JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1049–1053 Vol. 33, No. 5
0095-1137/95/$04.00+0

Two Markers, IS*901*-IS*902* and p40, Identified by PCR and by Using Monoclonal Antibodies in *Mycobacterium avium* Strains

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Received 7 July 1994/Returned for modification 20 August 1994/Accepted 18 January 1995

The occurrence of two markers, a newly identified 40-kDa protein (p40) and the insertion sequence IS*901*-IS*902*, in strains of *Mycobacterium avium* subspp. was evaluated. Analysis of 184 type and field strains of the *M. avium* complex from human, animal, and environmental sources by PCR specific to IS*901* and by a monoclonal antibody specific to p40 demonstrated the presence of the two molecular markers in all of the *M. avium* subsp. *silvaticum* strains examined and also in a number of *M. avium* subsp. *avium* strains (the latter isolated mainly from pigs). The appearance of the two markers was completely concurrent in all strains. Further, the marker-positive *M. avium* subsp. *avium* strains were mainly serotype 2, whereas *M. avium* complex strains of serotypes 4, 6, 8, 9, and 10 were marker negative. The *M. avium* subsp. *avium* type strain ATCC 25291 and approximately 50% of the *M. avium* subsp. *avium* field strains isolated from animals contained the markers, while only one strain of human origin was found to be marker positive. Therefore, IS*901* and p40 appear to have substantial potential to differentiate among isolates of the *M. avium* complex. This observation raises new issues regarding classification of strains, since the presence of the markers was found to be inconsistent with the present taxonomic grouping of *M. avium* subspp.

The *Mycobacterium avium* complex is a group of mycobacteria that are widespread in animals and the environment. The complex consists of *M. avium*, *M. intracellulare*, and *M. paratuberculosis*, as well as the wood pigeon bacillus. These slowly growing mycobacteria can infect and cause disease in lungs, lymph nodes, intestines, skin, bones, and soft tissues of animals and humans. *M. avium* infections have become the most commonly disseminated bacterial infections in immune-compromised populations such as patients with AIDS (12). *M. paratuberculosis* is the causative organism of paratuberculosis (Johne's disease), a debilitating chronic enteritis in ruminants, and it has been associated with Crohn's disease in humans (7, 25). The wood pigeon bacillus can cause enteritis in ruminants as well as disseminated infection in other hosts (8).

Taxonomic analysis of the *Mycobacterium* genus is complicated, and a number of schemes have been proposed to discriminate and classify organisms within the *M. avium* complex. The use of seroagglutination has enabled discrimination of *M. avium* and *M. intracellulare* (28, 29). Genetic analyses, particularly those examining the 16S rRNA genes, have been useful for discrimination of strains (23, 24) and even identification of new species (4). However, this approach has failed to discriminate among *M. avium*, *M. paratuberculosis*, and the wood pigeon bacillus (27, 32) and has led to suggestions that they were one species. Attempts to discriminate slowly growing mycobacteria with DNA probes have been reported, and considerable advances have followed the identification of the insertion elements IS*900* and IS*901*-IS*902* in *M. paratuberculosis* (10) and strains of *M. avium*, respectively (18, 22).

Each of the two insertion elements contains a unique sequence at the 5′ end of the gene which has formed the basis for PCR analysis of mycobacteria in several laboratories (6, 35, 36). IS*900* has been identified only in *M. paratuberculosis* strains (10), whereas IS*901*-IS*902* has been found in wood pigeon bacillus or in *M. avium* strains isolated mainly from animals (17, 18, 22).

Thorel et al. proposed *M. avium*, *M. paratuberculosis*, and the wood pigeon bacillus as a single species with three subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (31). This was based on genetic analysis and a number of culture characteristics such as mycobactin dependence, ability to grow on egg medium, and growth stimulation by pyruvate or pH 5.5.

Another grouping of strains was proposed by Kunze et al. (17) on the basis of the insertion elements IS*900* and IS*901*. These authors suggested that *M. paratuberculosis* was defined by the presence of IS*900. M. avium* consisted of two distinct groups, referred to as type A/I, containing IS*901* and type A not having this insertion element. The type A/I group corresponded mainly to *M. avium* subsp. *silvaticum* but also included *M. avium* subsp. *avium* strains, while type A corresponded to the remaining *M. avium* subsp. *avium* group. The division in type A and A/I was further supported by differences in plasmid possession and serotype antigens.

