

HPLC does not differentiate *Mycobacterium paratuberculosis* from *Mycobacterium avium*

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Abstract

HPLC, which is gaining its place as identification tool in mycobacteriology laboratories, has been proposed to distinguish *Mycobacterium paratuberculosis* from *Mycobacterium avium*. We had reported no significant difference between *M. avium* and *M. paratuberculosis* reference strain ATCC 19698. Because of the advantages offered by such a method, we enlarged our observations to include more isolates of *M. paratuberculosis*. Within the double cluster of peaks obtained by both *M. avium* and *M. paratuberculosis*, we could not find a consistent difference typical of *M. paratuberculosis*. Therefore, the present study confirmed that *M. avium* and *M. paratuberculosis* could not be distinguished by HPLC, raising doubts of a straightforward use of HPLC to identify *M. paratuberculosis*. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Mycobacterium paratuberculosis*; Diagnosis-bacteria; HPLC

1. Introduction

Mycobacterium paratuberculosis, the etiological agent of Johne's disease (JD) in ruminants, is no longer considered as a separate taxonomic species, but rather as *M. avium* subsp. *paratuberculosis* (Rogall et al., 1990; Thorel et al., 1990). Nevertheless, compared to *M. avium*, *M. paratuberculosis* grows more slowly, has stable a mycobactin requirement, is considered an obligate pathogen, and its natural host-range is more restricted (Cocito et al., 1994; Collins et al., 1982). Moreover, the insertion sequence

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IS900 is considered unique to *M. paratuberculosis* (Green et al., 1989), which can also be distinguished from *M. avium* by RFLP analysis of the *hsp65* gene (Eriks et al., 1996).

M. avium is occasionally responsible for a disease resembling paratuberculosis, and little is known about its transmission. In many countries animals infected with *M. paratuberculosis* are culled, while those infected with *M. avium* are not. Therefore, the need for reliable and rapid identification procedures (Collins, 1995; Eriks et al., 1996; McFadden et al., 1992). Among the innovative methods, genomic techniques (mostly IS900-based) are widely adopted, but also high-performance liquid chromatography (HPLC) profiling of mycolic acids has been considered for the identification of *M. paratuberculosis* (Collins, 1995).

Mycolic acids are used to identify mycobacteria, and HPLC analysis of mycolic acid profiles stands out as a simple and rapid alternative to more complex analytical methods to study those lipids. In addition, HPLC seems most practical when dealing with uncommon or new mycobacteria, for which probe-based commercial identification kits are not available (Butler et al., 1991, 1992; Cage, 1992; Glickman et al., 1994; Thibert and Lapiere, 1993; Tortoli et al., 1995; Tortoli and Bartoloni, 1996). The typical profiles of several mycobacterial species have been published, but, to our knowledge, few data are available on *M. paratuberculosis*. Collins et al. (1995) had reported that a difference between *M. avium* and *M. paratuberculosis* could be detected. (Collins et al., 1995). On the contrary, Tortoli et al. (1995), who included *M. paratuberculosis* ATCC reference strain 19698 in their study on less common mycobacteria, found that the *M. paratuberculosis* profile was very similar to that of *M. avium* (Tortoli et al., 1995). Because of that discrepancy, our previous work was enlarged to include other isolates of *M. paratuberculosis*.

2. Materials and methods

The *M. paratuberculosis* ATCC 19698 reference strain and six independent bovine isolates together with the human isolate ATCC 43544 were included in the study. The bacteria were sub-cultured in 7H11 medium supplemented with Mycobactin J (Rhône Mérieux, Lyon, France). The isolates were identified by standard conventional methods. All the strains were IS900 positive.

HPLC was carried out according to the procedure developed at the Centers for Disease Control and Prevention (Butler et al., 1991), as described by Tortoli and Bartoloni (1996). For pattern analysis, fractions eluted within the first 4 min and peaks less than 2% of the total peak height were not considered. Valid peaks were identified by their relative retention time (RRT), determined with an internal standard, according to the CDC scheme. The profiles were compared visually to our collection of profiles from *M. avium*, which were isolated from humans, mostly from HIV-positive subjects, and identified by conventional methods as well as a commercial probe.

3. Results and discussion

M. paratuberculosis and *M. avium* showed a double-cluster peak arrangement. The *M. paratuberculosis* chromatograms were, as expected, not perfectly matching. Compared

