Evaluation of Three Molecular Methods of Repetitive Element Loci for Differentiation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

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The aim of the present study is to evaluate the efficiency of three methods to determine the molecular diversity of 34 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) strains isolated from 17 cattle herds. The applied methods included the analysis of sequence polymorphism of the mononucleotide (G1 and G2) and trinucleotide sequences (GGT) of the Short Sequence Repeats (SSR) and the determination of size polymorphism of 9 different Mycobacterial Interspersed Repetitive Units (MIRU) and 6 Variable Number Tandem Repeats (VNTR). Sequence analysis of SSR of 34 isolates showed 4, 6, and 2 alleles of G1, G2, and GGT repeats, respectively. The amplification of the investigated 9 MIRU units revealed only two discriminatory genotyping systems (MIRU2 and MIRU3). Out of 6 VNTR PCR differentiation methods, only one method could be recommended for genotyping purposes. The profile 7g-12g-4ggt-II-b-2 of the combination systems G1-G2-GGT-MIRU2-MIRU3-VNTR1658 dominates among the examined isolates and was detected in 14.7% of the isolates. The use of certain repetitive loci of SSR, MIRU, and VNTR techniques in this study showed greater potential than others for the characterization of MAP isolates. The recommended loci can be used for the epidemiological tracing of MAP field strains and to determine the relationships between isolates in different herds.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), short sequence repeats (SSR), mycobacterial interspersed repetitive units (MIRU), variable number tandem repeats (VNTR), Johne's disease (JD)

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease (JD), which primarily infects cattle, sheep and other ruminants. The disease causes great economic losses for the dairy industry worldwide due to premature culling, reduced carcass value, decreased weight gain and decreased milk production (Collins, 1997; Collins 2003; Álvarez *et al.*, 2005; Corn *et al.*, 2005). Indeed, it is estimated that the average annual losses to the cattle industry in the United States alone exceed $1.5 billion (Whitlock, 1996). Furthermore, estimations based on epidemiological surveys of herds in different German regions indicate that 5 to 15% of cattle are currently infected (Stratmann *et al.*, 2005).

Different genotyping methods have previously been used to evaluate genetic variation among *Mycobacterium* species such as *M. tuberculosis*, *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis*. These molecular subtyping techniques have primarily included multiplex PCR for fingerprint analysis of the IS*900* integration loci (MPIL), IS*900*, IS*1311*, and IS*I245*-restriction fragment length polymorphism (RFLP), and amplified fragment length polymor-

phism (AFLP) (Pavlik *et al.*, 1999; Pavlik *et al.*, 2000). However, these techniques are inadequate for MAP genotyping and suffer from poor discriminatory power and low throughput. As a result, the data generated by such techniques are biallelic and therefore provide only limited information regarding the overall genetic diversity and evolutionary mechanisms within the species (Amonsin *et al.*, 2004). Other techniques based on repetitive elements and random single nucleotide polymorphisms (SNP) present on the MAP genome have been successfully applied (Bull *et al.*, 2003; Amonsin *et al.*, 2004; Overduin *et al.*, 2004; Möbius *et al.*, 2008; Thibault *et al.*, 2008). The short sequence repeats (SSR) method showed a high discriminatory value and can be used as a marker for the differentiation and subtyping of different bacteria, such as *Haemophilus*, *Mycoplasma*, and *Mycobacterium* species (Van Belkum *et al.*, 1998; Kremer *et al.*, 1999; Amonsin *et al.*, 2004). The mononucleotide (G-repeat) and the trinucleotide (GGT-repeat) markers have been found to be the most discriminatory markers for analysis by SSR (Ghadiali *et al.*, 2004). Supply *et al.* (1997) identified mycobacterial interspersed repetitive units (MIRU) in *M. tuberculosis* that were used later by Bull *et al.* (2003) to differentiate MAP strains from other species of the *Mycobacterium avium* complex. Bull *et al.* (2003) also described the use of the variable number tandem repeats (VNTR) based

on multilocus short repeat sequences as a new genotyping method for MAP.

