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First comprehensive study on molecular diversity of Austrian *Mycobacterium avium* subspecies *paratuberculosis* isolates from domestic and wild ruminants

Erste umfangreiche Studie zur molekularen Diversität von Mycobacterium avium subspecies paratuberculosis Isolaten von Haus- und Wildwiederkäuern aus Österreich

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Summary

The aim of this study was to gain knowledge of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) diversity, distribution and possible transmission in Austria. 249 isolates derived from 221 cattle (107 herds), 19 goats (one herd), one sheep and seven wild ruminants (red deer, roe deer, moufflon) were investigated. The isolates were subjected to Insertion Sequence *900* (IS*900*) based Restriction Fragment Length Polymorphism analysis (IS*900*-RFLP) and Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem-Repeat typing (MIRU-VNTR). Isolates originated from samples tested within the framework of the Austrian paratuberculosis control program and clinical diagnostics.
Using IS*900*-RFLP and restriction enzyme *Bst*EII seven types were found: C1, C14, C18, C28 and three new ones. All isolates belonged to the C-type. Using *Pst*I restriction six profiles were obtained, of which only P1 was already published before. Eight INRA Nouzilly MIRU-VNTR (INMV) profiles were detected by MIRU-VNTR (INMV 1, 2, 5, 6, 12, 13, 17, 80). The combination of IS*900*-RFLP and MIRU-VNTR allowed the discrimination between 20 combined MAP genotypes named AT (Austria) 1–20. AT 2 (C1-P1/INMV 2) was the most frequently found one, namely in 39% of the isolates. In nine out of 35 cattle herds with two or more isolates more than one combined MAP genotype was found. Examination of the seven wild ruminant isolates exhibited four combined genotypes also detected in local cattle and sheep herds suggesting MAP transmission among these species. The characterization of MAP using IS*900*-RFLP and MIRU-VNTR gives valuable information about diversity and distribution of MAP in Austria and of possible transmission routes.

Keywords: Paratuberculosis, Johne´s disease, genotyping, MAP transmission

Zusammenfassung

Ziel dieser Studie war, Erkenntnisse über die Diversität, die Verteilung und über mögliche Übertragungswege von *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Österreich zu erlangen. Es wurden insgesamt 249 Isolate von 221 Rindern aus 107 Herden, 19 Ziegen aus einer Herde, einem Schaf und sieben Wildwiederkäuern (Rotwild, Rehwild, Mufflon) untersucht. Die MAP-Isolate wurden mittels IS*900*-basierter Restriktions-Fragment-Längen Polymorphismus-Analyse (IS*900*-RFLP) und „Mycobacterial Interspersed Repetitive Unit-Variable-Number of Tandem-Repeat" Typisierung (MIRU-VNTR) differenziert. Die Isolate stammten aus dem österreichischen Paratuberkulose-Überwachungsprogramm und der Routinediagnostik.
Unter Verwendung von IS*900*-RFLP mit dem Restriktionsenzym *Bst*EII wurden sieben Typen gefunden: C1, C14, C18, C28 sowie drei bisher nicht veröffentlichte RFLP-Muster. Alle Isolate gehörten zum C-Typ. Die IS*900*-RFLP mit dem Restriktionsenzym *Pst*I zeigte sechs Profile, von denen fünf Profile neu waren. Mittels MIRU-VNTR wurden acht INMV-Profile (INMV 1, 2, 5, 6, 12, 13, 17, 80) nachgewiesen. Die Kombination von IS*900*-RFLP und MIRU-VNTR ermöglichte die Unterscheidung von 20 kombinierten MAP-Genotypen bezeichnet als AT (Austria) 1–20. AT 2 (C1-P1/INMV 2) trat am häufigsten auf (39% der Isolate). In neun von 35 Rinder-

herden von denen zwei oder mehrere Isolate untersucht wurden, wurde mehr als ein kombinierter MAP-Genotyp gefunden. Die Untersuchung der sieben Wildwiederkäuer-Isolate ergab vier kombinierte Genotypen, welche auch bei Rindern und einem Schaf detektiert wurden. Dies lässt auf eine MAP-Übertragung zwischen Haus- und Wildwiederkäuern schließen. Die Charakterisierung von MAP mit IS*900*-RFLP und MIRU-VNTR lieferte wertvolle Informationen zur Diversität und Verteilung von MAP in Österreich und zu möglichen Übertragungswegen.

Schlüsselwörter: Paratuberkulose, Johne´sche Erkrankung, Genotypisierung, MAP-Übertragung

Introduction

Paratuberculosis (Johne´s disease) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a chronic and therapy resistant infectious disease in domestic and wild ruminants spread worldwide (Chiodini et al., 1984; Barkema et al., 2010). Young animals are most susceptible for infection, mainly by oral ingestion of MAP via the contaminated udder of the dam or the intake of MAP from the young animal`s environment (Sweeney, 1996). Interspecies transmission between domestic ruminants and wildlife has been reported (Pavlik et al., 2000; Machackova et al., 2004; Kopecna et al., 2008; Fritsch et al., 2012). Wild species may even act as reservoir hosts or disseminators to farms (Kopecna et al., 2008; Stevenson et al., 2009; Fritsch et al., 2012; Gerritsmann et al., 2014). The results of Verdugo et al. (2014) suggested that cross-species transmission occurs, when close contact between animals is given. For instance, similar subtypes were shared by beef cattle and sheep grazing together. Pavlic et al. (2000) isolated identical RFLP types from cattle and wild ruminants sharing the same pasture. As the most probable reasons direct contact and faecal contamination of the grass have been mentioned.