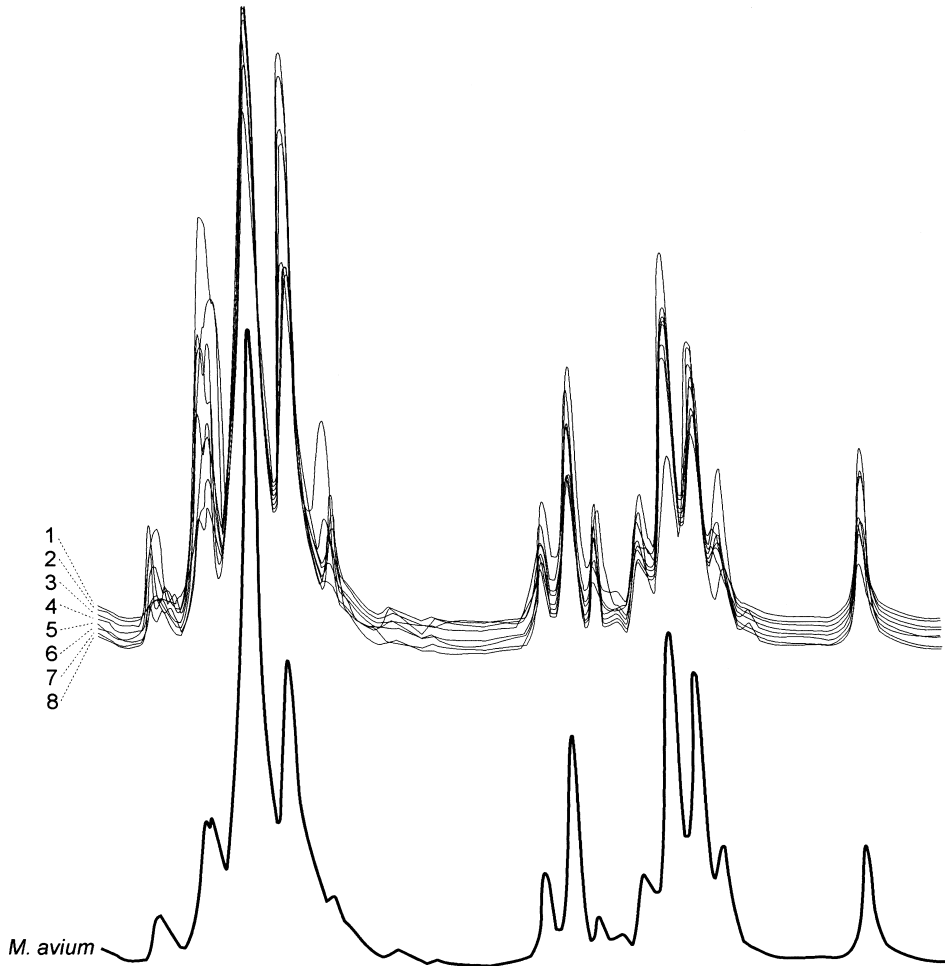


Fig. 1. Aligned HPLC profiles of mycolic acids from the eight *M. paratuberculosis* strains compared with the representative *M. avium* chromatogram. The profiles of ATCC 19698, ATCC 43455 and the six animal isolates are numbered 1 to 8.

with a collection of *M. avium* profiles, we could not detect a consistent difference either in relation to number, position, or height of the peaks. Thus, *M. paratuberculosis* could not be identified by a particular profile. The aligned profiles obtained with the *M. paratuberculosis* strains are shown in the Fig. 1 together with a representative *M. avium* profile.

Due to the overall similarity between *M. avium* and *M. paratuberculosis*, it is justified not to consider them as separate species, however the possibility to identify them separately is important to define biological properties, such as frequency and distribution in nature, or association with pathogenicity (Collins et al., 1982). Misidentification, when using traditional methods, can occur. McFadden et al. (1992) showed that about 10% of isolates from ruminants affected by JD and labelled as *M. paratuberculosis* were in fact

M. avium; the authors pointed out that the origin of the clinical sample as well as mycobactin-dependence might be a source of bias for the distinction between *M. avium* and *M. paratuberculosis*. The confirmation of IS900 is considered definitive for the identification of *M. paratuberculosis*, however, a few IS900-negative strains have been reported (Hines, 1997). Therefore, the possibility to identify *M. paratuberculosis* by HPLC was appealing (Collins et al., 1995).

We confirmed our previous observation obtained with the reference strain only, that HPLC cannot distinguish between *M. avium* and *M. paratuberculosis*: neither one major difference was present nor significant minor difference/s could be constantly detected. Referring to the Fig. 1, one has to point out that, although we showed a representative *M. avium* profile, the same amount of variability is present also with *M. avium*. Cage in his work on *Mycobacterium gordonae* shows the amount of peak variability, even in highly controlled conditions (Cage, 1992).

The discrepancy between Collins' and our results could be explained by differences in the origin of the strains, or by differences in the evaluation of the results. The recognition of profiles is indeed a critical step of HPLC profiling, and might require some expertise. The assessment of the chromatograms range from just the visual comparison to sophisticated computer-assisted analysis (Glickman et al., 1994; Tortoli and Bartoloni, 1996). By the visual evaluation we could not detect a differentiating key mycolic acid peak. As far as our experience is concerned, the visual comparison was always supported by other means of identification, and we have now a HPLC library of about 60 mycobacterial species (Tortoli et al., 1995, 1996; Tortoli and Bartoloni, 1996). According to Collins et al. (1995), "Initially the computer program could not distinguish the chromatograms. However, visual inspection of the HPLC patterns revealed subtle differences. Once the HPLC pattern was entered into the pattern recognition library, the system successfully recognized *M. paratuberculosis*." Thus, although computer-assisted evaluation of the chromatograms might facilitate recognition of significant differences, visual inspection by an experienced eye can still be helpful, especially when dealing with the less common species (Butler et al., 1992; Cage, 1992; Collins et al., 1995; Glickman et al., 1994; Tortoli and Bartoloni, 1996).