The present study was designed to compare the ability of three different genotyping methods, SSR, MIRU, and VNTR, to detect the degree of heterogeneity in MAP field isolates collected from numerous dairy cow farms in Hesse State, Germany.

Materials and Methods

Bacterial isolates

A total of 108 fecal samples were collected from 17 MAP positive dairy herds according to the sampling procedures recommended by the AVID (1996). The samples were then processed at the MAP laboratory, Department of Veterinary Microbiology, Division of Bacteriology and Mycology of Landesbetrieb Hessisches Landeslabor, Giessen, Germany. For the culture method, three tubes of HEYM agar (Harold's Egg Yolk Medium containing Mycobactin J and ANV, Becton

Dickinson, Germany) were used to culture aliquots of each fecal sample at 37°C for at least 16 weeks, during which time bacterial growth was tested once per week. After 4 weeks of incubation, bacterial colonies were stained with Ziehl-Neelsen (ZN) stain according to the manufacturer's instructions (Merck, Germany). A total of 34 growing isolates were found to be ZN positive; therefore, a single colony from each of the positive cultures was subcultured in a new tube of HEYM agar at 37°C for 4~12 weeks, after which they were evaluated by PCR.

Polymerase chain reaction for the identification of bacterial isolates

Bacterial strains including *M. avium* subsp. *avium* 7376/04, *M. avium* subsp. *avium* 6890/04, which were kindly obtained from National Reference Center (NRC) for Mycobacteria, Borstel, Germany, and *M. avium* subsp. *silvaticum* DSM 44175 and *M. intracellulare* DSM43223, which were kindly provided by the Friedrich-Loeffler-Institute Federal Research

Table 1. SSR, MIRU, and VNTR primers used for genotyping of the MAP isolates

Genotype system	Target region	Forward and reverse primers sequence (5'-3')	Amplification conditions[a] (×=cycles)	Reference
SSR	G1 repeat	SSR1: TCA GAC TGT GCG GTA TGG AA[b] SSR2: GTG TTC GGC AAA GTC GTT GT	35×: 94°C- 45 sec; 60°C- 60 sec; 72°C- 150 sec	Motiwala *et al.* (2005)
	G2 repeat	SSR5: GTG ACC AGT GTT CCC GTG TG SSR6: TGC ACT TGC ACG ACT CTA GG		
	GGT repeat	SSR3: AGA TGT CGA CCA TCC TGA CC SSR4: AAG TAG GCG TAA CCC CGT TC		Amonsin *et al.* (2004)
MIRU	MIRU1	M1.f: CGCGGACTTGATGGTCTC M1.r: ACTCCACCTGGACAACGG	35×: 94°C- 30 sec; 62°C- 30 sec, 72°C- 60 sec	Bull *et al.* (2003)
	MIRU2	M2.f: GAACGAAGATCCTGGGACTG M2.r: CGACGACGAACACCTCAAC		
	MIRU3	M3.f: ACATTCACCCTGTCCATTCC M3.r: CCTCCTTACGGAGCAGGAA		
	MIRU4	M4.f: CGTTCAGCCTGTGCATGG M4.r: CAAGTCGTCACGGGCAAC		
	MIRU4 ARG	M4b.f: ACG AGC AGT GTG TTT TTC AC M4b.r: CGT CGT GGA GGT CGT CAC	40×: 94°C- 60 sec; 57°C- 60 sec; 72°C- 90 sec	Romano *et al.* (2005)
	MIRU5 UK	M5.f: GTC ACA GCG TCA GCG TAG C M5.r: TGT TGG GTT GAC GAT CAG CA	40×: 94°C- 60 sec; 51°C- 60 sec; 72°C- 90 sec	
	MIRU6	M6.f: GGC GAT GAA ATG CCG TAT M6.r: GCG GAC ACA GGG TGA AAT		
	MIRU7	M7.f: GAC CAC CTG TGT CGG GTA TT M7.r: CCC TGC TGA CCC TGG AAT	40×: 94°C- 60 sec; 57°C- 60 sec; 72°C- 90 sec	
	MIRU11 UK/VNTR 11	M11.f: CTG TGG TCG TGG CTG GTG M11.r: CGG TCT TGG TGT CGG TGT AT	40×: 94°C- 60 sec; 57°C- 60 sec; 72°C- 90 sec	
VNTR	VNTR8	V8.f: CGC AGA TTC ATA CGC CAG AT V8.r: AGG TTG AGG TGG TCG CTG AT	40×: 94°C- 60 sec; 57°C- 60 sec; 72°C- 90 sec	Romano *et al.* (2005)
	VNTR1067	1067 Fw: CGC CGC CCG CCG AAA AAG 1067 Rv: CGG GAC ATC ACA AAT ACA GAA GAA	30×: 94°C- 60 sec; 50°C- 60 sec; 72°C- 60 sec	Overduin *et al.* (2004)
	VNTR1605	1605 Fw: CGA TGC CGC CGA AGG TTT TGG TG 1605 Rv: ACG TGA GGA TCG GGT TGG CAG TCG		
	VNTR1658	1658 Fw: CCC AAC CGT TCC CAA CGA GA 1658 Rv: CCC GGG GAG CAT CAG GTC		
	VNTR3249	3249 Fw: GCA GCA GGA CGA TCA GGC C 3249 Rv: CGC GAG TTC GGT GCC GTG A	30×: 94°C- 60 sec; 53°C- 60 sec; 72°C- 60 sec	
	VNTR3527	3527 Fw: GGC GCT CGC AGG AAA CCA AC 3527 Rv: ACG GCC TCA GCT CCC AGT AG	30×: 94°C- 60 sec; 55°C- 60 sec; 72°C- 60 sec	

