Epidemiol. Infect. (1997), **118**, 227–233. Printed in the United Kingdom

Comparative study of *Mycobacterium paratuberculosis* strains isolated from Crohn's disease and Johne's disease using restriction fragment length polymorphism and arbitrarily primed polymerase chain reaction

B. FRANÇOIS*, R. KRISHNAMOORTHY AND J. ELION†

INSERM U 458, Hôpital Robert Debré, 48 Bd Sérurier, 75019 Paris, France

(*Accepted 4 December 1996*)

SUMMARY

To obtain insights into the pathogenic mechanisms involving *Mycobacterium paratuberculosis* in Crohn's disease (CD) we questioned if the strains of *M. paratuberculosis* isolated from CD are distinguishable from those involved in Johne's disease (JD), a chronic granulomatous enteritis in cattle. Accordingly we compared human and animal strains at the DNA level, both by the analysis of restriction fragment length polymorphism (RFLP) in and around the insertion sequence IS 900 and by the arbitrarily primed polymerase chain reaction (AP-PCR). Results are in favour of a common clonal origin for the 4 strains isolated from CD and for 8 of the 11 strains isolated from cattle and sheep JD.

INTRODUCTION

Crohn's disease (CD) is one of the most frequent chronic enteritis in humans and its aetiology is still unknown. The proposal that *Mycobacterium paratuberculosis* might be involved in the pathogenesis of CD was based on two observations: (i) both clinical and histological features of CD resemble those of intestinal tuberculosis and (ii) *M. paratuberculosis* is a proven causative organism in Johne's disease (JD), a chronic granulomatous enteritis in cattle. *M. paratuberculosis* belongs to the *M. avium – intracellulare* complex. This species, as defined by DNA–DNA hybridization, includes a large variety of sub-species previously identified as *M. avium*, *M. intracellulare*, *M. wood-pigeon*, *M. paratuberculosis*, and *M. lepraemurium*. Among them, *M. paratuberculosis*, *wood-pigeon* mycobacteria and several serotypes of *M. avium* have been shown to be closely related, presenting greater than 90% hybridization level [1]. Progress in their classification was recently provided by the application of numerical taxonomy [2]. Among the genus, the subspecies *M. paratuberculosis* was initially distinguished on the basis of its very slow growth-potential and of the persisting dependence on mycobactin, an iron chelator [3]. An insertion DNA sequence, IS 900, was described as highly specific of *M. paratuberculosis*, which is repeated 15–20 times in its genome [4].

The identification of the IS 900 sequence, being both specific and present in multiple copies, has offered the possibility of developing sensitive strategies to examine the presence of *M. paratuberculosis* in CD's tissue, which overcome the cumbersome isolation procedure of such slow-growing micro-organisms [5–9]. Using specific oligonucleotide primers for IS 900 and DNA amplification by the polymerase chain reaction (PCR), several groups reported a positive correlation between the presence of IS 900 DNA (implying the presence of *M. paratuberculosis*) and CD [10–13]. However, frequency of detection of the IS 900 sequence differed from one study to another, ranging from 15% in CD and total absence in controls [10] to 65% in CD and 12% in controls [11]. Therefore *M. paratuberculosis* appears indeed to be involved in the pathogenesis of

* Present address: Unité des Agents Antibactériens, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15 France.
† Reprints: J. Elion, INSERM U458, Hôpital Robert Debré, 48 Bd Sérurier, 75019 Paris, France.

at least a subgroup of CD patients. No association has been made so far between CD in humans and Johne's disease (JD) in cattle, the common animal reservoir of the subspecies. However, in JD, two host-specific subgroups of *M. paratuberculosis* strains have been recognized respectively for bovines and sheep, based upon the restriction fragment length polymorphism (RFLP) profiles of genomic DNA [14]. This raises the possibility that a particular group of the subspecies *M. paratuberculosis* with host-specificity might be involved in CD. To clarify this issue, we have performed a comparative study of DNA polymorphism of *M. paratuberculosis* strains isolated from CD and JD by two independent methods, namely the analysis of RFLPs in and around the IS 900 sequence and arbitrarily primed PCR (AP-PCR). This second approach which involves study of DNA markers randomly dispersed over the bacterial chromosome has been shown to be highly discriminative in bacterial molecular epidemiology [15].