4. Conclusions

Both studies agree that the HPLC profiles of *M. paratuberculosis* and *M. avium* do not show any major difference, but, whereas Collins et al. (1995) report a minor but significant difference, we concluded that the possible difference was within the range of the variability and the reproducibility of the method. Therefore, our data question the use of using mycolic acid profiling to identify *M. paratuberculosis*; thus, more data will be needed to assess definitively the possibility of distinguishing *M. avium* from *M. paratuberculosis* by HPLC.

References

- Butler, W.R., Jost Jr., K.C., Kilburn, J.O., 1991. Identification of mycobacteria by high performance liquid chromatography. *J. Clin. Microbiol.* 29, 2468–2472.

- Butler, W.R., Thibert, L.M., Kilburn, J.O., 1992. Identification of *Mycobacterium avium* complex strains and some similar species by high performance liquid chromatography. *J. Clin. Microbiol.* 30, 2698–2704.
- Cage, G.D., 1992. High performance liquid chromatography patterns of *Mycobacterium gordonae* mycolic acids. *J. Clin. Microbiol.* 30, 2402–2407.
- Cocito, C., Gilot, P., Coene, M., De Kesel, M., Poupart, P., Vannuffel, P., 1994. Paratuberculosis. *Clin. Microbiol. Rev.* 7, 328–345.
- Collins, M.T., 1995. Diagnosis and control of paratuberculosis. Proc. 4th Int. Colloq. on Paratuberculosis, International Association for Paratuberculosis, Rehobot, MA, USA, pp. 325–344.
- Collins, M.T., Glickman, S.E., Kilburn, J.O., 1995. Identification of *Mycobacterium paratuberculosis* by high pressure liquid chromatography (HPLC) analysis of their mycolic acid extracts. Proc. 4th Int. Colloq. on Paratuberculosis, International Association for Paratuberculosis, Rehobot, MA, USA, p. 292.
- Collins, P., Matthews, P.R.J., McDiarmid, A., Brown, A., 1982. The pathogenicity of *Mycobacterium avium* and related mycobacteria for experimental animals. *J. Med. Microbiol.* 16, 27–35.
- Eriks, I.S., Munck, K.T., Besser, T.E., Cantor, G.H., Kapur, V., 1996. Rapid differentiation of *Mycobacterium avium* and *M. paratuberculosis* by PCR and restriction enzyme analysis. *J. Clin. Microbiol.* 34, 734–737.
- Glickman, S.E., Kilburn, J.O., Butler, W.R., Ramon, S.L., 1994. Rapid identification of mycolic acid patterns of mycobacteria by High-Performance Liquid Chromatography using pattern recognition software and a *Mycobacterium* library. *J. Clin. Microbiol.* 32, 740–745.
- Green, E.P., Tizard, M.L.V., Moss, M.T., Thompson, J., Winterbourne, D.J., McFadden, J.J., Hermon-Taylor, J., 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acid Res.* 17, 9063–9073.
- Hines, M.E., 1997. Nomenclature: *M. paratuberculosis* vs. *M. avium* subspecies *paratuberculosis* and strain definitions and differentiation. Proc. 5th Int. Colloq. on Paratuberculosis, International Association for Paratuberculosis, Rehobot, MA, USA, pp. 93–95.
- McFadden, J., Collins, J., Beaman, B., Arthur, M., Gitnick, G., 1992. Mycobacteria in Crohn's disease: DNA probes identify the wood pigeon strain of *Mycobacterium avium* and *Mycobacterium paratuberculosis* from human tissue. *J. Clin. Microbiol.* 30, 3070–3073.
- Rogall, T., Wolters, J., Flohr, T., Böttger, E.C., 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int. J. Syst. Bacteriol.* 40, 323–330.
- Thibert, L., Lapiere, S., 1993. Routine application of high-performance liquid chromatography for identification of mycobacteria. *J. Clin. Microbiol.* 31, 1759–1763.
- Thorel, M.F., Krichevsky, M., Levy-Frebault, V.V., 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int. J. Syst. Bacteriol.* 40, 254–260.
- Tortoli, E., Bartoloni, A., 1996. High-performance liquid chromatography and identification of mycobacteria. *Rev. Med. Microbiol.* 7, 207–219.
- Tortoli, E., Bartoloni, A., Burrini, C., Mantella, A., Simonetti, M.T., 1995. Utility of high-performance liquid chromatography for identification of mycobacterial species rarely encountered in clinical laboratories. *Eur. J. Clin. Microbiol. Infect. Dis.* 14, 240–243.
- Tortoli, E., Kirschner, P., Bartoloni, A., Burrini, C., Manfrin, V., Mantella, A., Scagnelli, M., Scarparo, C., Simonetti, M.T., Böttger, E.C., 1996. Isolation of an unusual mycobacterium from an AIDS patient. *J. Clin. Microbiol.* 34, 2316–2319.