[a] PCR programs included an initial denaturation step at 94°C for 10 min for *Taq* polymerase activation, and final extension step at 72°C for 7 min
[b] Oligonuclotide primers were synthesized by MWG Biotech (Germany)

Institute for Animal Health, Jena, Germany, were used as control strains. The 34 isolates and the control strains were processed for DNA extraction by suspending 3~5 colonies in 180 μl of TE lysis buffer [lysozyme 20 mg/ml (Merck) in 20 mmol/L Tris-HCl, 2 mmol/L EDTA, 1.2% Triton 100, pH 8.0] and then incubated for 1 h at 37°C. Subsequently, 35 μl of proteinase K (QIAGEN, Germany) and 200 μl of AL lysis buffer (QIAGEN) were added, after which the samples were briefly vortexed and incubated for 2 h at 56°C. The suspensions were then heated for 10 min at 100°C, after which they were cooled to 4°C. The DNA was then extracted using a DNeasy® Tissue kit according to the manufacturer's instructions (QIAGEN).

Two PCR systems were used for bacterial identification; IS*900* specific DNA fragments of MAP, which were amplified using the TJ1; GAA GGG TGT TCG GGG CCG TCG CTT AGG and TJ2; GGC GTT GAG GTC GAT CGC CCA CGT GAC primers described by Bull *et al.* (2003) and the f*57* DNA fragment, which was amplified using the F57; CCT GTC TAA TTC GAT CAC GGA CTA GA and R57; TCA GCT ATT GGT GTA CCG AAT GT, primers described by Vansnick *et al.* (2004). PCR amplification of IS*900* and f*57* was conducted in 30 μl reaction mixtures in 0.2 ml reaction tubes that contained the following: 3 μl GeneAmp 10× PCR Gold Buffer (150 mM Tris-HCl, 500 mM KCl, pH 8.0) (Applied Biosystem, Germany), 1.8 μl MgCl$_2$ (25 mM) (Applied Biosystem), 0.6 μl dNTP-mix (10 mmol) (MBI Fermentas, Germany), 1.0 μl of each primer (10 pmol/μl), 0.2 μl AmpliTaq Gold® polymerase (5 U/μl, Applied Biosystem), 19.9 μl sterile aqua dest and 2.5 μl DNA template. PCR was conducted by subjecting the samples to 1 cycle of 95°C for 10 min and then 40 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 3 min, followed by 1 cycle of 72°C for 7 min in a Biometra T3000 thermocycler (Biometra, Germany). The PCR products produced using the IS*900* and f*57* primers (12 μl) were then mixed with 2 μl loading dye (MBI Fermentas) and separated by 2% agarose gel electrophoresis (Biozym, Germany) at 120 V in 1× TBE buffer. A 100 bp DNA ladder marker (Roche, Germany) was electrophoresed in the same gel for comparison. The gels were then stained for 5 min with 5 μl/ml ethidium bromide solution (Sigma, Germany), after which the amplicons were visualized under a UV trans-illuminator (Biorad, Germany).

