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New Variable-Number Tandem-Repeat Markers for Typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* Strains: Comparison with IS*900* and IS*1245* Restriction Fragment Length Polymorphism Typing▽†

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Mycobacterium avium* subsp. *paratuberculosis*, the etiological agent of paratuberculosis, affects a wide range of domestic ruminants and has been suggested to be involved in Crohn's disease in humans. Most available methods for identifying and differentiating strains of this difficult species are technically demanding and have limited discriminatory power. Here, we report the identification of novel PCR-based typing markers consisting of variable-number tandem repeats (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs). Eight markers were applied to 183 *M. avium* subsp. *paratuberculosis* isolates from bovine, caprine, ovine, cervine, leporine, and human origins from 10 different countries and to 82 human isolates of the closely related species *M. avium* from France. Among the *M. avium* subsp. *paratuberculosis* isolates, 21 patterns were found by MIRU-VNTR typing, with a discriminatory index of 0.751. The predominant R01 IS*900* restriction fragment length polymorphism type, comprising 131 isolates, was divided into 15 MIRU-VNTR types. Among the 82 *M. avium* isolates, the eight MIRU-VNTR loci distinguished 30 types, none of which was shared by *M. avium* subsp. *paratuberculosis* isolates, resulting in a discriminatory index of 0.889. Our results suggest that MIRU-VNTR typing is a fast typing method that, in combination with other methods, might prove to be optimal for PCR-based molecular epidemiological studies of *M. avium*/*M. avium* subsp. *paratuberculosis* pathogens. In addition, presumably identical *M. avium* subsp. *paratuberculosis* 316F vaccine strains originating from the Weybridge laboratory and from different commercial batches from Mérial actually differed by one or both typing methods. These results indicate a substantial degree of genetic drift among different vaccine preparations, which has important implications for prophylactic approaches.

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of a severe gastroenteritis in ruminants known as Johne's disease, or paratuberculosis, since 1895 (11). Paratuberculosis is prevalent in domestic animals worldwide and has a significant impact on the economy. Recent studies have also described *M. avium* subsp. *paratuberculosis* isolation from wildlife (16), including rabbits (8). In addition, it has been suggested that *M. avium* subsp. *paratuberculosis* may be involved in Crohn's disease, a chronic enteritis in humans, but evidence for a causal link remains controversial (23). Therefore, paratuberculosis is considered a public health concern. Control of this disease requires a better knowledge of the causative agent and of its epidemiology, interspecies transmission, and biodiversity within *M. avium* subsp. *paratuberculosis* strains.

Study of *M. avium* subsp. *paratuberculosis* is hampered by the difficulty of growing and manipulating the organism in a laboratory setting. *M. avium* subsp. *paratuberculosis* is an extremely slow-growing organism and requires the addition of the iron chelator mycobactin for in vitro growth (2), and most bovine strains require 4 to 6 months of incubation. *M. avium* subsp. *paratuberculosis* strains are very difficult to isolate from sheep and humans and may require years to produce colonies. Therefore, small numbers of *M. avium* subsp. *paratuberculosis* isolates have been maintained in available collections, which has limited biodiversity studies.

Another limiting factor has been the lack of convenient discriminatory typing methods. The most widely used method to type *M. avium* subsp. *paratuberculosis* isolates is restriction fragment length polymorphism (RFLP), with detection of polymorphisms by hybridization to IS*900* (IS*900* RFLP typing) (24). As it is applicable only to cultivable strains, this method is slow and technically demanding. Moreover, it requires analysis of complex banding patterns and has limited discrimina-

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tory power. Therefore, rapid and discriminatory molecular typing methods need to be assessed as alternatives for studying the diversity of *M. avium* subsp. *paratuberculosis* strains. Motiwala et al. have recently reviewed the current genotyping methods used for determining genetic diversity within a population of *M. avium* subsp. *paratuberculosis* isolates (17).

Tandem-repeat (TR) sequences represent one of the rare categories of polymorphic structures in the genomes of highly monomorphic species, such as *Bacillus anthracis* and *Yersinia pestis* (12). Variable-number TRs (VNTRs), in particular those of genetic elements called mycobacterial interspersed repetitive units (MIRUs), have been discovered and used for typing of various mycobacterial species, including the *Mycobacterium tuberculosis* complex, *Mycobacterium marinum*, and *Mycobacterium ulcerans* (21, 25–27). Recently, partial genome screenings have identified a limited number of MIRU-VNTR loci in the *M. avium-Mycobacterium intracellulare* complex, providing very limited discrimination among *M. avium* subsp. *paratuberculosis* isolates (4, 18).