In Austria the number of seropositive cattle rose in the last years (Baumgartner et al., 2005); the cases of MAP positive wild ruminants increased as well (Deutz et al., 2005). As there are no international regulations concerning Johne's disease, in 2006 a compulsory paratuberculosis control program by government regulation started in Austria. Clinical paratuberculosis became a notifiable disease in cattle, sheep, goats and farmed deer. Suspicious animals that are tested MAP positive with either ELISA or PCR have to be culled, on farm level management and hygienic measures have to be implemented (Khol et al., 2007).

Up till now two studies on MAP genotyping of a limited number of animal isolates mainly originating from the federal province Styria, were carried out in Austria (Deutz et al., 2005; Gerritsmann et al., 2014). 25 isolates from different wild ruminants and three cattle exhibited all the identical IS*900*-RFLP(*BstE*II)-pattern C1, and in addition 17 isolates showed also identical RAPD (random amplified polymorphic DNA-analysis) profiles (Deutz et al., 2005). Using MIRU-VNTR analysis on 39 MAP isolates, 15 genotypes were detected including also two identical profiles for wildlife and cattle isolates (Gerritsmann et al., 2014). The results of both studies indicate interspecies transmission.

The aim of the present study was the molecular characterization of a significant higher number of MAP isolates (n=249) originating from seven federal provinces in order to gain deeper knowledge of diversity, distribution and possible transmission routes on a national scale in Austria. The isolates were sampled from cattle, sheep, goats and wild ruminants within the framework of the Austrian paratuberculosis control program and from clinical diagnostics. In order to increase the discriminatory power of typing two typing techniques (IS*900*-RFLP and MIRU-VNTR) were used in combination.

Material and Methods

Origin of isolates

249 MAP isolates were selected from paratuberculosis control program samples and from samples sent to AGES IVET Linz for clinical diagnostics between 2006 and 2013. MAP was isolated from faecal and tissue samples of 221 cattle from 107 herds, of 19 goats from one herd, of one sheep and of seven wild ruminants (red deer, roe deer, moufflon). Animals originated from seven Austrian provinces: Vorarlberg (32 animals), Tyrol (37 animals), Salzburg (14 animals), Carinthia (eleven animals), Upper Austria (41 animals), Lower Austria (six animals) and Styria (107 animals) (Table 3).

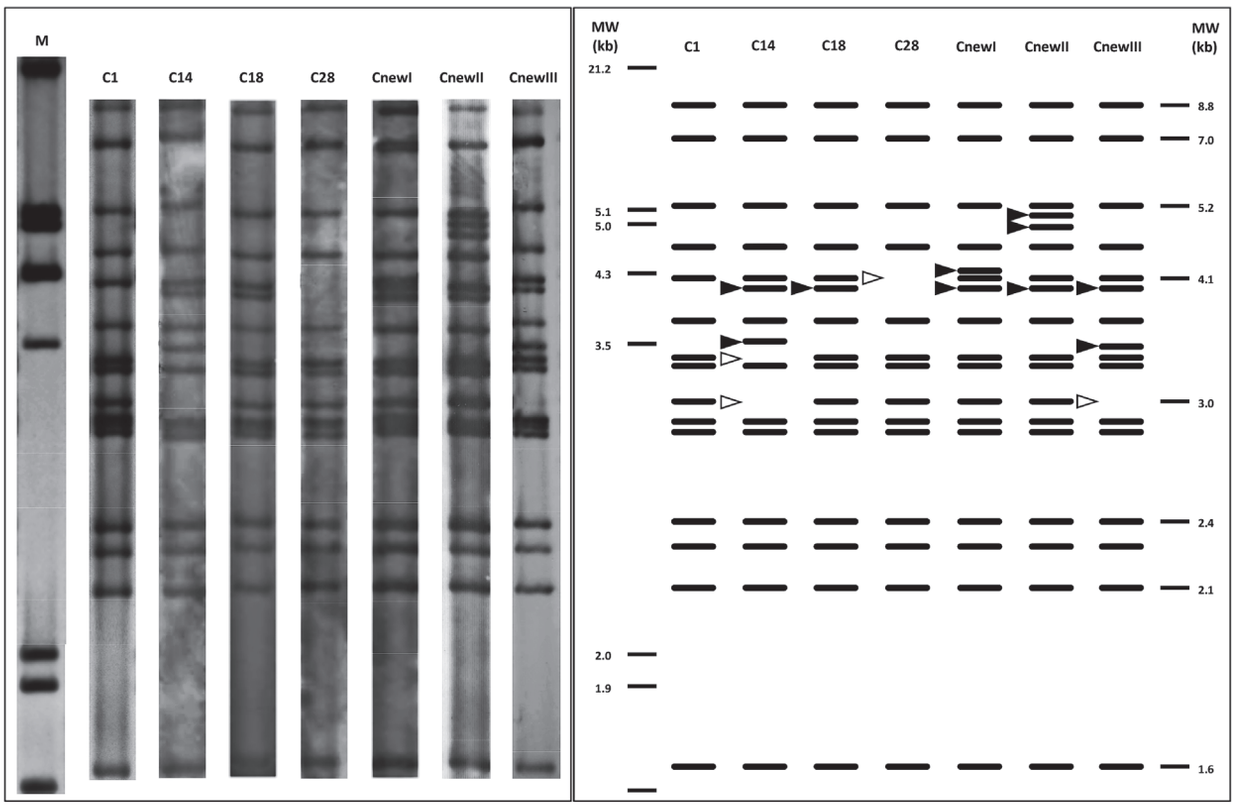
Bovine samples derived from four Austrian cattle breeds (Austrian Brown Mountain, Austrian Fleckvieh, Murbodner, Tyrolean Grey Mountain) as well as from six imported cattle breeds (Aberdeen Angus, Blonde d´Aquitaine, Holstein, Jersey, Limousin, Piedmontese). Data about sheep and goat breeds were not available.