Genotyping of the bacterial isolates
SSR analysis

The short sequence mononuclotide G1 and G2 repeats were amplified by PCR using primer pairs SSR1-SSR2 and SSR3-SSR4 described by Motiwala *et al.* (2005), repectively. For the trinuclotide SSR GGT repeat, PCR amplification was conducted using primers SSR5 and SSR6 as described by Amonsin *et al.* (2004). The sequences of the primers and the PCR conditions are summarized in Table 1. Each PCR mixture had a final volume of 30 μl and contained 1.0 μl of each primer (10 pmol/μl), 1.5 μl dNTP-mix (10 mmol) (MBI Fermentas), 3 μl GeneAmp 10× PCR Gold Buffer (150 mM Tris-HCl, 500 mM KCl, pH 8.0) (Applied Biosystem), 1.8 μl MgCl$_2$ (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. PCR

products (6 μl) were separated on 2% agarose gel and then stained by ethidium bromide solution as described above. The remaining templates were purified using commercial kits according to the manufacturer's instructions (QIAGEN). The purified DNA (30 μl) was sequenced by SEQLAB Sequence Laboratories (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., USA) and CLUSTAL W (http://clustalw.genome.ad.jp/).
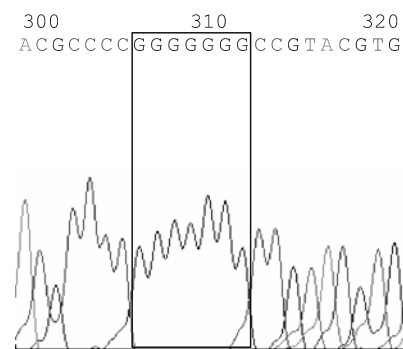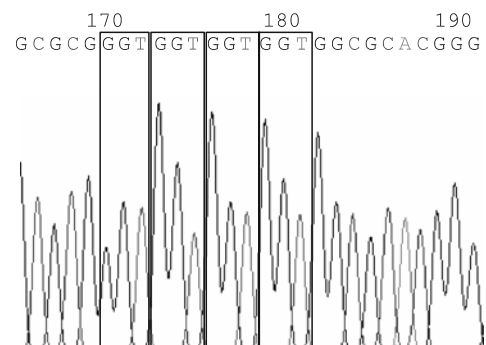
(A) G1 Repeat
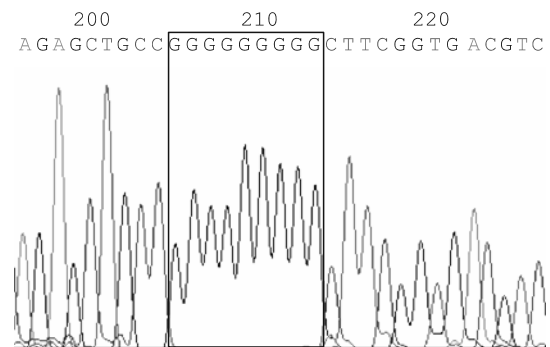
(B) G2 Repeat

(C) GGT Repeat

Fig. 1. Example of three sequences of SSR results (A=G1 repeat), (B=G2 repeat), and (C=GGT repeat) were involved in the present study.