The aim of this study was to identify novel MIRU-VNTR loci based on an exhaustive screening of TR loci in the *M. avium* subsp. *paratuberculosis* genome and to study their variability in a large collection of *M. avium* subsp. *paratuberculosis* and *M. avium* isolates obtained from different hosts and from different geographic origins. The discrimination provided by the novel MIRU-VNTR loci was compared to that achieved by IS*900* RFLP and IS*1245* RFLP typing.

MATERIALS AND METHODS

Strains. *M. avium* subsp. *paratuberculosis* isolates were isolated on Herrold's egg yolk medium containing mycobactin J, amphotericin B, and nalidixic acid (Becton Dickinson, Le Pont de Claix, France) according to the method of Whipple et al. (29). Mycobacterial isolates were subcultured in Middlebrook 7H9 broth supplemented with Middlebrook albumin-dextrose-catalase enrichment medium (Becton Dickinson, Le Pont de Claix, France) and 2 μg/ml of mycobactin J (Institut Pourquier, Montpellier, France) when required. A panel of 183 *M. avium* subsp. *paratuberculosis* isolates was assembled (see Table S1 in the supplemental material) from 10 countries and different host species. The *M. avium* isolates included in the present study were obtained from the Institute Pasteur of Paris, Laboratoire de Référence des Mycobactéries, Paris, France (see Table S6 in the supplemental material). These isolates were all isolated from blood samples recovered from 93 AIDS patients over several months (14) and were typed by serotyping, by IS*1245* RFLP analysis, in some cases by RFLP analysis with plasmids pVT2 and pLR7 as probes, and by pulsed-field gel electrophoresis (20).

The *M. avium* subsp. *paratuberculosis* vaccine strains analyzed in the present study were obtained from the Veterinary Laboratories Agency Weybridge laboratory and from various batches of the vaccine Néoparasec (Mérial, Bourgelat, France).

Preparation of mycobacterial DNA. Mycobacterial DNA was obtained according to the method of Baulard et al. (3). *M. avium* DNA for PCR amplifications was obtained from strains preserved at −20°C in Youmans medium as follows: 0.2 ml of the medium was centrifuged for 10 min at 6,000 × *g*, and the pellet was washed twice with 0.2 ml of Tris-EDTA buffer and then resuspended in 0.2 ml of Tris-EDTA buffer. The bacteria were heat killed for 30 min at 95°C, and the DNA from the supernatant was directly used as a template.

Molecular identification of *M. avium* and *M. avium* subsp. *paratuberculosis*. All *M. avium* subsp. *paratuberculosis* isolates were screened for the presence or absence of IS*900* and IS*901* insertion sequences. Synthetic oligonucleotides (Sigma), described by Sanderson et al. (22) for IS*900* primers and by Inglis et al. (10) for IS*901* primers, were used.

IS*900* RFLP typing. IS*900* RFLP typing of *M. avium* subsp. *paratuberculosis* DNA was performed as previously described by van Soolingen et al. (28), with some modifications. The IS*900* DNA probe was prepared by PCR amplification of a 707-bp fragment of the IS*900* insertion sequence specific for *M. avium* subsp. *paratuberculosis* using the primers described by Overduin et al. (18). PCRs were performed starting from 10 ng of chromosomal DNA of *M. avium* subsp. *paratuberculosis* strain ATCC 19698 by using a Bio-Rad iCycler thermal cycler. The PCR product was purified on Qiaquick spin columns (QIAGEN) according to the manufacturer's instructions. The probe was biotin labeled with the NEBlot Phototope kit (New England Biolabs) by following the instructions of the manufacturer.

Digestion was performed with 3 μg of DNA prepared as described above and 7 U of BstEII (Promega) at 37°C for at least 4 h. Fragments were resolved by agarose gel electrophoresis and transferred onto Immobilon-S nylon membranes (Millipore) by vacuum transfer with the Vacu-Gene system (Pharmacia LKB Biotechnology). Detection of DNA fragments hybridizing with the biotinylated probe was performed with the Phototope-Star detection kit for nucleic acids (New England Biolabs), according to the manufacturer's instructions. A photobiotinylated mixture of HindIII-digested lambda DNA and HaeIII-digested ϕX174 DNA at a concentration of 100 ng/μl (New England Biolabs) was used as a molecular size marker.