Data collection

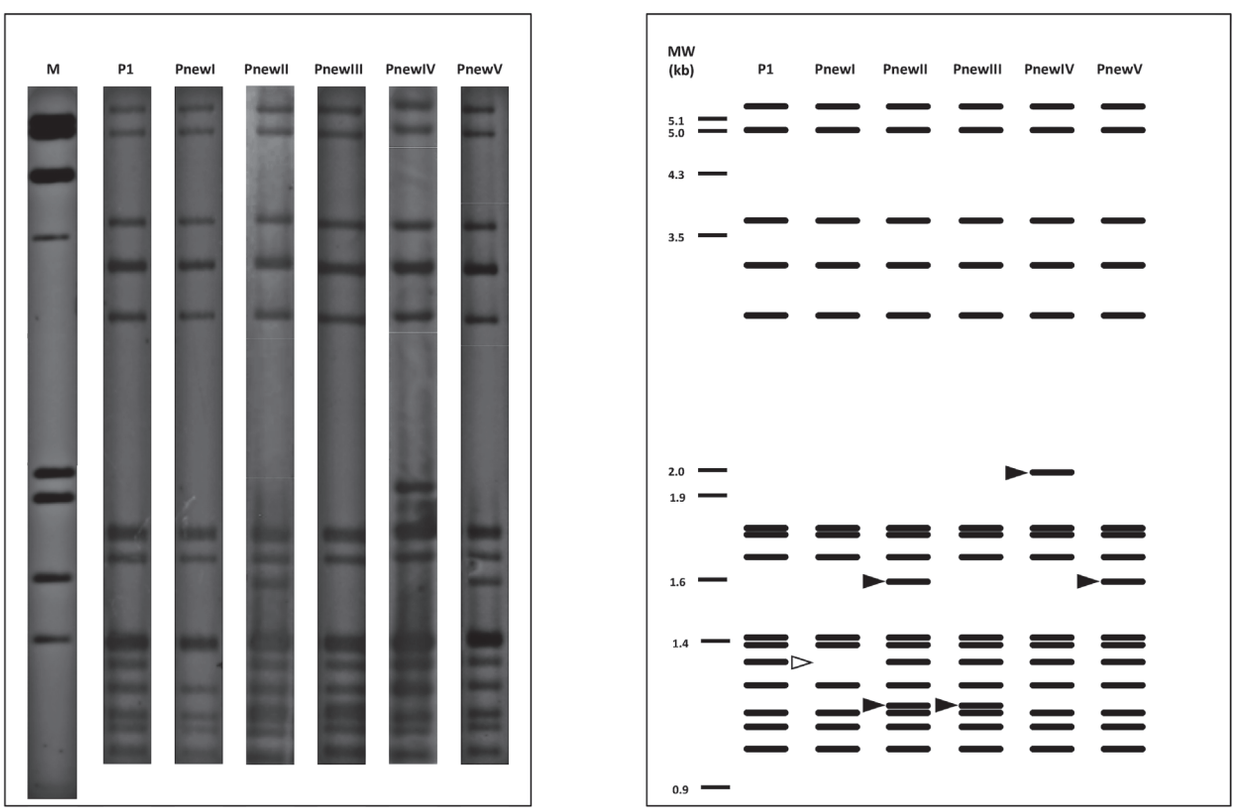
Information on the individual animal (ear tag number, date of birth, date of death, holding and breed) was gathered from the completed forms accompanying the samples. In cattle, information (date of birth, date of death, holding, breed and movements) could additionally be collected from eama (https://www.eama.at/), an internet service portal provided by Agrarmarkt Austria (AMA).

Detection of MAP positive samples

Faecal and tissue samples were tested for the presence of the DNA insertion segment IS*900* with the Adiavet® ParaTBC real-time PCR kit (Adiagene, Bio-X Diagnostics S.A., B; Donaghy et al., 2008) on a Light Cycler 2.0 (Roche Diagnostics International AG, CH). Samples with a Ct (Threshold cycle) below 40 were considered positive. Extraction of DNA from 1 g faeces/5 g tissue was performed combining the QIAamp DNA Mini Kit (Qiagen GmbH, G) with disruption by grinding using glass beads (Retsch GmbH, G) and a TissueLyser (Qiagen GmbH, G) following the instructions provided by Adiagene.



(A) *Bst*EII



(B) *Pst*I

FIGURE 1: *IS900-RFLP profiles of MAP isolates from Austria after digestion with BstEII (A) and after digestion with PstI (B). The numbers above the lanes are the type designations. Lane M represents the molecular weight marker III (Roche Diagnostics). The arrowheads in the schematic diagram show the differences between type C1 or P1 and the other profiles. Black arrowheads designate additional or shifted bands; white arrowheads mark the absence of individual bands within the specific patterns. The marker lane on the right side in (A) indicates the sizes of reference bands according to Overduin et al. (2004).*

