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Comparison of *Mycobacterium avium* complex (MAC) strains from pigs and humans in Sweden by random amplified polymorphic DNA (RAPD) using standardized reagents

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Abstract

Infections with atypical mycobacteria belonging to the *Mycobacterium avium/intracellulare* complex (MAC) can cause infection in both animals and humans. Using a standardized reagents commercial kit for random amplified polymorphic DNA (RAPD) analysis, 49 MAC strains isolated from 32 slaughter pigs and 17 humans in Sweden were identified and sorted out, yielding 6 RAPD types. By combining the results of RAPD primers 4 and 5 and the primer IS1245A, we found that pigs and humans may be infected with the same types of MAC strains, since 14 strains from humans and 8 strains from pigs were essentially identical and together, comprised RAPD type 2, the largest group of strains (44.8% of strains). With respect to grouping of strains, serotype and RAPD type were uncorrelated, except for serotype 20 and RAPD type 6. Using standardized beads, RAPD analysis is a reproducible technique for typing MAC strains, as the indistinguishable banding patterns obtained with repeated analyses of two isolates from each strain in this study demonstrate. However, primer selection and DNA purity were crucial for differentiating closely related strains.

Keywords: *Mycobacterium avium*; Complex; Pig-bacteria; Zoonoses; RAPD; Sweden

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1. Introduction

Mycobacterium avium complex (MAC), which can cause lymphadenitis with local granulomatous lesions in pigs (Thorel et al., 1997), can also cause infections in humans (Hoffner et al., 1990). Moreover, previous studies, using restriction fragment length polymorphism (RFLP), have shown that pigs and humans share a high degree of IS1245-based polymorphism, indicating that pigs may be an important vehicle for *M. avium* infections in humans (Bono et al., 1995; Komijn et al., 1999).

New molecular techniques, such as pulse-field gel electrophoresis (Mazurek et al., 1993; Bono et al., 1995) and RFLP, applying IS900, IS901, IS1311, IS1245 repetitive insertion sequences as probes (Collins et al., 1997), or IS1245 alone (Van Soolingen et al., 1998; Komijn et al., 1999), appear to be suitable epidemiological tools for studying MAC infections. However, these techniques require from 1 to 2 μg of DNA to give informative results.

There is though an alternative typing technique available now for many species of bacteria, including *Mycobacterium* (Richner et al., 1997; Scheibl and Gerlach, 1997; Zhang et al., 1997), namely, random amplified polymorphic DNA (RAPD) (Williams et al., 1990, 1993), or arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and Mccleland, 1990, 1993), which requires only nanograms of the target DNA, and no prior knowledge of its genetic structure. Moreover, by priming with an arbitrary-sequence primers, the entire DNA information is utilized.

At the same time, despite recent successes with RAPD analysis, such as revealing the genetic diversity in *M. avium* strains obtained from AIDS patients (Matsiota-Bernard et al., 1997), reproducibility of results within and between laboratories can be a problem (Meunier et al., 1993; Tyler et al., 1997). This can result from differences in DNA quality and inconsistencies in reagent concentrations, DNA polymerase, or annealing temperatures (Meunier and Grimont, 1993; Power, 1996). However, by using pre-formulated RAPD analysis beads that contain all PCR reaction buffers, all nucleotides and two DNA polymerases (AmpliTaq and Stoffel fragment), Vogel et al. (1999) achieved stable banding patterns and within-laboratory reproducibility, and Grundmann et al. (1997) achieved inter-laboratory reproducibility. Therefore, because suitable use of these standardized RAPD beads not only provides reproducible results, but is also inexpensive and fast to use, we employed them to identify and compare *M. avium* complex strains, taken from humans and slaughtered pigs in Sweden.

2. Material and methods

2.1. Bacterial strains

Thirty-two MAC strains were taken from conventionally reared infected fattening pigs (5–6-months-old) that, at meat inspection in different slaughterhouses, were found to have gross lesions of generalized mycobacteriosis. The pigs came from medium to large scale farms, mainly in the southern part of Sweden. Tissue samples were taken mostly from lymph nodes and liver. Another 17 MAC strains were collected from Swedish AIDS

and non-AIDS patients in the Stockholm area. All 49 isolates were identified as MAC strains by standard methods described by (Hoffner et al., 1990) and all strains were also serotyped, using the standard Schaefer method (Schaefer, 1980).