Analysis of RFLP patterns was performed according to the methods of Overduin et al. (18) and other studies (5, 19). Conserved bands of 8.8, 5.2, 3.0, 2.4, 2.1, and 1.6 kb in the IS*900* RFLP pattern were used as internal standards for the normalization of RFLP patterns.

Identification of TR and MIRU loci. The published genomic sequence of *M. avium* subsp. *paratuberculosis* strain K10 (13) (GenBank accession number NC_002944 [http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=380]) was used to identify MIRU and potential VNTR sequences. TRs were identified by using the Tandem Repeats Finder software of the Laboratory for Biocomputing and Informatics, Boston University (http://tandem.bu.edu/trf/trf.submit.options.html), under the default settings of the program. MIRU loci were identified by searching sequences homologous to those of previously described MIRU loci in the *M. tuberculosis* H37Rv chromosome (27) using the BLAST 2.2.11 software at the NCBI website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

MIRU-VNTR typing. Primers designed to target flanking regions of the MIRU-VNTRs and the conditions of the PCR amplification are listed in Table 1 and Table S2 in the supplemental material. The PCR mixture was composed as follows using the Go *Taq* Flexi DNA polymerase (Promega). Five microliters from fivefold-diluted DNA solution was added to a final volume of 25 μl containing 0.1 μl of Go *Taq* Flexi DNA polymerase (5 U/μl), 5 μl of betaine (Sigma), or 1 μl of dimethyl sulfoxide (Sigma); 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Promega); 5 μl of 5× PCR buffer supplied by the manufacturer; 1 μM of primers; and 1.5 mM of $MgCl_2$. The primers were designed using Oligo 5.0 software (National Biosciences). The reactions were carried out using an iCycler thermal cycler (Bio-Rad). PCR conditions were as follows: 1 cycle of 5 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 1 cycle of 7 min at 72°C. To detect differences in repeat numbers, the PCR products were analyzed by electrophoresis using 1.5% agarose gels (agarose electrophoresis grade; Invitrogen).

Calculation of discriminatory power. The discriminatory index (DI) described by Hunter and Gaston (9) was used as a numerical index for the discriminatory power of each typing method. The DI was calculated using the following formula:

$$DI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1) \right]$$

where N is the total number of strains in the typing scheme, s is the total number of distinct patterns discriminated by each typing method and strategy, and n_j is the number of strains belonging to the jth pattern.

RESULTS

IS*900* RFLP typing. For relevant study of *M. avium* subsp. *paratuberculosis* TR variability, a representative panel of isolates from diverse geographic regions and host origins was assembled based on a preliminary IS*900* RFLP typing analysis. All of the *M. avium* subsp. *paratuberculosis* isolates analyzed in this study were found to be positive for IS*900* and negative for IS*901* by PCR, confirming them as *M. avium* subsp. *paratuberculosis* strains. Over 183 *M. avium* subsp. *paratuberculosis* isolates were subjected to IS*900* RFLP typing. Twenty-six different RFLP types were found (see Tables S1 and S3 in the

TABLE 1. Polymorphic TRs, positions, and primer sequences

TR no.	Position of primer on *M. avium* subsp. *paratuberculosis* genome		Predicted size of PCR product (bp)	Primer		T_m[a] (°C)	Buffer used[b]	
	Start	Stop		Forward	Reverse		1 μl DMSO	5 μl betaine
32	1125707	1126004	298	CCACAGGGTTTTTGGTGAAG	GGAAATCCAACAGCAAGGAC	55	+	+
292	3253590	3253889	300	CTTGAGCAGCTCGTAAAGCGT	GCTGTATGAGGAAGTCTATTCATGG	58	+	−
X3	4441875	4442070	196	AACGAGAGGAAGAACTAAGCCG	TTACGGAGCAGGAAGGCCAGCGGG	58	−	−
25	3665598	3665947	350	GTCAAGGGATCGGCGAGG	TGGACTTGAGCACGGTCAT	58	+	−
3	131320	131527	208	CATATCTGGCATGGCTCCAG	ATCGTGTTGACCCCAAAGAAAT	60	+	−
7	3711417	3711619	203	GACAACGAAACCTACCTCGTC	GTGAGCTGGCGGCCTAAC	60	+	−
10	4279553	4279855	303	GACGAGCAGCTGTCCGAG	GAGAGCGTGGCCATCGAG	60	−	+
47	4128604	4128821	217	CGTTGCGATTTCTGCGTAGC	GGTGATGGTCGTGGTCATCC	64	+	−