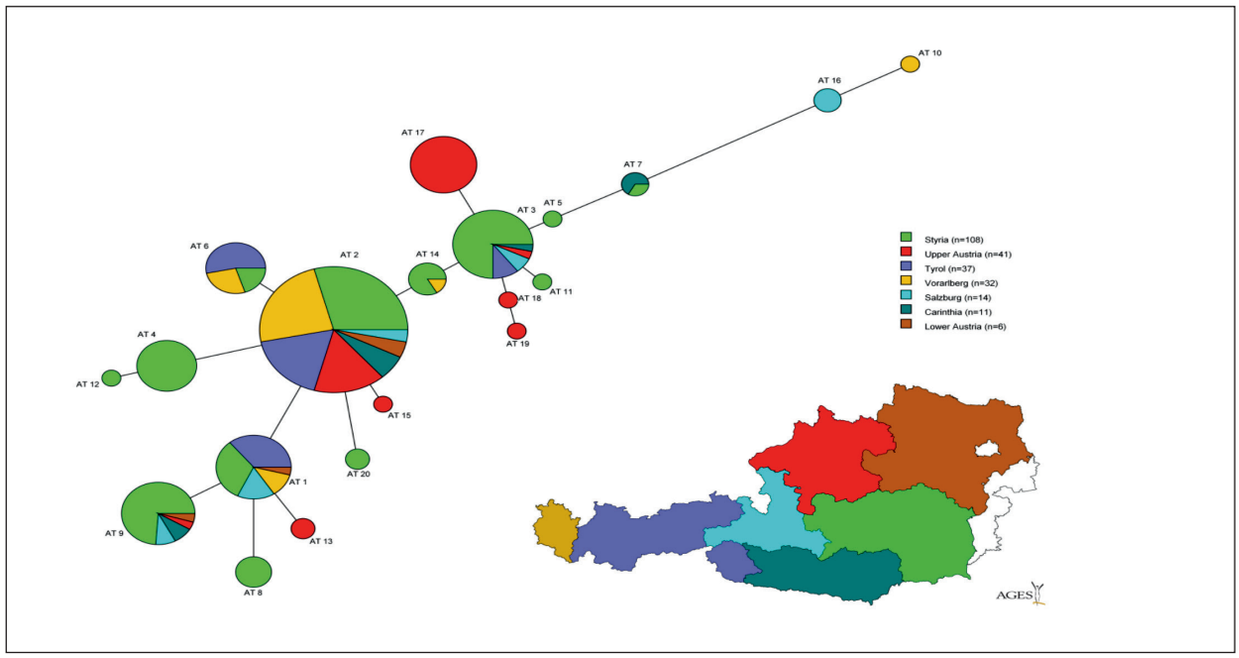


FIGURE 2: *Minimum spanning tree based on IS900 RFLP typing with two enzymes (BstEII, PstI) and MIRU-VNTR typing of eight loci of 249 isolates with map of the Austrian federal provinces. Each combined genotype is displayed as a node, dimensioned proportional to the number of isolates. Different colours represent the origin of isolates in seven Austrian federal provinces.*

Isolation of MAP by culture

IS*900* PCR positive tested samples were cultivated on Herrold's Egg Yolk agar slants with mycobactin J (HEYM) (Becton, Dickinson and Company, USA). The decontamination procedure was as follows: 2 g of faeces or thinly cut tissue were put into a 50 ml falcon tube with 30 ml of 0.75% hexadecylpyridinium chloride monohydrate (HPC) (Sigma-Aldrich Inc., USA), mixed for 30 min on a rotator and incubated for 24 to 48 h at room temperature. For better disruption tissue suspensions were filled into a blender bag, homogenized for 2 min in a lab blender and returned to the falcon tube prior to mixing. After the removal of crude particles by filtering through sterile gaze the samples were centrifuged at 2000 g for 15 min. About 200 µl of the sediment were inoculated onto HEYM, the agar slants were incubated at 37°C and checked for mycobacterial growth once a week for 3 months. Colonies were identified as MAP by mycobactin J-dependency, cultivation time, morphology and IS*900* PCR. Subsequently, subcultures for the preparation of genomic DNA were performed on HEYM. One colony per animal was used for subculture, except for one culture from cattle faeces showing morphologically quite distinct colonies. Here two isolates were subcultured and typed separately.

Isolation of genomic DNA

DNA was isolated by cetyltrimethylammonium bromide (CTAB) (Sigma-Aldrich Inc., USA) method according to van Soolingen et al. (1991). Concentration of genomic DNA was determined with an UV/VIS biophotometer (Eppendorf AG, G).

IS*900*-RFLP

For the preparation of the digoxygenin (DIG)-labeled IS*900* probe, DNA from the MAP reference strain DSMZ 44135 was isolated as described above and used as matrix in the labeling reaction. With primers published by Kunze et al. (1992) (Forward: 5`-TGG ACA ATG ACG GTT ACG GAG GTG G-3` and Reverse: 5`-GAT CGG AAC GTC GGC TGG TCA GGA T-3`) PCR was perfomed with DIG DNA Labeling Mix (Roche Diagnostics International AG, CH) according to the manufacturer`s instruction. After electrophoresis in a 1% agarose gel at 40 V for one hour the amplification product was cut out and purified using the QIAquick Gel Extraction Kit (Qiagen GmbH, G).

IS*900*-RFLP was performed as described by Moebius et al. (2008) using two restriction enzymes: *Bst*EII and *Pst*I (Fermentas by Thermo Fisher Scientific Inc., USA).

MIRU-VNTR

For PCR based MIRU-VNTR typing according to Thibault et al. (2007) eight polymorphic genomic loci were chosen: MIRU 292, X3 and VNTR loci 25, 47, 3, 7, 10, 32 (Biet et al., 2005; Thibault et al., 2007).

Analysis of typing results

IS*900*-RFLP patterns were analysed visually and compared with those published by Pavlik et al. (1999), Overduin et al. (2004), Moebius et al. (2008) and Fritsch et al. (2012). Patterns without matches were named Cnew and Pnew, respectively.

The number of repeats at MIRU-VNTR loci 292, X3, 25, 47, 3, 7, 10 and 32 yielded patterns were converted into INMV profiles (http://mac-inmv.tours.inra.fr) as defined by Thibault et al. (2007).

A minimum spanning tree was constructed using BioNumerics version 7.6 created by Applied Maths NV (available from http://applied-maths.com) to display the phylogenetic clustering of the combined genotypes (IS*900*-RFLP and MIRU-VNTR) and their distribution among seven different Austrian federal provinces.