Table 2. Summary of the number of strains distributed in the most applicable genotyping methods used in this study

Distribution of 34 isolates in genotyping methods					
Amplicon with sequence polymorphisms and number of isolates (n)			Amplicon with size polymorphismus in base pair and number of isolates (n)		
G1	G2	GGT	MIRU2	MIRU3	VNTR1658
7g (27)	9g (3)	4ggt (26)	330 (8)	350 (8)	370 (8)
10g (2)	10g (7)	5ggt (8)	280 (20)	290 (26)	300 (26)
12g (3)	11g (11)		250 (6)		
14g (2)	12g (7)				
	13g (4)				
	14g (2)				

(n) = number of isolates showed the results with genotype method

MIRU and VNTR analysis

All 34 MAP isolates were amplified using 9 different MIRU and 6 different VNTR primers (Table 1). The PCR mixture had a final volume of 30 μl and contained primers, dNTP-mix, GeneAmp Gold Buffer, MgCl$_2$, and AmpliTaq Gold® polymerase in the same quantities described for the SSR analysis methods above, as well as 1.5 μl of dimethyl-sulfoxide (DMSO) (Roche Diagnostics) and 18.4 μl of sterile aqua dest. In addition, 2.5 μl of DNA were added to the PCR mixture as a template. The PCR conditions for each of the methods are described in Table 1. PCR amplicons (10 μl) were separated in 2% (w/v) agarose gel alongside a 100 bp DNA ladder marker for 2 h at 120 V. The gel was stained with ethidium bromide and visualized by ultraviolet trans-illumination. The number of repeats was then calculated based on the size of the amplicon.

Statistic analysis

Simpson's index and Simpson's Index of Diversity were used to detect the genetic diversity among MAP strains (Hunter and Gaston, 1988). Simpson's index measures the probability that two isolates selected at random belong to the same subgroup.

Results

In the present study, a total of 34 MAP isolates were evaluated. The isolates were confirmed to be MAP through microscopic examination of Ziehl-Neelsen stained samples. In addition, the colonies were identified as MAP by application of the MAP specific sequence IS900 and the f57 gene. All isolates produced amplicons with a size of 356 bp (IS900) and 432 bp (f57). No product was recovered from

Table 3. The 18 different MAP profiles detected in the present study. The typing power of the applied genotyping methods is compared based on Simpson's Index and Simpson's Index of Diversity

Type of profile	Number of isolates	Farms number	Genotyping methods					
			SSR sequence analysis of repeat			PCR products size bp (allelic group)		
			G1	G2	GGT	MIRU2	MIRU3	VNTR 1658
1	2	9, 11	7g	10g	4ggt	330 (I)	350 (a)	370 (1)
2	1	3	7g	11g	4ggt	330 (I)	350 (a)	370 (1)
3	1[a]	5	7g	11g	4ggt	330 (I)	350 (a)	370 (1)
4	2	15	7g	12g	4ggt	330 (I)	350 (a)	370 (1)
5	2	9, 15	7g	13g	4ggt	330 (I)	350 (a)	370 (1)
6	2	2, 17	7g	9g	4ggt	280 (II)	350 (a)	370 (1)
7	1	7	7g	10g	5ggt	280 (II)	350 (a)	370 (1)
8	2	1, 12	7g	10g	4ggt	280 (II)	350 (a)	370 (1)
9	2	8	10g	11g	5ggt	280 (II)	350 (a)	370 (1)
10	3	17, 14, 16	7g	11g	4ggt	280 (II)	290 (b)	300 (2)
11	1[b]	10	7g	11g	4ggt	280 (II)	290 (b)	300 (2)
12	5	10, 13, 12, 6, 12	7g	12g	4ggt	280 (II)	290 (b)	300 (2)
13	2	6, 12	7g	13g	4ggt	280 (II)	290 (b)	300 (2)
14	2	4	12g	14g	5ggt	280 (II)	290 (b)	300 (2)
15	1	14	7g	9g	4ggt	250 (III)	290 (b)	300 (2)
16	2	4	14g	10g	5ggt	250 (III)	290 (b)	300 (2)
17	2	14, 4	7g	11g	4ggt	250 (III)	290 (b)	300 (2)
18	1	14	12g	11g	5ggt	250 (III)	290 (b)	300 (2)
Simpson's index			0.496	0.196	0.629	0.415	0.629	0.629
Simpson's index of diversity			0.504	0.804	0.371	0.585	0.371	0.371

[a] When MIRU4-ARG was used, this strain produced a smaller fragment (80 bp) than the remaining strains
[b] When VNTR3527 was used, this strain produced a smaller fragment (80 bp) than the remaining strains

PCR analysis of *M. avium* subsp. *avium* 7376/04, *M. avium* subsp. *avium* 6890/04, *M. avium* subsp. *silvaticum* DSM 44175, and *M. intracellulare* DSM43223, which were used as negative controls.