[a] T_m, melting temperature.
[b] DMSO, dimethyl sulfoxide. +, used; −, not used.

supplemental material). The vast majority (131 isolates; 72%) were of type R01 (Fig. 1). Interestingly, all caprine strains were grouped in this profile. The R09 type was represented among 5.5% of the isolates, followed by R10 (3.8%); C18 (2.2%); R13, R24, and R34 (1.6%); and R04, R20, and R27 (1.1%) (Fig. 1). The other 16 profiles were found in single isolates. Thus, 167 isolates belonged to 10 cluster patterns, whereas 16 patterns were unique (see Table S3 in the supplemental material).

In silico identification and characterization of TRs and MIRU loci. The genome sequence of *M. avium* subsp. *paratuberculosis* strain K10 was analyzed for the presence of TRs. Over 363 TR sequences were identified in the genome. We focused on TRs of the minisatellite category, defined by a repeat unit size in the range of 10 to 100 bp, as their corresponding allelic differences can be easily resolved by agarose gel electrophoresis. Thirty-three TRs present in more than two copies and with 85% or more nucleotide identity among individual repeat units in the reference strain were selected for experimental analysis. The use of these two criteria was based on the observation that the presence of at least two identical or nearly identical repeats is necessary and sufficient to generate TR variability in the case of *M. tuberculosis* MIRU minisatellites (27).

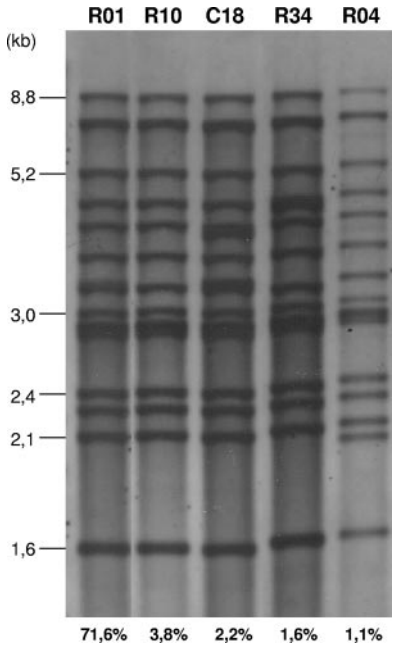

FIG. 1. Selected IS*900* RFLP profiles represented in our collection of *M. avium* subsp. *paratuberculosis* strains. The percentages indicate the proportion of each IS*900* RFLP profile in our collection. R types are designated according to the nomenclature of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

In addition, two MIRU loci were identified in *M. avium* subsp. *paratuberculosis* strain K10 by BLAST searches using as templates the sequences of the flanking genes of two polymorphic MIRU-VNTR loci in *M. tuberculosis*. These two MIRU loci were called *M. avium* subsp. *paratuberculosis* SenX3-RegX3 and *M. avium* subsp. *paratuberculosis* 2920c-2921c. The repeat units of these MIRUs in the *M. avium* subsp. *paratuberculosis* K10 genome have a length of 53 bp and are present with copy numbers of two and three in the SenX3-RegX3 and 2920c-2921c loci, respectively. These loci containing TRs of MIRUs were added to the above selection of TR loci for further experimental analysis.

Polymorphism in repeat numbers among *M. avium* subsp. *paratuberculosis* isolates. The polymorphism of the 35 TR loci selected by in silico analysis was initially investigated using a subset of *M. avium* subsp. *paratuberculosis* isolates selected for diversity based on IS*900* RFLP types and geographic and host origins (see Table S1 in the supplemental material). Only the eight TR and MIRU loci that showed size polymorphism after PCR among the isolates in this subset were used for typing the total collection of 183 isolates.

Twenty-one different MIRU-VNTR types were found in the total collection (see Tables S1 and S4 in the supplemental material). Patterns INMV1 and INMV2 represented the majority of the isolates (36% and 34%, respectively), followed by 11 patterns representing from 1 to 5.5% of the isolates. In total, MIRU-VNTR grouped 175 isolates into 13 clusters, whereas 8 MIRU-VNTR patterns were unique (see Table S4 in the supplemental material). All ovine strains in our collection have the same type: INMV2.