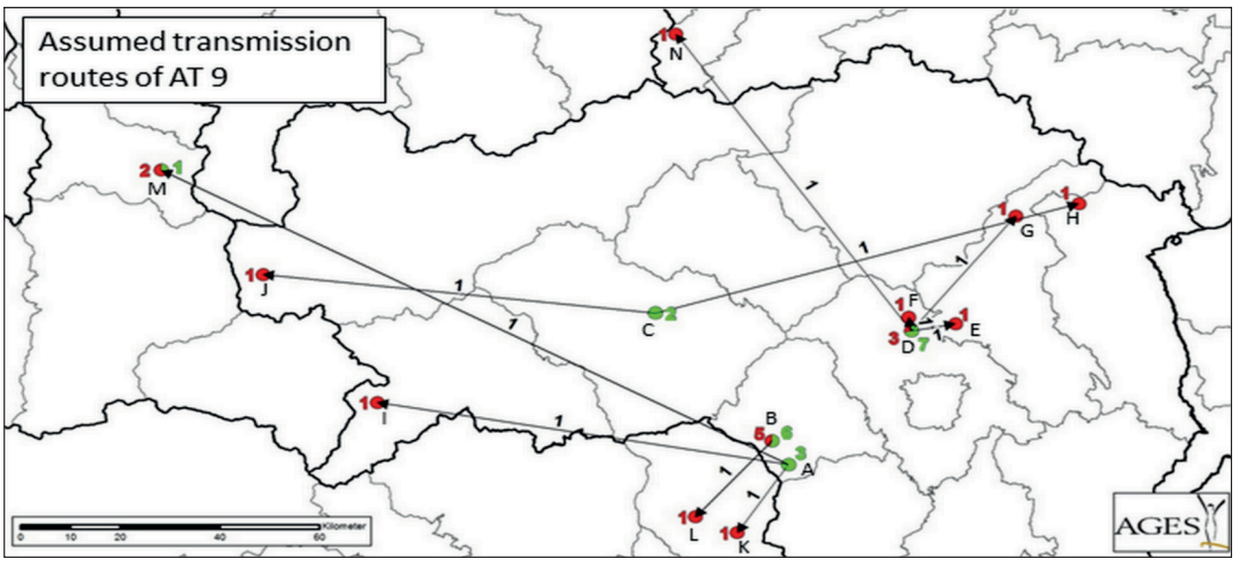


FIGURE 3: *Assumed transmission routes of genotype AT 9 via cattle from four farms (A–D) – holding of birth (localisation shown as green points) to 10 other farms (E–N) – holding of perished and culled animals (shown as red points) based on individual animal data, also collected by eama (https://www.eama.at/). Numerics in green: Number of AT 9 infected cattle born on farms A-D and M. Numerics in black: Number of AT 9 infected animals that were shipped from farms A–D to farms E–N. Numerics in red: Number of perished and culled animals on farms B, D–N.*

Results

Molecular characterization of isolates

Altogether, 249 MAP isolates were subjected to molecular characterization by IS*900*-RFLP and MIRU-VNTR. Seven *Bst*EII IS*900*-RFLP types were found. Four of them (C1, C14, C18, C28) were already described (Pavlik et al., 1999; Bartos et al., 2002), whereas three new profiles could be detected (CnewI, CnewII, CnewIII) (Fig. 1). Hence, all investigated isolates belong to the C-type. C1 was detected in 66% of the tested isolates (n=163), including all wild ruminant isolates and in 74% of the herds. IS*900*-RFLP using *Pst*I resulted in six profiles with P1 already described by Whipple et al. (1990) and Moebius et al. (2008), and five new ones (PnewI, PnewII, PnewIII, PnewIV, PnewV) (Fig. 1, Tab. 1). In seven herds more than one IS*900*-RFLP genotype (combination of *Bst*EII and *Pst*I digestion results) was detected (herd no.: 21, 24, 62, 64, 77, 95, 111) (Tab. 1, 3).

The MIRU-VNTR typing resulted in eight different INMV profiles (Tab. 1). The predominant pattern was INMV 2 in 63 herds and two wild ruminants, followed by INMV 1 in 31 herds and three wild ruminants. In three cattle herds more than one MIRU-VNTR pattern (herd no. 43 and no. 111: INMV 1 and 2, herd no. 80: INMV 2 and 17; see Tab. 3) were found.

Combination of IS*900*-RFLP and MIRU-VNTR

The combination of IS*900*-RFLP and MIRU-VNTR analysis results allowed a differentiation between 20 combined MAP genotypes designated as AT types 1 to 20, shown in Table 1. The order was chosen chronologically.

The distribution of genotypes in different animal species, herds and isolates are shown in Table 2. AT 2 (C1-P1/INMV2) was found most often, followed by AT 1 (C1-P1/INMV1), AT 9 (C18-PnewIII/INMV1), AT 4

TABLE 1: *AT type designation of combined genotypes (results of MIRU-VNTR and IS900-RFLP analysis) detected in this study and the number of herds and isolates exhibiting these genotypes*

INMV profile[a]	MIRU-VNTR repeats[b]	IS900-RFLP pattern	AT type	No. of herds/ regional origins[c]	No. of isolates[d]
1	42332228	C1-P1	**1**	17	25
1		C18-PnewIII	**9**	15	23
1		C28-PnewI	**13**	2	2
2	32332228	C1-P1	**2**	51	96
2		C1-PnewIV	**15**	1	1
2		C1-PnewV	**6**	5	15
2		C14-PnewIII	**18**	1	1
2		C18-P1	**14**	3	6
2		C18-PnewII	**5**	1	1
2		C18-PnewIII	**3**	9	28
2		CnewI-PnewIII	**11**	1	1
2		CnewII-PnewIII	**17**	1	19
2		CnewIII-PnewIII	**19**	1	1
5	42332218	C1-P1	**8**	3	5
6	32332128	C1-P1	**20**	2	2
12	22522228	C1-P1	**16**	1	3
13	22332228	C18-PnewII	**7**	2	3
17	31332228	C1-P1	**4**	11	15
17		C18-P1	**12**	1	1
80	22522226	C1-P1	**10**	1	1