Recently, a 40-kDa protein (p40) was identified in a *M. avium* subsp. *silvaticum* field strain but could not be detected in isolates of *M. avium* subsp. *paratuberculosis*. Preliminary sequence analysis of this protein revealed that the amino-terminal 20 amino acids were not homologous to any known protein. Further, polyclonal sera from sheep and deer naturally or experimentally infected with *M. avium* subsp. *silvaticum* reacted with the 40-kDa antigen in immunoblots (5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated that p40 is one of the most abundant antigens in strains in which it is found to be present (12a). The function of the protein is not known. In the present study, 184 mycobacterial strains were examined by PCR specific to IS*900* and IS*901*-IS*902*, by a monoclonal antibody (MAb) raised against the 40-kDa protein, and by serotyping in order to evaluate the distribution of the two markers IS*901* and p40.

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TABLE 1. Source and origin of 184 *M. avium* complex strains used in this study and distribution of IS*900*, IS*901*, and p40 in all strains and serotypes of *M. avium* subsp. *avium* type, and reference strains[a]

Strain designation	Total no.	Origin	No. of strains showing the presence of:			Serotype[b]	Source
			IS*900*	IS*901*	p40		
Reference strains							
M. avium subsp. *paratuberculosis*							
ATCC 19698	1	Bovine	1	0	0		
M. avium subsp. *avium*							
ATCC 19075	1	Human	0	1	1	2	
ATCC 25291	1	Avian	0	1	1	2	
ATCC 15769	1	Avian	0	0	0	1	
NCTC 8551	1	Porcine	0	1	1	2	
NCTC 8552	1	Bovine	0	1	1	2	
NCTC 8558	1	Ovine	0	1	1	2	
NCTC 8559	1	Avian	0	1	1	2	
NCTC 8562	1	Avian	0	1	1	2	
Field strains							
M. avium subsp. *paratuberculosis*	25	Bovine	25	0	0		NVL
	7	Cervine	7	0	0		NVL
	6	Caprine	6	0	0		FS
M. avium subsp. *avium*	42	Human	0	1	1		SSI
	62	Porcine	0	31	31		NVL
	6	Cervine	0	2	2		NVL
	20	Environment	0	0	0		NVL
	1	Bovine	0	0	0		NVL
	1	Avian	0	1	1		NVL
M. avium subsp. *silvaticum*	5	Wild animals	0	5	5		FS, NVL

[b] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; FS, Finn Saxegaard, National Veterinary Institute; NVL, National Veterinary Laboratory; SSI, Statens Seruminstitut.

[b] The serotypes of the *M. avium* subsp. *avium* field strains are listed in Table 2.

MATERIALS AND METHODS

Mycobacterial strains and growth conditions. The mycobacterial strains used in this study are listed in Table 1. The collection of strains consisted of a number of type strains from the American Type Culture Collection, Rockville, Md., and the National Collection of Type Cultures, London, United Kingdom; a number of human isolates (provided by Kurt Fuursted, Statens Seruminstitut, Copenhagen, Denmark); and isolates from goats and wild animals (provided by Finn Saxegaard, National Veterinary Institute, Oslo, Norway) as well as a number of field isolates from pigs, cattle, farm deer, and environmental sources collected at the National Veterinary Laboratory, Copenhagen, Denmark. The *M. avium* subsp. *paratuberculosis* strains were isolated from feces, mesenteric lymph nodes, or intestinal mucosa. Other strains from humans or animals were isolated from various organs.

Fecal samples were decontaminated by the method described by Beerwerth (3), while tissue samples were decontaminated with 5% sulfuric acid for 10 min.

Mycobacteria were grown on slopes of modified Lövenstein-Jensen medium (14) or Middlebrook 7H11 medium. The identification of field strains was based on growth rate, colonial morphology, mycobactin dependence, growth stimulation by pyruvate, resistance to isoniazid, and serotyping. Furthermore, 66 of the *M. avium* subsp. *avium* field strains from all sources were tested by the Gen-Probe (San Diego, Calif.) Rapid Diagnostic System according to the manufacturer's recommendations for confirmation of *M. avium* identity.

The five wood pigeon strains have previously been identified as such by F. Saxegaard (26a) and by J. B. Jørgensen (15).

Serotyping. Serotyping was performed as a tube agglutination test with 25 antisera as described by J. B. Jørgensen (13). In cases of cross-reactions, an antibody absorption test was performed (28). No further examination was made of strains that were nonagglutinable or that showed spontaneous agglutination.