Comparison of IS*900* RFLP and MIRU-VNTR typing and a combination of the two methods. Interestingly, the major RFLP type R01, representing 131 isolates, could be subdivided

TABLE 2. Discriminatory powers of IS*900* RFLP and MIRU-VNTR typing used alone and in combination

Typing method	No. of different patterns	No. of clusters	No. of clustered isolates	No. of unique isolates	No. of isolates in each cluster	DI
RFLP	26	10	167	16	2–131	0.483
MIRU-VNTR	21	13	175	8	2–66	0.751
RFLP + MIRU-VNTR	51	18	150	33	2–53	0.855

into 15 different MIRU-VNTR types. Likewise, the seven isolates with identical RFLP types (R10) were divided into five different VNTR types. RFLP types R09 and C18 could be divided into three VNTR types, while RFLP types R13, R24, and R27 could each be divided into two different VNTR types.

Conversely, several major and minor MIRU-VNTR types were also subdivided by IS*900* RFLP typing. For instance, MIRU-VNTR type INMV2, comprising 66 isolates of *M. avium* subsp. *paratuberculosis*, and MIRU-VNTR type INMV1, comprising 62 isolates, were subdivided into 11 and 10 IS*900* RFLP types, respectively. At the other extreme, the minor MIRU-VNTR types INMV5 to -8, INMV11, and INMV13 could each be divided into two RFLP types.

In total, the combination of the two methods distinguished 51 distinct patterns, including 18 cluster patterns comprising 150 isolates and 33 unique patterns (Table 2; see Tables S5 and S6 in the supplemental material). Therefore, a maximal DI (9) of 0.855 was achieved for the 183 isolates by using IS*900* RFLP and MIRU-VNTR typing in combination compared to 0.483 for IS*900* RFLP typing alone and 0.751 for VNTR typing alone.

Polymorphism among 316F vaccine strains. The *M. avium* subsp. *paratuberculosis* strain 316F is one of the strains used for vaccination against paratuberculosis. In this study, we analyzed the clonal identity between 316F vaccine batches from various origins or from the same origin by using both IS*900* RFLP and MIRU-VNTR typing. The results shown in Fig. 2 and Fig. 3 demonstrate that Weybridge-316F differs from Mérial-316F by both genotyping methods. The two Weybridge-316F batches analyzed by MIRU-VNTR typing displayed the same INMV17 pattern, but it differed from that of the all Mérial-316F batches by a variation in the single SenX3-RegX3 locus (Fig. 3). By IS*900* RFLP analysis, the two Weybridge-316F batches displayed identical R01 patterns, which clearly differed by three bands from the C7 pattern detected for four Mérial-316F batches in accordance with that described in the past (6). In addition, a fifth analyzed Mérial-316F vial displayed an R10 pattern differing from C7 by two bands (Fig. 2). Furthermore, two isolates from two different Mérial vials identified by identical batch numbers (L68451) produced in the same year, 2001, showed the same MIRU-VNTR type but two RFLP profiles differing by two IS*900* bands, suggesting a degree of clonal heterogeneity among commercial preparations of the vaccine strain.

INVM polymorphism in *M. avium*. In order to study the levels of polymorphism of the eight MIRU-VNTR loci in other members of the *M. avium-M. intracellulare* complex, 82 *M. avium* isolates previously serotyped and typed by IS*1245* RFLP were typed using the same primers as those targeting the eight MIRU-VNTR loci of *M. avium* subsp. *paratuberculosis*. This analysis (see Tables S6 and S7 in the supplemental material) showed that MIRU-VNTR typing may be applied to *M. avium* isolates using the same conditions as those defined for *M. avium* subsp. *paratuberculosis*. Thirty MIRU-VNTR types were found for the 82 *M. avium* isolates (see Table S7 in the supplemental material), yielding a DI of 0.889. None of the *M. avium* MIRU-VNTR profiles matched those of *M. avium* subsp. *paratuberculosis* isolates.

DISCUSSION

VNTRs of the minisatellite class are valuable markers used for genotyping several mycobacterial species (4, 18, 21, 25, 26).