[a] Designation according to the INRA Nouzilly MIRU-VNTR nomenclature for MIRU-VNTR patterns (Thibault et al., 2007)

[b] MIRU-VNTR repeats at loci 292, X3, 25, 47, 3, 7, 10, 32 (Thibault et al., 2007)

[c] Number of herds for cattle, sheep and goats; regional origin for wild ruminants. Seven herds with two and two herds with four different combined genotypes were detected

[d] One cattle found to be simultaneously infected with two MAP isolates with different combined genotypes. 249 isolates from 248 animals were investigated

TABLE 2: *Distribution of combined genotypes (AT type) on isolates originating from cattle, sheep, goats and wild ruminants*

AT type[a]	Cattle No. of		Sheep No. of		Goats No. of		Wild ruminants No. of	
	herds	isolates	herds	isolates	herds	isolates	regional origins	isolates
1	14	22					3	3
2	49	94					2	2
3	9	28						
4	9	13	1	1			1	1
5	1	1						
6	5	15						
7	2	3						
8	2	4					1	1
9	15	23						
10	1	1						
11	1	1						
12	1	1						
13	2	2						
14	3	6						
15	1	1						
16	1	3						
17					1	19		
18	1	1						
19	1	1						
20	2	2						

[a] Combined genotype designation (Tab. 1)

(C1-P1/INMV17), AT 3 (C18-PnewIII/INMV2), and AT 6 (C1-PnewV/INMV2). Nine genotypes were identified only in single herds. Altogether, 19 genotypes were detected in cattle (AT 1–16, 18–20), four genotypes in wild ruminants (AT 1, 2, 4 and 8), genotype AT 4 in sheep, and genotype AT 17 in all 19 isolates from the single goat herd. Consequently, the genotypes detected in wild ruminant and sheep isolates were also found in cattle.

In Figure 2 the phylogenetic clustering of the combined genotypes among Austrian federal provinces is depicted. AT 2 was found in all seven, AT 1, AT 3, and AT 9 in five, AT 6 in three, AT 7 and AT 14 in two federal provinces. The other 13 genotypes were each detected only in one province.

In nine herds more than one combined genotype was found: two AT types in seven herds (herd no. 21, 24, 43, 62, 64, 77, 80) and four AT types in two herds (herd no. 95, 111) (Tab. 3).

Out of 222 cattle isolates, the majority of the genotyped isolates originated from Limousin (n=93), Holstein (n=55) and Austrian Fleckvieh (n=38) (Tab. 4). As found for the distribution of genotypes in all federal provinces, AT 2 was also the most frequently detected one in these three cattle breeds, whereas AT 9 was found in 22 Limousin cattle and only one Austrian Fleckvieh (Tab. 4).

Transmission routes

Using individual animal data from eama it was possible to trace transmission routes of specific MAP genotypes between cattle herds in Austria. As one example, Figure 3 shows the assumed transmission routes of genotype AT 9 (C18-PnewIII/INMV1). This genotype was isolated as mentioned before from 23 cattle. All these animals perished due to the main symptoms of paratuberculosis (emaciation and diarrhea) or were culled within the framework of paratuberculosis control program. Twenty-two out of these 23 cattle (21 Limousin and one Austrian Fleckvieh) from 15 farms, had their origin in one federal province. Eighteen out of these 22 cattle were born on only four different farms (Fig. 3, farm A–D). Additionally, two of these four farms are located in the same village (Fig. 3, farm A and B). Ten animals from farm A-D were shipped to ten farms (farm E-N) in four federal provinces, where all of them were notified as suspicious of clinical paratuberculosis by the district veterinarian either on the farm or in the abattoir and confirmed as MAP-infected by ELISA and/or PCR (Fig. 3, Farm E–N). Furthermore, on Farm M, seven years after the purchase of a Limousin bull from Farm A in 2006 and its culling in 2008 after confirmation of paratuberculosis, another case of paratuberculosis in a Limousin cow, born in 2010, was diagnosed (Fig. 3). Isolates of all these animals were typed as AT 9.

The second example concerns genotype AT 13. AT 13 was only found twice, in two animals of the Limousin breed of two different farms. Using data provided by eama it could be shown that both cattle had their origin in one individual herd.

As third, for genotype AT 1 (C1-P1/INMV1) isolated from four investigated Tyrolean Grey Mountain cattle and two red deer in the same federal province, epidemiological links could also be presumed based on data from eama. The four cattle were born on three farms. Two of these farms located in the same village. Two animals of the same age group born on these two farms, spent the summer months of two subsequent years together on the same mountain pasture. In addition, one red deer found in the same district and another one in the same federal province were also infected with genotype AT 1.

