp	N (%)	bp	N (%)	bp	N (%)	bp	N (%)	bp	N (%)	bp	N (%)
Distribution of 143 isolates in subgroups obtained by the use of VNTR genotyping methods													
250	1 (0.7%)	250	16 (11.2%)	**200**	**99 (69%)**	200	1 (0.7%)	180	5 (3.5%)	170	1 (0.7%)	190	24 (16.7%)
270	1 (0.7%)	**300**	**84 (58.7%)**	250	43 (58.7%)	300	4 (2.8%)	**200**	**136 (9%)**	190	42 (29.4%)	**210**	**112 (78%)**
300	**99 (69%)**	350	41 (28.7%)	300	1 (0.7%)	**350**	**113 (79%)**	NR	2 (1.4%)	**220**	**100 (69.9%)**	230	7 (4.9%)
370	40 (28%)	NR	2 (1.4%)			480	22 (15.4%)						
400	1 (0.7%)					NR	3 (2.1%)						
NR	1 (0.7%)												
S.I.	**0.55**		**0.44**		**0.57**		**0.65**		**0.91**		**0.57**		**0.64**

N, number of isolates; (%), percentage of each group in correlation to the total number (143 isolates), NR, no amplicon could be obtained, S.I., Simpson's index = $\sum n(n-1)/N(N-1)$ where n = the number of every subgroup and N = the total number of isolates.

nance of isolates showing amplicon sizes of 280 bp, 280 bp and 300 bp for MIRU2, MIRU3 and VNTR 1658, respectively, among Map isolates from. On the other hand, the SSR results differed from those obtained by El-Sayed et al. [15]. While only isolates with 11 G repeats dominated in our previous work, isolates with 10 G shared the dominance with those having 11 G in the present work and were represented by 49 and 48 isolates out of 143 isolates, respectively. Conversely, most Map field isolates isolated from dairy herds in Ohio, USA, are reported to contain only 7 G [5].

The present investigation provides an outlook on the genetic diversity among Map isolates originated from dairy herds in a relatively limited geographic area of approximately 20.000 km^2. These results are in disagreement with the results obtained by Motiwala et al. [5,8] who stated the absence of great genetic differences among Map isolates recovered from cows regardless of their geographic location. This may be due to either the difference in the nature of local field Map isolates in Germany/USA or due to differences in discrimination of the different genotyping methods used in the two studies. Because of the high differentiation capability of both G repeat (SSR) and MIRU2 based on SI in the current investigation, it is recommended to combine both methods as an efficient tool for molecular genotyping of Map.

In a recent work, Thibault et al. [20] combined the use of MIRU–VNTR–SSR and IS*900* RFLP for Map genotyping in France. In parallel, Möbius et al. [21] combined the use of MIRU–VNTR and the IS*900* fingerprinting for the same purpose in Germany. In opposite to Thibault et al. [14,20]. Isolates included in the present study originated from a limited geographic region and only from a single host species with the objective to assess the current informative value of the post genomic era most popular genotyping methods in the epidemiology of Map. Furthermore, the high discriminatory value of the loci number 2, 3, 292, 7, 25 and 47 – as shown in the present work – confirms results obtained previously [21]. Since the combination of SSR with MIRU–VNTR could be done in more laboratories as routine work. The data presented here agrees with some previously published reports that combing of more than one fingerprinting technique is the best practice for epidemiological studies targeting differentiation among the Map population [14,15,20,21].

Types with 11G and 280 bp, 300 bp, 200 bp, 200 bp, 210 bp, 350 bp, 210 bp, 300 bp and 300 bp for the SSR5–6, MIRU2, MIRU3, VNTRX3, VNTR3, VNTR7, VNTR25, VNTR47, VNTR292 and VNTR1658, respectively, were detected in 24 isolates (16.8%). On the other hand, the obtained results showed that 56 different genetic groups were represented by a single isolate each while others by only 2 or 3 members. This variation in the distribution of Map field isolates among the different genetic groups was not very surprising.

5. Conclusion

The cluster of high number of Map dairy cattle naïve-field isolates in a major genotype helps to emphasize the old hypothesis that the origin of Johne's disease in cattle was due to an epidemic infection with a single clone of *M. a.* subspecies *paratuberculosis*. It is important to compare the obtained results with similar data from diverse continents in order to identify the geographic distribution, virulence factors or special invasive elements of the world most dominant and rare Map genotypes that might be reasonable candidates for further epidemiological studies and disease control programs for the ruminant industry. Answers to whether there is an association between the new genotypes and the clinical or the sub-clinical disease are still open speculations.

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