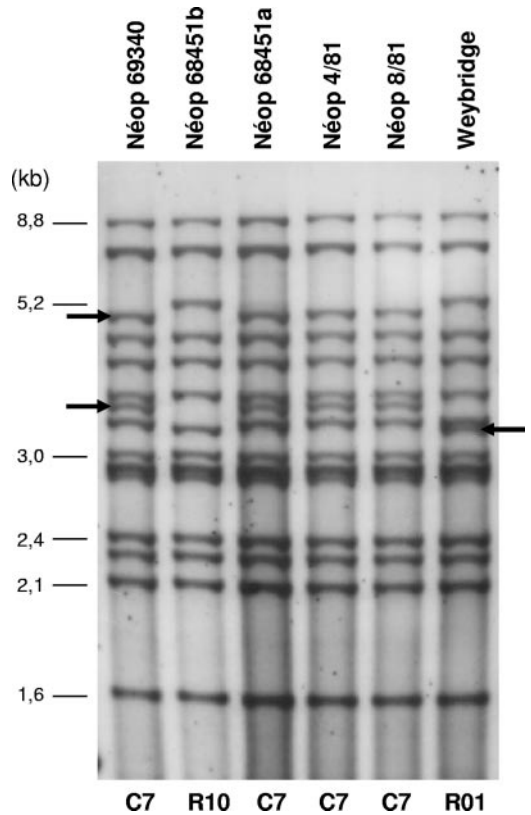

FIG. 2. IS*900* RFLP profiles of *M. avium* subsp. *paratuberculosis* 316F strains. Profiles from five different cultures of Mérial-316F (Néoparasec) corresponding to four batches, Néop 69340, Néop 68451, Néop 4/81, and Néop 8/81, as well as from one culture from a 316F strain from Weybridge, are represented. Néop 68451a and Néop 68451b correspond to cultures from two different Mérial-316F vials identified by the same batch number, 68451. R and C types are designated according to the nomenclature of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands, and Collins et al. (5) and Pavlik et al. (19), respectively. The arrows indicate polymorphic bands among the different profiles.