The genotypic properties of the MAP isolates were characterized using 18 pairs of primers belonging to three genotypic systems (Table 1). PCR amplification of the G1 (primers SSR1 and SSR2), G2 (primers SSR3 and SSR4), and GGT (primers SSR5 and SSR6) SSR fragments of the isolates revealed amplicons of 450 bp, 430 bp, and 440 bp, respectively. Subsequent sequence analysis of the PCR products of G1, G2, and GGT revealed 4 different profiles of poly-

morphisms for G1 (7g, 10g, 12g, and 14g repeats) and 6 different profiles of polymorphisms for G2 (9g, 10g, 11g, 12g, 13g, and 14g repeats). Two different polymorphisms were also identified in the GGT repeat (4ggt and 5ggt repeats). The distributions of these profiles are summarized in Tables 2 and 3. Amplification of the MIRU fragments, including MIRU1, MIRU4, MIRU4-ARG, MIRU5-UK, MIRU6, MIRU7, and MIRU11-UK/VNTR-11, did not reveal any size polymorphisms and showed universal amplicon sizes of approximately 230 bp, 200 bp, 720 bp, 160 bp, 190 bp, 200 bp, and 600 bp, respectively. The PCR products of MIRU2 were found to be 330, 280, or 250 bp, whereas those of MIRU3 were only 350 or 290 bp (Fig. 2A and B, Tables 2 and 3). The use of MIRU4-ARG yielded limited polymorphisms. For example, only one strain showed an amplicon that was 80 bp smaller than the remaining strains. PCR of the VNTR systems, including fragments of VNTR8, VNTR 1067, VNTR1605, and VNTR3249, did not reveal any size polymorphisms and yielded universal amplicons with sizes of approximately 500 bp, 380 bp, 530 bp, 350 bp, and 280 bp, respectively. Only the product of PCR conducted using primers specific for VNTR1658 showed size polymorphisms, with two different amplicon sizes being observed (370 bp and 300 bp: Fig. 2C). Additionally, one isolate produced an amplicon that was approximately 80 bp smaller than the others when examined by the VNTR3527 reaction. Based on the alleles at the investigated loci, the 34 isolates examined in the present study could be divided into 18 distinct genotypes (Table 3) and the profile 7g-12g-4ggt-II-b-2 of the combined G1-G2-GGT-MIRU2-MIRU3-VNTR1658 systems was the dominant genotype (14.7%) of the examined isolates. Furthermore, the Simpson's diversity indices were 0.72, 0.8, 0.32, and 0.33 for loci 1, 2, 8, and 9, respectively (Table 3). In this study, the detection of 2 different MAP genotypes in one single cow was observed in 3 out of 4 cows from which 2 colonies per cow were examined.

Discussion

There are significant gaps in our understanding of the use of specific MAP subtypes to evaluate the characteristics of infections and the velocity of their spread in animal populations. There is also still much to learn about the molecular epidemiology of Johne's disease. Indeed, this lack of knowledge hinders rapid identification and discrimination of epidemic, widely distributed or eventual zoonotic strains, which in turn affects the success of control programs (Rideout *et al.*, 2003).