2.2. Preparation of chromosomal DNA from MAC strains

The 49 strains (Table 2) were cultured on Lowenstein–Jensen medium. Loopfuls of cultured bacteria were transferred to conical tubes containing 0.5 ml sterile water, one loopful per tube, then incubated at 94°C for 15 min. Later, 0.5 ml of 100 μ sterile glass beads (Sigma) were added. The mixture was vortexed vigorously for 5 min, cooled by ice bath and later centrifuged at 13,000 RPM for 5 min in an Eppendorf centrifuge 5042. The DNA from the supernatant was purified by phenol:chloroform extraction and ethanol precipitation, a method described by (Sambrook et al., 1989). Afterwards, the resulting purified DNA pellet was dissolved in 100 μl sterile distilled water in order to determine its purity and concentration, spectrophotometrically, using Gene Quant RNA/DNA calculator (Pharmacia Biotech, Sweden).

2.3. Selection of RAPD primers

DNA samples were obtained from 3 *M. avium* reference strains, namely, IWGMT49, R13 and Benkowa, and also from 3 strains randomly selected from our 49 MAC strains. These samples were amplified with 6 different primers from the commercial RAPD kit (Amersham Pharmacia Biotech) and in addition, with primers IS1245A and IS1245B (Table 1). RAPD primers 4 and 5 from the commercial RAPD kit yielded the largest number of strong band amplicons over a broad molecular size range and differentiated the 3 MAC reference strains. Hence, these 2 primers were selected for RAPD analysis of the remaining 46 of our original 49 strains. In addition, we tested the discriminatory power of primer IS1245A using all 49 MAC strains.

Table 1
The random amplified polymorphic DNA primers screened for this study

Primer	Sequence (5′ to 3′)	Number of bands	Banding molecular size range (bps)	RAPD profile[a]
RAPD analysis primer 1	5′-d[GGTGCGGGAA]-3′	8	230–1000	LD
RAPD analysis primer 2	5′-d[GTTTCGCTCC]-3′	8	400–2000	LD
RAPD analysis primer 3	5′-d[GTAGACCCGT]-3′	9	420–1700	LD
RAPD analysis primer 4[b]	5′-d[AAGAGCCCGT]-3′	9	230–1800	GD
RAPD analysis primer 5[b]	5′-d[AACGCGCAAC]-3′	10	150–2000	GD
RAPD analysis primer 6	5′-d[CCCGTCAGCA]-3′	10	175–1800	LD
IS1245A[b]	5′-d[GCCGCCGAAACGATCTAC]-3′	7	250–1800	LD
IS1245B	5′-d[AGGTGGCGTCGAGGAAGAC]-3′	7	250–1800	LD

[a] GD — strong band, good discrimination, LD — strong band, low discrimination.
[b] After screening, primers 4, 5 and IS1245A were used for the RAPD analysis of all the MAC strains.

2.4. RAPD-polymerase chain reaction conditions and RAPD data analysis

The "Ready-To-Go RAPD Analysis Beads" commercial kit (Amersham Pharmacia Biotech, Sweden) was used, which contains thermostable polymerases (AmpliTaq DNA polymerase and Stoffel fragment), dNTPs (0.4 mM, each dNTPs in a 25 μl reaction volume), bovine serum albumin (2.5 μg) and buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris, pH 8.3 in a 25 μl reaction volume). We added 10 μl of primer (2.5 pmol/μl), 8 μl of template DNA (10 ng/μl) and 7 μl of sterile water, yielding a total volume of 25 μl. The reaction mixture was run in a Gene Amp PCR system, 2400 thermocycler (Perkin–Elmer), for 45 cycles (1 min at 94°C, 1 min at 36°C, and 2 min at 72°C). The amplicons were visualized by means of ethidium bromide staining after electrophoresis in a 2% Nusieve agarose gel. Photographs were taken under UV light using a transilluminator. To determine the reproducibility of our results, PCR was done at least twice on all samples. The *M. avium* reference strain "Benkowa" was used as internal control for measuring variability of RAPD patterns among experiments. RAPD patterns of the individual strains were analyzed with respect to the presence or absence of electrophoresis DNA bands. The index of similarity (F_{xy}) between samples was calculated using the formula (Nei and Li, 1979)

$$F_{xy} = \frac{2n_{xy}}{(n_x + n_y)},$$

where n_{xy} is the number of RAPD bands shared by the two samples and n_x and n_y are the number of RAPD bands scored in each sample. The genetic distance (d) was calculated using the equation $d = (1-F_{xy})$ given by Hillis and Moritz (1990).