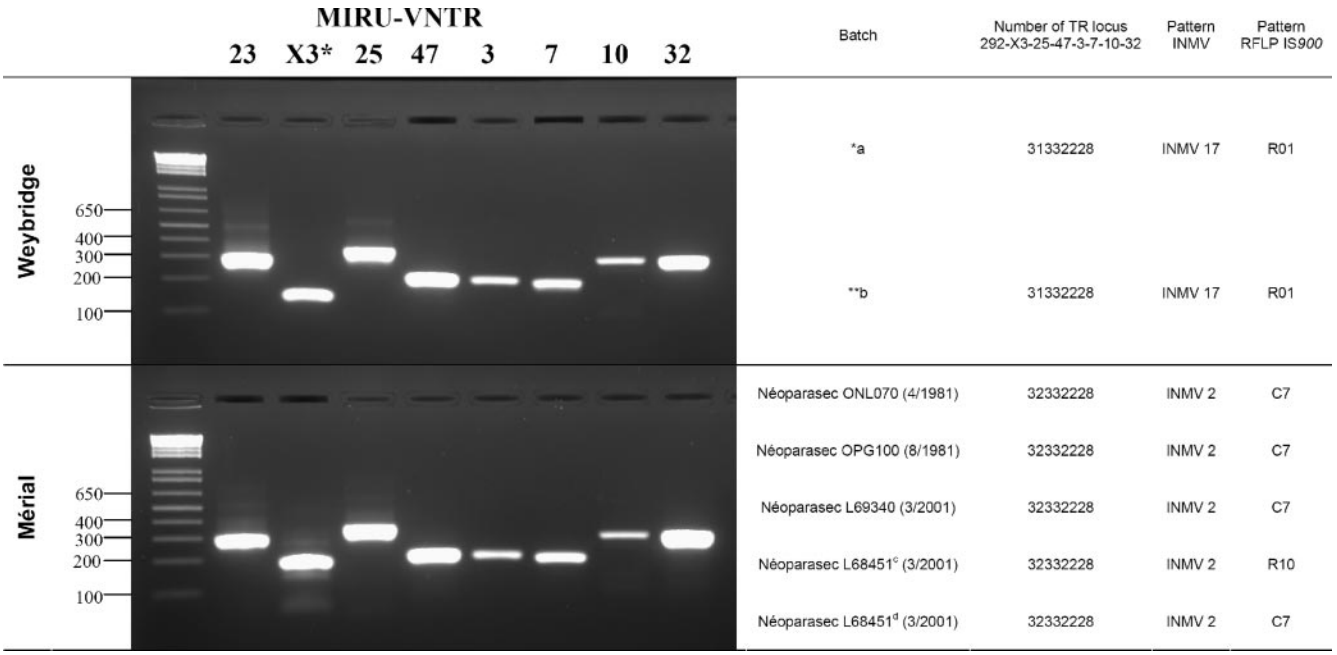

FIG. 3. MIRU-VNTR profiles of *M. avium* subsp. *paratuberculosis* 316F strains from Weybridge laboratory and Mérial. The PCR products were analyzed by electrophoresis using agarose gels, as described in Materials and Methods. The positions of size standard bands and designations of MIRU-VNTR loci are indicated on the left and at the top, respectively. a, analysis of Weybridge 316F strain; b, analysis of Mérial 316F strain. A large asterisk indicates the locus (X3) that varies between the two strains. *a, provided by P. Willemsen (Central Institute for Animal Disease Control, Department of Bacteriology and TSEs, 8203 AA Lelystad, The Netherlands); **b, provided by K. Stevenson (Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Scotland, United Kingdom). The batches marked [c] and [d] were cultured from two different Néoparasec vials identified by the same batch number on the same date.

Two previous studies (4, 18) identified a few VNTR loci in *M. avium* subsp. *paratuberculosis* isolates based on partial screenings of the *M. avium* subsp. *paratuberculosis* K10 genome. In this study, we performed an exhaustive screening of potential VNTR loci in this genome based on in silico identification of TRs in the whole *M. avium* subsp. *paratuberculosis* K10 genome and experimental testing of the polymorphism of the most interesting TR candidates using a reference set of *M. avium* subsp. *paratuberculosis* isolates with diverse IS*900* RFLP types and geographic and host origins. By this means, we have identified eight VNTR loci, seven of which are novel and one of which (*senX3-regX3*) has been previously identified by Bull et al. (4).

When used alone, this eight-locus-based typing system distinguished slightly fewer types of *M. avium* subsp. *paratuberculosis* isolates in this collection than IS*900* RFLP (21 versus 26), but the MIRU-VNTR types were more equally distributed in this *M. avium* subsp. *paratuberculosis* collection than the IS*900* RFLP types (i.e., with the single R01 type comprising 71.5% of the isolates). Interestingly, this R01 RFLP type, representing the vast majority of the *M. avium* subsp. *paratuberculosis* isolates found in this study and in the other studies, could be successfully divided into 15 subgroups by MIRU-VNTR typing. On the other hand, 10 VNTR types could also be subdivided by IS*900* RFLP, six of which were subdivided into only two IS*900* RFLP subgroups. Therefore, the highest resolution was achieved when the two typing methods were combined.

We analyzed the eight MIRU-VNTR loci in different batches of 316F *M. avium* subsp. *paratuberculosis* vaccine in order to investigate both the degree of clonality of the presumably identical corresponding strains and the clonal stability of MIRU-VNTR markers. The stability of *M. tuberculosis* complex MIRU-VNTR loci was analyzed in a similar manner using the genealogically distant *Mycobacterium bovis* BCG strain (27). Rather surprisingly, both typing methods distinguished Weybridge-316F batches from Mérial-316F batches. In the case of MIRU-VNTR typing, the batches from Weybridge and Mérial differed by a single locus, namely, *senX3-regX3*. Interestingly, the same locus was shown to display some degree of VNTR polymorphism among BCG sister strains cultivated separately for more than 30 years (27). This single MIRU-VNTR locus difference was corroborated by differences of three IS*900* RFLP bands between batches from Weybridge and four batches from Mérial. These results confirm the IS*900* RFLP profiles of the vaccine strains described by Collins et al. (6). Furthermore, a polymorphism involving two other IS*900* RFLP single-band differences was detected, not only between different Mérial batches, but also between two cultures from two different Mérial vials identified by the same batch number. In the latter case at least, the vials can be assumed to originate from the same seed stock. In contrast, the eight MIRU-VNTR loci remained unchanged among these different commercial preparations. These observations suggest that there has been a substantial degree of genetic drift between the Weybridge- and Mérial-316F strains, which most likely results from separate culturing after the exchange of the presumed original strain decades ago. The outcome of this process has been two closely related but now clearly distinct clones in the Weybridge and

commercial preparations, as judged by two fully independent genotyping methods. Remarkably, this genetic drift appears to be ongoing for the commercial vaccine, as indicated by IS*900* RFLP observed between preparations from different batches or even from the same batch. Because MIRU-VNTR types remained the same among tested commercial preparations, this degree of clonal heterogeneity appears to be less than that observed between these commercial preparations and those from Weybridge. The conservation of the eight MIRU-VNTR loci among these apparent clonal variants with slightly different IS*900* RFLP types thus suggests a slightly lower evolutionary rate for these eight-locus-based genotypes than those of IS*900* RFLP fingerprints.