Preparation of MAbs. The 40-kDa antigen was purified by high-performance liquid chromatography from a *M. avium* subsp. *silvaticum* field strain (5). BALB/c mice were immunized intraperitoneally with the purified antigen adsorbed to $Al(OH)_3$. The mice were immunized twice at intervals of 2 weeks and were boosted with the same amount of antigen 3 days before the fusion with the myeloma cell line P3 X63 Ag8-653 (16) by standard procedures (9). Screening of supernatant from growing hybridomas was performed by an enzyme-linked immunosorbent assay (ELISA) essentially as described below. Hybridoma supernatants diluted 1:10 were screened against crude antigen from *M. avium* subsp. *avium* ATCC 25291 and *M. avium* subsp. *paratuberculosis* ATCC 19698. The corresponding hybridomas were cloned by limiting dilution.

ELISA. An ELISA was used for the examination of antigen from different mycobacterium strains. Microtiter plates were coated with 50 μl of crude antigen diluted in 50 mM sodium carbonate buffer (pH 9.6). Each antigen was tested with the hybridoma supernatant diluted 1:10 and 1:100 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin and was incubated for 2 h at 20°C.

Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) diluted 1:1,000 was added for 1 h. The substrate-dye solution (0.07% 1,2-phenylenediamine, 0.04% H_2O_2 in 10 mM citrate [pH 5.0]) was allowed to react for 20 min; the reaction was stopped by the addition of 0.5 M H_2SO_4. The optical density at 490 nm was measured with 650 nm as the reference.

In addition to the specific MAb, the ELISA analysis included as a positive control a MAb directed against an epitope present in all *M. avium* subsp. isolates (16a) and as a negative control a MAb raised against *Actinobacillus pleuropneumoniae.*

Sera from immunized mice were diluted 1:1,000 and were used as mentioned above.

Antigen preparation. Crude antigen was produced from cultured mycobacteria. The mycobacteria were harvested in 10 mM PBS (pH 7.2) containing 0.5% phenol, washed three times in PBS, disrupted with zirconium beads in a bead beater (Biospec Products, Bartlesville, Okla.) four times for 50 s, and clarified by centrifugation for 30 min at 20,000 × *g*. Finally, the suspension was filtered through a 0.22-μm-pore-size filter.

SDS-PAGE and immunoblotting. Antigens from different mycobacteria strains were separated on 12.5% polyacrylamide gels (19) and transferred onto nitrocellulose membranes by standard procedures (33). The protein concentrations of the individual samples were adjusted according to visual judgment of silver-stained gels (21).

The nitrocellulose membranes were cut into strips and incubated overnight with MAbs or serum and were visualized by peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:1,000 and then by staining with tetramethylbenzidine and hydrogen peroxide.

PCR. Cultured mycobacteria were disrupted by bead beating. A 2-μl sample was inoculated into 48 μl of a prepared reaction mixture containing 65 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, the four deoxyribonucleotide triphosphates (100 μM each), 65 pmol of each of the oligonucleotide primers, and 0.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany, or Perkin-Elmer Cetus, Norwalk, Conn.), and the mixture was covered with 50 μl of paraffin oil. Samples were subjected to an initial denaturation step 94°C for 3 min and then to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min in a thermal cycler.

The primers for IS*901* PCR were GTCTGGGATTGGATGTCCTG (primer 901a) and CACCACGTGGTTAGCAATCC (primer 901c), which were derived from positions 19 to 394 of the IS*901* sequence (18). Primers for IS*900* PCR were CGTCGTTAATAACAATGCAG (primer 11) and GGCCGTGCGTTAGGCT TCGA (primer 36), which were derived from positions 27 to 304 of the IS*900* sequence (10). Amplification products were analyzed by PAGE. Five microliters of the reaction product was subjected to electrophoresis through a 7.5% polyacrylamide gel in a Tris-phosphate buffer (pH 7.5). The gel was fixed in 10% ethanol–0.5% acetic acid and was silver stained according to standard protocols (21). Samples were considered IS*901*-IS*902* positive when a single band of DNA, 375 bp in size, was evident in the gels. IS*900* was identified by the appearance of a 279-bp band of DNA. Amplification products were digested with the restriction enzymes *Msp*I and *Rsa*I and were analyzed on a 10% polyacrylamide gel, fixed, and silver stained as mentioned above. Agarose gel electrophoresis and Southern blotting were performed by standard procedures. Nitrocellulose filters were hybridized with a DNA probe labelled with ^{35}S-dATP (Amersham International) as described earlier (1). The hybridization probe was the 375-bp PCR amplification product using DNA from *M. avium* subsp. *avium* ATCC 25291 as a target. DNA cycle sequencing (30) was performed as prescribed by the supplier (CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit; New England Biolabs, Beverly, Mass.).