3. Results

By serotyping, 9 (18%) of the 49 MAC strains were untypeable (Table 2). In the 32 MAC strains from pigs, only 3 serotypes, 1, 2 and 8, were found. Serotype 2, by far the most prevalent, was found in 17 (53%) of the pig strains, whereas serotypes 8 and 1 were found in 6 (18%) and 4 (12%) strains, respectively. Results for the 17 MAC strains from humans were quite different, serotypes 4, 6 and 20 were found and serotype 4, found in 6 (35%) of the strains, was the most prevalent.

In contrast to serotyping, by the RAPD technique, using RAPD primers 4, 5 and IS1245A, all strains could be typed, based on the presence and size of their RAPD products. Amplification reactions generated informative arrays of bands composed of a minimum of 4 bands and a maximum of 10, with the molecular size ranging from 150 to approximately 2000 bps (Table 1). The 49 MAC strains fell into the following profiles, 6 genomic RAPD profiles (A1–A6), 4 RAPD profiles (B1, B2, B3, B4), and 2 RAPD profiles (C1, C2), as shown in Table 3. By combining the results of the 3 mentioned primers, all 49 strains fell into 6 RAPD types, where RAPD type 2, represented by 22 strains (44.8%), was predominant, followed by RAPD type 1 (13 strains), then RAPD type 3 (5 strains), RAPD type 4 (3 strains) and RAPD type 5 (3 strains), as shown in Tables 2 and 3.

Table 2
Strain number/collected year, origin, serotype, RAPD type and group type of *M. avium* complex (MAC) isolates from humans and slaughter pigs in Sweden