In the present study, a comparative analysis of different SSR, MIRU, and VNTR loci was conducted to assess the contribution of polymorphisms to the allelic diversity of field isolates. A total of 18 alleles were identified with one profile 7g-12g-4ggt-II-b-2 being observed in 14.7% of the investigated isolates. Knowledge of the extent of strains shared across herds within the same region is vital to understanding the dynamics of MAP transmission. The results of the present study are in agreement with those of a study conducted by Harris *et al.* (2006), who found that the use of SSRs provided high discriminatory value for the characterization of MAP strains. The isolates investigated in this study

(A)

(B)

(C)
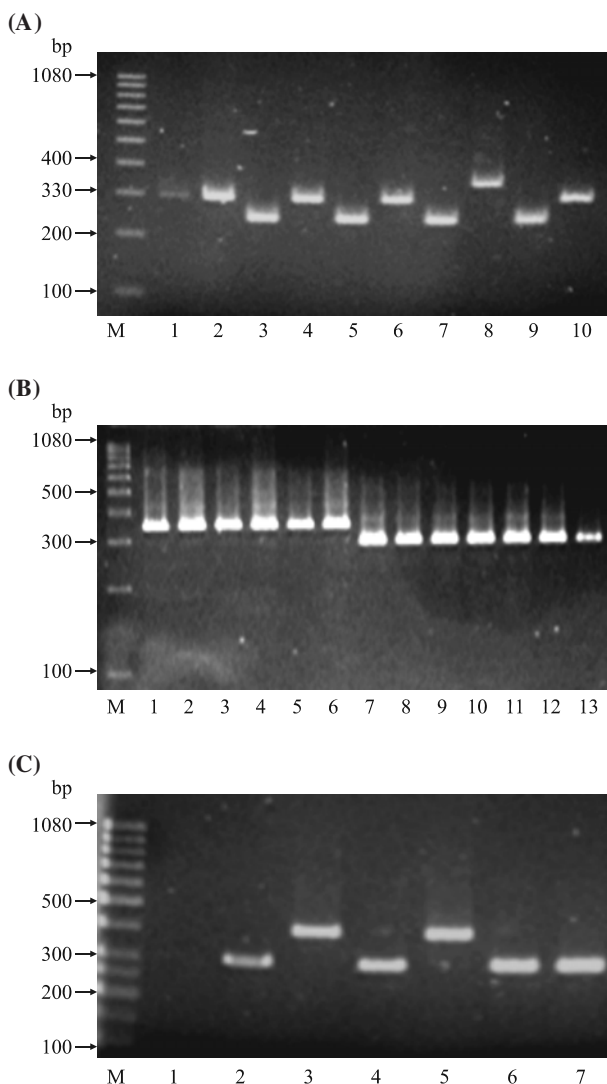
Fig. 2. (A) Amplicons obtained with MIRU 2 (lanes 3, 5, 7, 9) with the size of 250 bp, amplicons in lanes 1, 2, 4, 6, 10 with 280 bp and in the lane 8 with 330 bp. (B) Amplification products obtained by MIRU 3, lanes 1~6 with PCR product of 350 bp and the lanes 7~13 with 290 bp. (C) Amplification products obtained with VNTR1658, in lanes 2, 4, 6, 7 with PCR product of 300 bp, and finally in lanes 3, 5 with 370 bp. The lane 1 is the negative control used. M=100 and 50 bp GeneRuler DNA ladder (Fermentas).

showed a large diversity, even though all of the isolates were collected from one source (dairy cow farms) in a restricted geographic area (Hesse County). These results are in disagreement with those of a study conducted by Motiwala *et al.* (2005), who identified a high degree of genetic similarity among MAP isolates recovered from cows, regardless of their geographic origin.

In the current investigation, the use of the G2 repeat was shown to have the highest discriminatory value among all investigated SSRs. This agrees with the results of a study conducted by Motiwala *et al.* (2005), in which the use of both G2 and TGG were recommended for MAP genotyping. However, these findings differ from those of Motiwala *et al.* (2006), who found that application of the G2 repeat as a single SSR (G-repeat) locus was insufficient to differentiate MAP isolates from the same animal species. The combination of both G2 Loci and MIRU2 in the present study reveals a promising and efficient method for molecular genotyping of MAP. Our results showed that 29 isolates contained a similar 7g-4ggt profile that was also found to dominate cattle herds in Ohio (Motiwala *et al.*, 2005). It is also worth reporting that MAP isolates suspected to cause Crohn's disease have a similar profile (7g-4ggt) (Ghadiali *et al.*, 2004). Such clustering of human isolates and isolates derived from animal species indicates the presence of inter- and intra-species transmission and correlates the animal MAP isolates with the pathobiology of Crohn's disease (Motiwala *et al.*, 2006). However, it is still not known if these strains have different virulence mechanisms or even zoonotic.