RESULTS

Sera and MAbs. ELISA screening of hybridoma supernatants led to the identification of 10 clones producing MAbs to p40. One of these, MAb 6.34, reacted in ELISA as well as in immunoblotting and was chosen for this study.

The molecular specificity of MAb 6.34 and of murine serum raised against the 40-kDa protein was demonstrated by immunoblot analysis (Fig. 1). The murine serum (Fig. 1A) bound strongly to a 40-kD protein present in the *M. avium* subsp. *avium* serotype 2 strains ATCC 19075 and ATCC 25291 (lanes 3 and 4, respectively), whereas this protein was not identified in the *M. avium* subsp. *avium* serotype 1 strain ATCC 15769 (lane 2) or in *M. avium* subsp. *paratuberculosis* ATCC 19698 (lane 1). Minor reaction was seen with other antigens (especially seen in lane 4). The reaction of MAb 6.34 is displayed in Fig. 1B. This MAb reacted only with a 40-kDa antigen present in the *M. avium* subsp. *avium* strains ATCC 19075 (lane 3) and ATCC 25291 (lane 4) as well as the field strains 42 (*M. avium* subsp. *silvaticum* [lane 5]) and FGR41 (*M. avium* subsp. *avium* serotype 2 [lane 6]). There was no reaction with *M. avium* subsp. *paratuberculosis* (lane 1) and *M. avium* subsp. *avium* ATCC 15769 (lane 2).

Field strains. The specificities of the MAbs were further investigated by ELISA with MAb 6.34 and by immunoblot analyses of field strains. Antigens from 64 strains were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with MAb 6.34. A single 40-kDa band was observed for 15 strains, while 49 strains gave negative results. The ELISA analysis demonstrated that identical results was obtained by the two immunological methods (data not shown).

Antigens from 39 of the above-mentioned 64 strains were also tested by ELISA with murine serum instead of MAb 6.34. Murine serum showed results identical to those obtained with the MAb. This suggested that the specificity of p40 is not confined to a single epitope.

PCR. The published DNA sequences of the insertion sequences IS*901* (18) and IS*902* (22) were compared for selection of primers for specific amplification of a 375-bp fragment. The specificity of the PCR was examined by amplification with type strains of mycobacteria (Table 1). The insertion sequence was identified in the *M. avium* subsp. *avium* strains ATCC 19075, ATCC 25291, NCTC 8551, NCTC 8552, NCTC 8558, NCTC 8559, and NCTC 8562 by the appearance of a 375-bp fragment, whereas no amplification was observed for strain ATCC 15769 or for the *M. avium* subsp. *paratuberculosis* strain ATCC 19698.

The amplification products were further examined by restriction fragment analysis. Amplified DNAs from ATCC 25291 and ATCC 19075 digested with *Rsa*I showed fragments of 241 and 134 bp, while digestion with *Msp*I showed fragments of 282, 73, and 21 bp, as could be predicted from the published sequence of IS*901* (not shown).