Traditional techniques or comparative genomics used for studying the genetic structures of *M. avium* subsp. *paratuberculosis* and *M. avium* populations has shown the very close relatedness, as well as the distinctiveness, of these mycobacterial species (13). Consistent with the first feature, our results show that the flanking sequences and the polymorphisms of the eight MIRU-VNTR loci are sufficiently conserved between the two species to use the same PCR primers and loci for MIRU-VNTR typing of *M. avium* isolates. In accordance with the second feature, the MIRU-VNTR types identified for the *M. avium* isolates were all distinct from any of those identified for *M. avium* subsp. *paratuberculosis*. Interestingly, 30 MIRU-VNTR types were obtained for the 82 *M. avium* isolates, although they all came from a single country (France) and host (human), while only 21 types were obtained for the 183 *M. avium* subsp. *paratuberculosis* isolates from different hosts and settings. Although these results, based on limited samples, must be considered preliminary, this higher degree of MIRU-VNTR diversity among *M. avium* isolates is consistent with the higher genetic diversity in *M. avium* strains seen by using other markers (20).

In addition, the use of these markers could shed new light on molecular studies of *M. avium* subsp. *paratuberculosis* epidemiology. For instance, it is interesting to note that two *M. avium* subsp. *paratuberculosis* strains in our series that shared a rare MIRU-VNTR pattern (INMV9) and an IS*900* RFLP pattern (R01) were isolated from humans and cattle from the same geographical origin (France), raising the question of a common source.

In conclusion, we have described here the identification of novel MIRU-VNTR markers for more specific differentiation of *M. avium* subsp. *paratuberculosis* isolates. Our preliminary analyses suggest that MIRU-VNTR typing provides us with a discriminatory power close to that obtained with the IS*900* RFLP method. Parts of the respective discriminatory powers provided by these two independent methods are nonredundant, resulting in higher resolution when the two typing approaches are combined. This result should be verified with a larger panel of isolates with different IS*900* RFLP patterns and geographic origins and from hosts other than cattle, such as sheep. However, it is already clear that these markers constitute very useful additional tools for typing *M. avium* subsp. *paratuberculosis* (as well as *M. avium*), especially because MIRU-VNTR typing is PCR based. As suggested by previous results (4, 18), the MIRU-VNTR loci could be further subjected to DNA sequence analysis to detect possible sequence polymorphisms among repeat units, in addition to the variation in the number of repeats among *M. avium* subsp. *paratuberculosis* isolates. This potential supplementary polymorphism and the addition of the few nonredundant VNTR loci described by the authors of the previous studies may further improve the DI of this typing method. According to the results for allelic diversity (Table 3), some markers are more polymorphic than others. These markers must be applied in priority for genotyping. Recently, a multilocus short sequence repeat sequencing approach was described for discriminatory genotyping of *M. avium* subsp. *paratuberculosis* strains (1, 7). Eventually, a combination of this method with VNTR-MIRU-based typing might prove to be optimal for PCR-based molecular epidemiological studies of this pathogen. Last but not least, the phenomena of genetic drift and clonal heterogeneity discovered among vaccine preparations from different origins, or even a single origin, have implications that must be taken into account for evaluating and ensuring the stability of protective effects of paratuberculosis vaccine preparations over time and across settings.

TABLE 3. MIRU-VNTR allelic distribution among *M. avium* subsp. *paratuberculosis* and *M. avium* isolates

Isolate and locus	No. of isolates with the specified MIRU allele										Allelic diversity (h)[a]
	0	1	2	3	4	5	6	7	8	9	10	
M. avium subsp. *paratuberculosis*												
32									177	5	1	0.59
292			6	108	69							0.51
7		12	164	3	2	2						0.19
10		19	164									0.18
25				176	1	6						0.07
47			5	178								0.05
X3	3	179	1									0.04
3	1		182									0.005
M. avium												
X3		1	31	10	26	12	2					0.72
47			63	19								0.35
25		2	66	4	7	3						0.33
32						1		6	67	8		0.3
292	8	1	70	3								0.27
10		7	75									0.15
7		2	80									0.04
3		82										0

[a] MIRU-VNTR allelic distribution was calculated as described by Mazars et al. (15).

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