	Strain number/ collected year	Origin	Serotype[a]	RAPD profile[b]			RAPD type	Group type[c]
				Primer 5	Primer 4	IS1245A		
1	S 147/85	Pig liver	1	A1	B1	C1	1	1
2	S 289/86	Pig liver	2	A1	B1	C1	1	
3	S 304/86	Pig liver	2	A1	B1	C1	1	
4	S 210/86	Pig liver	2	A1	B1	C1	1	
5	S 293/86	Pig liver + lymph node	2	A1	B1	C1	1	
6	S 294/86	Pig lymph node	2	A1	B1	C1	1	2
7	S 307/86	Pig liver	2	A1	B1	C1	1	
8	S 315/86	Pig liver	2	A1	B1	C1	1	
9	S 51/87	Pig liver	2	A1	B1	C1	1	
10	S 306/86	Pig liver	2	A1	B1	C1	1	
11	S 302/86	Pig liver	2	A1	B1	C1	1	
12	S 55/87	Pig	2	A1	B1	C1	1	
13	S 53/87	Pig	8	A1	B1	C1	1	3
14	S 316/86	Pig liver	1	A2	B2	C1	2	4
15	S 312/86	Pig lymph node	1	A2	B2	C1	2	
16	S 295/86	Pig liver + lymph node	2	A2	B2	C1	2	5
17	S 52/87	Pig	8	A2	B2	C1	2	6
18	S 60/87	Pig	8	A2	B2	C1	2	
19	S 286/86	Pig liver	UNT	A2	B2	C1	2	
20	S 303/86	Pig liver	UNT	A2	B2	C1	2	7
21	S 288/86	Pig liver	UNT	A2	B2	C1	2	
22	S 285/86	Pig liver	2	A3	B1	C1	3	
23	S 145/85	Pig lung tissue	2	A3	B1	C1	3	8
24	S 151/85	Pig lymph node	2	A3	B1	C1	3	
25	S 301/86	Pig	2	A3	B1	C1	3	
26	S 305/86	Pig liver	1/2	A3	B1	C1	3	9
27	S 309/86	Pig lymph node	8	A4	B3	C1	4	10
28	S 311/86	Pig lymph node	8	A4	B3	C1	4	
29	S 300/86	Pig lymph node	No GPLs	A4	B3	C1	4	11
30	S 299/86	Pig liver + lymph node	1	A5	B3	C1	5	12
31	S 296/86	Pig liver + lymph node	2	A5	B3	C1	5	13
32	S 287/86	Pig liver	8	A5	B3	C1	5	14
33	S 311/90	Human AIDS (blood)	6	A2	B2	C1	2	15
34	S 308/90	Human (sputum)	6	A2	B2	C1	2	
35	S 290/90	Human (bone marrow)	4	A2	B2	C1	2	
36	S 309/90	Human (blood)	6	A2	B2	C1	2	
37	S 112/87	Human AIDS (blood)	6	A2	B2	C1	2	16
38	S 34/87	Human AIDS (blood)	6	A2	B2	C1	2	
39	S 71/87	Human AIDS (blood)	6	A2	B2	C1	2	
40	S 56/88	Human AIDS (blood)	6	A2	B2	C1	2	
41	S 89/87	Human AIDS (lymph node)	UNT	A2	B2	C1	2	
42	S 136/87	Human AIDS	UNT	A2	B2	C1	2	
43	S 44/87	Human AIDS (faces)	UNT	A2	B2	C1	2	7
44	S 54/91	Human (blood)	UNT	A2	B2	C1	2	
45	S 10/87	Human (sputum)	UNT	A2	B2	C1	2	
46	S 49/87	Human (bronchial secretion)	UNT	A2	B2	C1	2	
47	S 291/90	Human (sputum)	20	A6	B4	C2	6	
48	S 298/90	Human (bronchial secretion)	20	A6	B4	C2	6	17
49	S 364/90	Human (sputum)	20	A6	B4	C2	6	

[a] UNT — not typeable, No GPLs — no glycolipids.

[b] RAPD profiles are identified by a letter indicating the profile's major group and a digit indicating its subgroup (i.e. minor differences).

[c] Group type from combining serotype and RAPD type.

Table 3
Similarity coefficient *F* (above diagonal) and genetic distance *d* (below diagonal) for the 6 RAPD types of MAC from pigs and humans in Sweden and the RAPD type C of *M. avium* "Benkowa," used as an internal-control reference strain[a]

RAPD types	1	2	3	4	5	6	C
1	–	0.86	0.93	0.57	0.61	0.43	0.93
2	0.14	–	0.77	0.50	0.54	0.85	0.92
3	0.07	0.23	–	0.61	0.46	0.54	0.86
4	0.43	0.50	0.39	–	0.91	0.66	0.50
5	0.39	0.46	0.54	0.09	–	0.83	0.54
6	0.57	0.15	0.56	0.34	0.17	–	0.36
C	0.07 0.08	0.14	0.50	0.46	0.64	–	
Human (strain)	–	14	–	–	–	3	–
Pig (strain)	13	8	5	3	3	–	–

[a] Average *F* of RAPD type C = 69%, average *d* of RAPD type C = 0.31.

The banding pattern of RAPD type 1 was highly similar to that of RAPD type 3, as indicated by a similarity index of 93%, and likewise, RAPD types 4 and 5 were highly similar (similarity index, 91%). The thirty-two strains from pigs fell into RAPD types 1 through 5, and the 17 strains from humans fell into RAPD types 2 and 6. We found that 14 (82%) of the strains from human and 8 (25%) of the strains from pigs belong to RAPD type 2 (Tables 2 and 3). The *M. avium* "Benkowa" reference strain, used as an internal control, fell into RAPD type C, which has 93, 92, 86, 50, 54 and 36% genetic similarity with RAPD types 1, 2, 3, 4, 5 and 6, respectively. The average genetic similarity between RAPD type C and RAPD types 1 through 6 for all 49 strains was 69%, with an average genetic distance of 0.31 (Table 3).