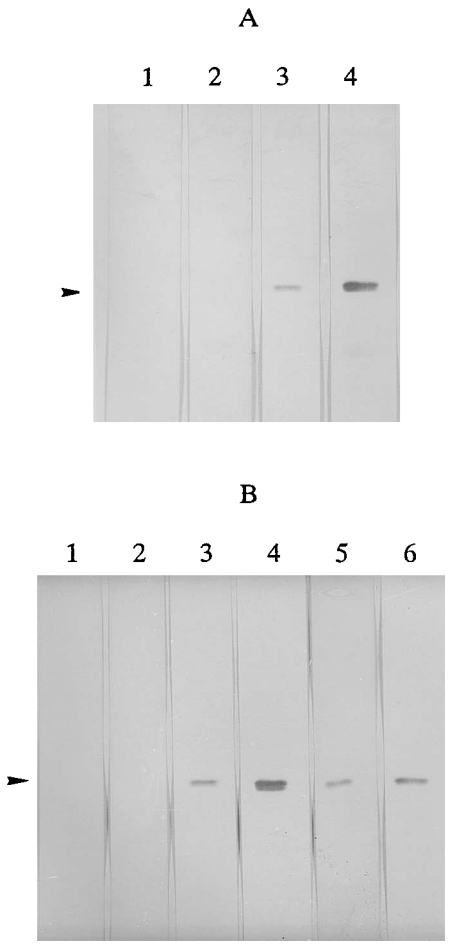

FIG. 1. Immunoblot of antigens from strains of *M. avium* subspp. showing the reactivities of murine serum to p40 (A) and MAb 6.34 (B). Lanes: 1, *M. avium* subsp. *paratuberculosis* ATCC 19698; 2, *M. avium* subsp. *avium* ATCC 15769; 3, *M. avium* subsp. *avium* ATCC 19075; 4, *M. avium* subsp. *avium* ATCC 25291; 5, *M. avium* subsp. *silvaticum* field strain; 6, *M. avium* subsp. *avium* field strain. The arrows indicate the positions of a 40-kDa band.

For verification of the PCR, the amplification products from 12 IS*901* positive strains were examined by digestion with the restriction enzymes *Msp*I and *Rsa*I, respectively. The amplification products from all strains showed *Rsa*I and *Msp*I fragments of identical sizes which were indistinguishable from the expected size (*Rsa*I, 241 and 134 bp; *Msp*I, 282, 73, and 21 bp [data not shown]).

The identities of the amplification products were further confirmed by Southern blotting with purified amplification product from *M. avium* subsp. *avium* ATCC 25291 as probe (not shown). Primers 11 and 36 directed the amplification of a 279-bp fragment with ATCC 19698 but not with any of the other type or reference strains (Table 1).

Partial DNA sequence determinations (average, 166 nucleotides) of the amplification products of ATCC 25291 and ATCC 19698 and six field strains confirmed the specificity of the amplification.

TABLE 2. Serotypes of 132 *M. avium* subsp. *avium* field strains

Origin	No. of isolates of the following serotypes[a]:															
	1	2	3	4	6	8	9	10	1/6	2/3	2/8	3/9	4/8	NA	SA	Total
Human	3	3 (1)	1	8	5	2	4			9	1			4	2	42
Porcine	2 (1)	30 (29)		4	8	3	8	1	1	1 (1)		3	1			62
Cervine	1	1 (1)	1 (1)			2								1		6
Environment				1	5		4					4		3	3	20
Bovine				1												1
Avian	1 (1)															1
Total	7 (2)	34 (31)	2 (1)	14	18	7	16	1	1	10 (1)	1	7	1	8	5	132

[a] Numbers of IS*901*- and p40-positive isolates are given in parentheses. NA, not agglutinable; SA, spontaneous agglutination.

Distribution of IS*900*, IS*901*, and p40. A total of 184 type and field strains were analyzed by PCR specific to IS*901* or to IS*900* and by ELISA with MAb.

All of the 38 strains identified by culture as *M. avium* subsp. *paratuberculosis* showed positive reactions with the IS*900* PCR, whereas all other strains were found negative. Of the 184 strains analyzed, 47 were IS*901* positive and 137 were IS*901* negative. All strains that were found to contain IS*901* were also shown to be positive by monoclonal ELISA, whereas IS*901*-negative strains did not react with MAb 6.34. This suggests that the two markers IS*901* and the 6.34 epitope do not segregate independently ($P < 0.001$ [χ^2 test]).

Association of IS*901* and p40 with serotypes. With the exception of the *M. avium* subsp. *paratuberculosis* strains, all of the isolates were serotyped. Of the five *M. avium* subsp. *silvaticum* strains, two were untypeable, one was serotype 2, and two strains were agglutinated with both type 2 and type 3 sera.

The serotypes as well as the presence of p40 and the insertion elements IS*900* and IS*901* in type and reference strains are listed in Table 1.

The distribution of serotypes of the 132 *M. avium* subsp. *avium* strains is shown in Table 2. Of 34 field strains identified as serotype 2, 31 were positive for both markers, while 3 strains did not possess any of the two markers. Furthermore, the markers were identified in 2 of 7 serotype 1 isolates, in 1 of 2 serotype 3 isolates, and 1 of 10 serotype 2/3 isolates. All but one of the strains that contained IS*901* and p40 were isolated from animals.

DISCUSSION

The *M. avium* complex consists of a group of microorganisms whose members are not readily distinguishable from one another. They are important pathogens in animals and humans, and the AIDS epidemic has drawn increasing attention to this group of bacteria. The establishment of a taxonomic relationship among isolates has proved difficult. Different and incomparable schemes have been used for classification, and this may evoke some confusion.