TABLE 3: *Origin of MAP isolates from different federal provinces in Austria and AT type*

Federal province	Herd designation/Regional origin[a]	No. of herds/ regional origins	No. of isolates	Host animal	Cattle breed[b]	AT type[c]
Vorarlberg	1, 2, 3, 5, 9, 10, 11, 12, 13, 14, 15, 17, 19	13	23	c, wr	BM, FV, H, J	2
Vorarlberg	4	1	1	c	H	10
Vorarlberg	6, 8	2	3	c	H, BM	1
Vorarlberg	7	1	1	c	J	14
Vorarlberg	16, 18	2	4	c	H, BM	6
Tyrol	20, **21**[d], 23, **24**[d], 26, 27, 28, 30, 31, 34, 36	11	17	c	FV, H, J,	2
Tyrol	**21**[d]	1	3	c	J	3
Tyrol	22, 25, 29, 32, 33, 35, 37, 38	8	9	c, wr	GM, FV, BM	1
Tyrol	**24**[d]	1	8	c	H	6
Salzburg	39	1	3	c	H	16
Salzburg	40	1	2	c	FV, H, J,	3
Salzburg	41, 42, **43**[d]	3	3	c	L	2
Salzburg	**43**[d]	1	4	c	L	1
Salzburg	44	1	2	c	L	9
Carinthia	45	1	2	c	BA	7
Carinthia	46, 47, 48, 50, 52, 54	6	6	c	FV, L, H	2
Carinthia	49	1	1	c	FV	3
Carinthia	51, 53	2	2	c	L	9
Upper Austria	55	1	1	c	L	18
Upper Austria	56	1	2	c	L	19
Upper Austria	57	1	19	g		17
Upper Austria	58, 65	2	2	c	L	13
Upper Austria	59, 60, 61, **62**[d], 63, **64**[d]	6	14	c	FV, AA, J, L, H	2
Upper Austria	**62**[d]	1	1	c	J	15
Upper Austria	**64**[d]	1	1	c	FV	3
Upper Austria	66	1	1	c	L	9
Lower Austria	67	1	1	c	BA	1
Lower Austria	68, 69, 71, 72	4	4	c	L, FV	2
Lower Austria	70	1	1	c	L	9
Styria	73, 94, 106, 108, 113	5	8	c, wr	FV, J, L, P	1
Styria	74, 91	2	3	c	BA	6
Styria	75, 81, 83, 88, 89, 92, 105, 109, 110, **111**[d]	10	17	c	L, FV	9
Styria	76, **77**[d], 78, 79, **80**[d], 87, 96, 97, 99, 112, 116	11	15	c, wr, s	FV, L, M	4
Styria	**77**[d]	1	1	c	M	12
Styria	**80**[d], 85, **95**[d], 101, 102, 103, **111**[d], 114	8	28	c, wr	AA, FV, L	2
Styria	82, 93, **95**[d], 98, **111**[d]	5	21	c	L, M	3
Styria	84, 107, 115	3	5	c, wr	FV	8
Styria	86, 100	2	2	c	L, FV	20
Styria	90	1	1	c	FV	5
Styria	**95**[d]	1	1	c	L	11
Styria	**95**[d], **111**[d]	2	5	c	L	14
Styria	104	1	1	c	H	7

[a] Herd designation 1-18, 20-36, 39-56, 58-111 was used for description of individual origin of cattle (c), 112 for sheep (s), 57 for goats (g); Regional origin 113-116 was applied for wild ruminants (wr)

[b] Cattle breed: Aberdeen Angus (AA), Austrian Brown Mountain (BM), Austrian Fleckvieh (FV), Blonde d´Aquitaine (BA), Holstein (H), Jersey (J), Limousin (L), Murbodner (M), Piedmontese (P), Tyrolean Grey Mountain (GM)

[c] Combined genotype designation (Tab. 1)

[d] Herds found to be sumultaneously infected with more than one strain

Discussion

IS*900*-RFLP (*BstE*II) C1 type was the most common genotype found in the present study. This corresponds with results of Deutz et al. (2005) who detected exclusively C1 patterns and of Stevenson et al. (2009), who found this type most frequently in isolates from other European countries. Moebius et al. (2008) reported about 43% of German cattle isolates and 79% of the tested herds exhibiting type C1. In the current study the combination with *Pst*I digestion divided this dominant type into three (C1-P1, C1-PnewIV, C1-PnewV) while the combination with MIRU-VNTR gave seven subtypes, altogether nine combined genotypes. The second most common IS*900*-RFLP profile in this study was C18, detected in 25% of all isolates and 26% of the herds and only in cattle. It was divided into six combined genotypes applying MIRU-VNTR. In Germany C18 was only reported once in one isolate (Moebius et al., 2008).

Gerritsmann et al. (2014) yielded 15 MIRU-VNTR genotypes for only 39 isolates, mainly from different wild ruminant species from the eastern Alpine region in Styria, Austria. In the current study six of these profiles (INMV 1, 2, 5, 6, 13, 17) were also found in isolates from cattle, wild ruminants and sheep originating from Styria (Tab. 1, Tab. 3).

TABLE 4: *Distribution of combined genotypes (AT) in different Cattle breeds*[a]

AT type[b]	total No. of		L No. of		H No. of		FV No. of		J No. of		BA No. of		BM No. of		AA No. of		GM No. of		M No. of		P No. of	
	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates	herds	isolates
1	14	22	2	5	1	2	4	6	1	1	1	1	2	2			3	4			1	1
2	49	94	16	32	22	37	8	13	3	4			2	3	2	5						
3	9	28	4	20			3	4	1	3									1	1		
4	9	13	3	3			5	8											2	2		
5	1	1					1	1														
6	5	15			3	11					2	3	1	1								
7	2	3			1	1					1	2										
8	2	4					2	4														
9	15	23	14	22			1	1														
10	1	1			1	1																
11	1	1	1	1																		
12	1	1																	1	1		
13	2	2	2	2																		
14	3	6	2	5					1	1												
15	1	1							1	1												
16	1	3			1	3																
17																						
18	1	1	1	1																		
19	1	1	1	1																		
20	2	2	1	1			1	1														