For each strain included in the study, the banding patterns obtained with the 3 selected primers were indistinguishable in repeated analyses (data not shown). Also, the resolution of our typing procedure was strengthened by the fact that between RAPD types and serotypes, there was no correlation with respect to grouping of strains, except that 3 MAC strains within serotype 20 belonged to RAPD type 6 (Table 2).

4. Discussions

The serotype pattern for MAC in pigs and humans in Sweden accords with previously reported patterns (Hoffner et al., 1990) in that serotype 2 was the most prevalent in pigs, and serotype 4, the most prevalent in humans. In this study, besides serotype 4, humans were infected with MAC serotypes 6 and 20. The latter is considered to be *M. intracellulare* (Schaefer, 1980). Note also that we differentiated MAC serotypes 1, 2, 4, 6 and 8 (RAPD types 1–5) from *M. intracellulare* (serotype 20/RAPD type 6), as shown in Tables 2 and 3.

In this study, the utility of using RAPD for typing of MAC strains can be summarized as follows. Using serotyping technique, 9 strains (18%) were untypeable, but all 49 MAC

strains were typed by means of RAPD, using all 3 of our selected primers, which allowed us to clearly distinguish, serotype 20, as stated. Also, it was possible to differentiate strains of the same serotype (Table 2). For example, the RAPD technique subdivided MAC strains with serotype 1 into RAPD types 1, 2 and 5, and strains with serotype 2 into RAPD types 1, 2 and 3, and finally, strains with serotype 8 into RAPD types 2 and 4. At the same time, in reverse, strains with same RAPD type could be subtyped by serotyping, as in the case of RAPD type 2, which included strains with serotype 1, 2, 4, 6 and 8 (Table 2). This means that the RAPD technique cannot replace serotyping in epidemiological studies, and that the best way to identify MAC strains is a combination of serotyping and RAPD typing. Indeed, by combining the information from both typing techniques, we were able to group more informatively, and with greater detail (into 17 groups), the 49 MAC strains of our study (Table 2).

We also found that 14 (82%) MAC strains from human and 8 (25%) strains from pigs were included in RAPD type 2, which supports the idea that pigs and humans may be infected with the same type of MAC strains, as other studies of MAC in pigs and humans corroborate. For instance, in Switzerland and the Netherlands, using RFLP technique, they found that pigs and humans shared a high degree of IS1245-based polymorphism (Bono et al., 1995; Komijn et al., 1999).

Although our results confirm the finding of Matsiota-Bernard (1997) that the primers IS1245A and IS1245B amplify insertion element IS1245, which is specific for *M. avium* strains and in high stringency conditions can be used for identification of *M. avium* strains by RAPD analysis, at the same time, we found that these primers gave less discriminatory power than using short (10 bps) arbitrary-sequence primers, such as RAPD primers 4 and 5 (Table 3). In fact, RAPD primer 5 was, by itself, sensitive enough to differentiate all 49 MAC strains. Our results for *M. tuberculosis* followed suit. We ran RAPD using the PGRS (polymorphic guanine–cytosine-rich) primer, which is normally used as a probe in RFLP analysis, and compared it with primer 5, our short (10 bps) arbitrary-sequence RAPD primer. We found, for all selected *M. tuberculosis* strains, that primer 5 discriminated better (unpublished data).

It is noteworthy that the reproducibility of RAPD bands was affected by DNA purity. Before purification of the DNA, we observed slight variation in banding pattern for the same MAC strain that came from two different DNA extraction methods (result not shown), but after we purified the DNA, from either extraction method and ran RAPD again, we obtained an identical and reproducible banding pattern. Hence, we suspect that purification of DNA before running RAPD is needed, and that using an inexpensive method is sufficient, such as phenol chloroform extraction and ethanol precipitation.

In summary, our results demonstrate that RAPD analysis using Ready-To-Go beads is a reproducible method for identifying MAC strains and that RAPD can be simple, cheap and fast and also that large numbers of samples can easily be handled. In other words, RAPD can be used as a productive alternative subtyping technique for MAC strains, especially when samples contain low amounts of DNA, at nanogram levels. Nonetheless, confirmation of our findings is needed, along with further study of large scale MAC typing, using more extensive primer screening and comparing results with other typing techniques.

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