In this study, a MAb directed against a newly identified protein p40 and a PCR test specific for the insertion sequence IS*901*-IS*902* were used for the analysis of type and field strains of the *M. avium* complex. The insertion sequence appeared concurrently with the MAb 6.34 epitope in all of the analyzed strains. The nature of this close linkage between the two molecular markers remains to be clarified. Only *M. avium* subsp. *silvaticum* strains, most *M. avium* subsp. *avium* serotype 2 strains, and some serotype 1 and serotype 3 strains from animals were found to contain the two markers. The only human marker-positive strain was the reference strain ATCC 19075.

The correct assignment of isolates within the *M. avium* complex is by no means simple. In recent years, the use of DNA probes has gained widespread acceptance as the method of choice. However, these probes do not distinguish among *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*. The insertion sequence IS*901*-IS*902* has previously been suggested to be specific for *M. avium* subsp. *silvaticum* (22). The data presented here demonstrate that this insertion element was present in all strains of *M. avium* subsp. *silvaticum* but also in a number of *M. avium* subsp. *avium* strains. IS*901* was identified not only in field strains but also in reference strains, including the type strain of *M. avium* subsp. *avium* (ATCC 25291). The correct assignment of these strains can hardly be questioned. This demonstrates that *M. avium* subsp. *silvaticum* could not be defined by IS*901*.

Another study suggested that IS*901* is a marker for strains differing in host range and virulence from other isolates of the *M. avium* complex (17). In the present study, the majority of the marker-positive strains were isolated from pigs. Nevertheless, about 50% of the porcine strains were found to be marker negative. Marker-positive and marker-negative strains could not be distinguished by cultivation characteristics such as growth rate and colonial morphology.

In the present study, the presence of p40 was identified by ELISA using a specific MAb. Nevertheless, all of the strains analyzed by ELISA using polyclonal serum only confirmed the results obtained by the MAb. Thus, it is likely that not only the MAb 6.34 epitope but also the 40-kDa protein are specific to a certain group of isolates.

The different appearance of IS*901* and p40 in human and animal isolates could support the idea that livestock is not the primary source of human infections.

The function of p40 is not known. Partial amino acid sequencing of p40 has failed to demonstrate homology with any known protein, including the putative protein from the open reading frame of IS*901* (5). Therefore, we have no leads regarding the apparently different host ranges of the two markers.

In the present study, the majority of the marker positive strains were serotype 2. The finding of variant prevalence of IS*901*-IS*902* in different hosts could simply reflect the variant prevalence of serotype 2. Kunze and coworkers (17) examined a number of strains for the presence of IS*901* and found the insertion element present in serotypes 1, 2, 3, 6, and 8. In the present study, the insertion element was found only in serotypes 1, 2, and 3.

A different distribution of serotypes in human and animal isolates has been described previously. Serovars 4, 8, 1, 9, and 6 are the most prevalent among human isolates in Western countries (2, 11, 34), while among animal isolates serotype 2 is

more prominent. The distribution of serotypes among porcine isolates seems to differ considerably among countries. Serotype 2 constituted 7.3% of the isolates in Norway (26), an earlier study found a result of 86.3% in Denmark (13), and in Japan serotype 2 was not found at all (20, 37). This highly variant distribution of *M. avium* subsp. *avium* serotype 2 strains could indicate that the molecular markers p40 and IS*901* appear with variant frequencies in different countries.

Taxonomy within the *M. avium* complex has been confused by the lack of uniform characters. The taxonomic scheme proposed by Thorel et al. (31) is not in full agreement with the system based on insertion sequences used by Kunze et al. (17).

The presence of the two markers described in the present paper could be demonstrated without laborious DNA purification or time-consuming biochemical tests. Although further work is needed to elucidate the linkage between the two markers, we believe that the system will prove helpful in the characterization of *M. avium* complex isolates.

ACKNOWLEDGMENTS

We thank Kurt Fuursted, Statens Seruminstitut, and Finn Saxegaard, National Veterinary Institute, Oslo, Norway, for providing us with strains; and B. Berg, G. Funch, and K. Vestergaard for skillful technical assistance.

This study was supported by a grant from the Commission of the European Communities (Competitiveness of Agriculture and Management of Agricultural Resources, no. 8001-CT90-0014), by the Scottish Office, Agriculture and Fisheries Department, and by the Animal Disease Research Association.

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