[a] Cattle breed: Aberdeen Angus (AA), Austrian Brown Mountain (BM), Austrian Fleckvieh (FV), Blonde d´Aquitaine (BA), Holstein (H), Jersey (J), Limousin (L), Murbodner (M), Piedmontese (P), Tyrolean Grey Mountain (GM)

[b] Combined genotype designation (Table 1)

As expected, the application of two restriction enzymes in combination with MIRU-VNTR results in a better discrimination; altogether 20 combined genotypes were identified in this study. Up till now, in Germany more than 60 combined MAP genotypes have been found (Moebius, unpublished results). In Austria, the close trade relation between farms and the common grazing of cattle from different herds may have a major impact on the lower heterogeneity of MAP isolates in cattle. All in all, combined genotype AT 2 (C1-P1/INMV 2), detected in 39% of the Austrian isolates and 46% of the tested cattle herds, was found in all seven included federal provinces, AT 1 (C1-P1/INMV 1), AT 3 (C18-PnewIII /INMV 2) and AT 9 (C18-PnewIII /INMV 1) in five federal provinces (Fig. 2).

Three combined genotypes: AT 1, 2 and 8 (C1-P1/INMV 5) were found in cattle as well as in wild ruminants. In addition, combined genotype AT 4 (C1-P1/INMV 17) was detected in cattle, wild ruminants and also sheep. These findings point to a possible interspecies transmission. In Austria the practice of common grazing on mountain pastures has a considerable impact on transmission between herds and local wildlife (Deutz et al., 1995; Khol et al., 2007).

In the present study goats from one herd were found to be infected all with one combined genotype, AT 17 (CnewII-PnewIII/INMV 2), exhibiting new IS*900*-RFLP *Bst*EII and *Pst*I profiles, not found in any cattle, sheep or wild ruminant isolate. No information could be gathered about the origin of these animals. More caprine isolates have to be tested to verify if goats in Austria exhibit specific genotypes or share also common ones. In contrast to these findings, Djonne et al. (2005) found identical genotypes (IS*900*-RFLP patterns) among Norwegian goat and cattle isolates, suggesting interspecies transmission.

Molecular characterization is a useful tool for answering particular epidemiological questions (Collins, 2010); three examples for tracing transmission routes of combined genotypes (AT 1, AT 9 and AT 13) supported by individual cattle movement data are presented here. These data demonstrate that identical combined genotypes of MAP isolates originating from different herds or regions can reflect MAP transmission in the past. So these applied genotyping methods (MIRU-VNTR + IS*900*-RFLP) present a useful tool for tracing transmission.

National and international trade of animals plays an important role in spreading the disease. For epidemiological studies on a larger scale, standardization of typing methods would be essential. Currently, comparing the results of publications on MAP typing is rendered difficult due to different typing techniques.

42% of the genotyped isolates differentiated in eleven combined genotypes originate from Limousin cattle. About 41% of MAP positive tested cattle within the paratuberculosis control program belong to this breed (data not yet published). Taking into account that only approximately 2% of the cattle in Austria are Limousin (https://zar.at/Rinderzucht-in-Oesterreich/Rinderrassen/weitere-Rassen/Limousin.html) it seems that paratuberculosis may present a considerable problem in Austrian Limousin farming. Deutz et al. (1995) described an increased occurrence of clinical paratuberculosis in Styrian Limousin farms in the early 1990s. The import of serologically positive cattle from Luxembourg, Denmark and France was mentioned as the probable cause. Limousin cattle are usually raised in suckler cow husbandry, where the calves are kept together with the cows; Newborn calves are most susceptible to infection, especially when they are suckled by dams shedding the organism (Sweeney, 1996). According to Sweeney (1996), most of the herds acquire MAP because of the purchase of infected animals, which is later a source of infection in the new herd. As shown in Figure 3, plausible routes of transmission of MAP between Limousin farms could be demonstrated with isolates of combined genotype AT 9.

In this study, nine out of 35 herds were infected with more than one combined MAP genotype: Two herds with four and seven herds with two combined genotypes. These results correspond with various studies (Moebius et. al, 2008; Sevilla et al., 2008; van Hulzen et al., 2011; Fritsch et al., 2012), where more than one MAP strain within one herd were observed.

Furthermore, from one faecal sample of a cattle two different combined MAP genotypes AT 2 (C1-P1/INMV 2) and AT 3 (C18-PnewIII/INMV 2) were isolated. The cattle came from herd no. 95, where four combined genotypes (AT 2, AT 3, AT 11, AT 14) were detected (Tab. 3). Gerritsmann et al. (2014) reported 15% of the MAP isolates to be multiple strains exhibiting multiple bands in single MIRU-VNTR-PCR products. In contrast, there was no occurrence of double bands for isolates genotyped by MIRU-VNTR in the present study. The two genotypes AT 2 and AT 3 showed different RFLP results but the same MIRU-VNTR profile.

Conclusion

The Austrian paratuberculosis control program made it possible to collect a large number of MAP isolates from different regions and four ruminant species over eight years. Furthermore, this is the first comprehensive study providing data about characterization of bovine MAP strains on a national scale in Austria. The combination of MIRU-VNTR and IS*900*-RFLP analysis allowed a differentiation of 249 MAP isolates into twenty combined genotypes. Four identical combined genotypes were found in domestic as well as wild ruminants indicating interspecies transmission. AT 2, the most common genotype in Austria (C1-P1/INMV 2) was found in 46% of all investigated herds. Some herds and also one individual cattle were infected by more than one strain. Based on data on individual animals and their movements, combined genotyping revealed complex transmission ways of three combined MAP genotypes. In summary, combined genotyping of MAP provides valuable information about diversity and possible epidemiological links.

Conflict of interest

The authors declare that they have no conflict of interest.

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