A higher degree of discrimination among MAP isolates can be achieved by combining the use of both MIRU2 and SSR. This may provide the foundation for development of a highly discriminatory typing approach for the differentiation of strains among isolates of MAP and indicates that the focus of future studies should be the development of planned control programs and management strategies designed to interrupt the transmission of the pathogen to susceptible cattle. The presence of a major shared genotype among MAP isolates within herds and the stability of these loci suggests that SSRs can be used in epidemiological studies to track transmission pathways of Johne's disease (Harris *et al.*, 2006).

The results obtained here revealed a relationship between MIRU3 and VNTR 1658. Another correlation between group 1 (VNTR1658), group I (MIRU2), and group a (MIRU3) was also observed. Furthermore, all strains with a G1 profile of greater than 7 were found to have the same GGT profile (5GGT). Such relationships may indicate different evolutionary lines among MAP strains in Germany; however, the pathological importance of such relationships is not clear. Both the MIRU4-ARG and VNTR3527 reactions are not recommended for genotyping due to their limited polymorphism and poor discriminatory role. In addition, the other genotyping reactions of VNTR and MIRU used in this study appear to be of limited discriminatory value. Although MIRU4-ARG yielded size polymorphisms in one strain, which is in disagreement with results of a study conducted by Romano *et al.* (2005), MIRU5-UK, MIRU6, and MIRU7 did not show size variations in any investigated strains, in disagreement with the results of the same study. These 4

MIRUs are not suitable for MAP genotyping, but are useful for identifying *M. avium* subsp. *hominissuis*. These results suggest that SSR is more discriminative than MIRU and VNTR, as indicated by the Simpson index. Harris *et al.* (2006) speculated that mixed infection of single animals with different MAP genotypes at the same time is possible, but rare. This may occur when introducing an infected, but apparently healthy, animal into dairy herds during herd replacements. This animal will later serve as a source of infection in the farm (Sweeney, 1996). Rodents, insects and wild animals also serve as a hidden source for MAP transmission into MAP free dairy herds. The presence of more than one MAP genotype in a sample obtained from an animal may also indicate pass through phenomena. In such cases, the MAP infected animal ingests feed contaminated with another MAP genotype and the organism is later shed in the feces without infecting the animal (Crossley *et al.*, 2005).

While the present study was being conducted, Thibault *et al.* (2008) evaluated the combined use of MIRU-VNTR with the use of SSR and IS*900* RFLP for the typing of MAP isolates in France. Contrary to the study conducted by Thibault *et al.* (2007, 2008), our MAP field strains were isolated from a limited geographical region and from only one species of animals to compare the efficiency of different genotyping methods in closely related MAP isolates. The combination of 8 MIRU-VNTR used by Thibault *et al.* (2007, 2008) could be used to divide the investigated MAP isolates (n=127) into 13 different groups, while the use of 5 SSRs for the same purpose yielded 19 different groups. Conversely, the use of 3 different MIRU-VNTR and 3 SSRs in the present study resulted in classification of the MAP isolates (n=34) into 3 and 10 groups, respectively. Although Thibault and his team used different MIRU-VNTR sequences from those used in the present study, their results are still in agreement with ours. Overall, the results of this study demonstrate that the combined use of MIRU-VNTR and SSR for the genotyping of MAP is promising and can be used to replace the classical techniques currently in use. However, the high cost of sequencing and the absence of a DNA sequencer from most laboratories may limit the widespread use of SSR in the near future. Finally, based on the results of the present study, the use of MIRU2 and the SSRs (G1, G2, and GGT) is recommended for genotyping of MAP. A combination of these systems can be used as a useful model for epidemiological studies.

Additional studies to evaluate the applicability of the recommended combination of SSR-MIRU2-MIRU3-VNTR1685 systems for the large epidemiological screening of MAP in dairy herds in Hesse County, Germany are currently being conducted.

Acknowledgements

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