MATERIALS AND METHODS

Mycobacterial strains

Four strains isolated from CD (strains 64–68) were collected from the American Type Culture Collection (ATCC). Strains were primarily isolated from human tissue culture. Geographic origin was USA for three strains and Europe (Netherlands) for one strain. Nine strains isolated from JD were chosen from the ATCC (strain 63) and the United States Department of Agriculture (USDA) collections (strains 71–78), based on results of previous RFLP studies [16], in order to get the largest level of genetic heterogeneity. Two European strains (strains 79–80) were added, in order to have a geographic heterogeneity similar to that of CD strains. They were provided by the École Vétérinaire de Maison-Alfort, France. These JD strains were isolated from faeces. All strains were cultured in Middlebrook 7H9 medium supplemented with 10% Dubos oleic albumine complex enrichment, 2 mg/l mycobactin J (Institut Mérieux, Lyon, France) and 0·05% Tween 80.

DNA preparation

DNA extraction was carried out according to the method reported by Bose and colleagues [17]. Briefly, mycobacteria were washed in a solution of 50% Tris-HCl pH 8·0 and 50% saturated cesium chloride with 1% Triton-X, centrifuged and resuspended with 30 volumes of sterile water to create an osmotic shock. Enzymatic lysis was performed for 1 h at 4 °C by adding 0·5 mg/ml of lysozyme, and then 4 h at 55 °C with 100 μg/ml of proteinase K in sodium dodecyl sulphate (final concentration 0·5%) at 55 °C. 1 ml of a 4 M guanidium isothiocyanate cocktail (5·5 g/ml guanidium isothiocyanate, 0·05 M Tris-HCl (pH 7·5), 12 mM EDTA (pH 8·0), 0·2 M NaCl, 2% *N*-lauroyl sarcosine, 0·15 M 2-mercaptoethanol) and 1% cetyl trimethyl ammonium bromide were added and the mixture was incubated at 65 °C for 30 min. Further steps consisted in phenol–chloroform extraction followed by isopropanol precipitation.

For AP-PCR, DNA was extracted using the Insta Gene Purification Matrix (Bio-Rad, Hercules, CA, USA) as follows: one colony was incubated in 200 μl of Insta Gene Matrix for 60 min at 56 °C, boiled 12 min at 100 °C, centrifuged and the supernatant was stored at −20 °C.

Southern blotting

For each strain, 0·5–1 μg of DNA was digested overnight with 20 units of *Bst*E II, *Pst* I or *Pvu* II under the conditions recommended by the manufacturers (Pharmacia, Uppsala, Sweden). DNA fragments were separated by electrophoresis on 1% agarose gels at 30 V for 36 h and transferred to Hybond N+ membranes (Amersham, UK). A 400 bp fragment of the 5′ region of IS 900 (nucleotides 22 to 421) was amplified by PCR as described [10]. The PCR product was cloned into pCR™ II using the TA cloning kit (Invitrogen, San Diego, CA, USA) and sequence of the insert was confirmed. 50 ng of insert was labelled with [α^{32}P]dCTP using the Random Primed DNA Labeling Kit (Boehringer, Mannheim, Germany). Membranes were prehybridized for 2 h at 65 °C in 15 ml of 1% sodium dodecyl sulphate, 1 M NaCl, 50 mM Tris-HCl (pH 7·5), 5 × Denhardt's solution (1 × solution is 0·1% polyvinylpyrrolidone, 0·1% Ficoll, 0·1% bovine serum albumine) and 100 μg per ml of denatured salmon sperm. Hybridization was carried out overnight at 65 °C in a solution containing the prehybridization solution with 5% dextran sulphate and the labelled probe. Membranes were washed 2 × 15 min in 2 × SSC (1 × SSC is 0·15 M NaCl and 0·015 sodium citrate, pH 7·0) at room temperature, and twice for 30 min in 1 × SSC, 0·5%

https://doi.org/10.1017/S0950268897007486 Published online by Cambridge University Press

sodium dodecyl sulphate, at 65 °C. Autoradiographies were exposed for 1–4 days.

Arbitrarily primed-PCR

1 and 10 μl of total DNA extract were amplified in 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 4·5 mM $MgCl_2$, 0·2 mM each dNTP, 2·5 μCi [^{35}S]dATP, 75 pmole of primer and 0·75 unit of *Taq* polymerase (Gibco BRL, Life Technologies, Eragny, France) in a final volume of 25 μl. Primer sequences were: P1: 5′CTGTGGGGCGGTGTACTGAT3′, P2: 5′AGCCGAAGGTCAAAGCCATT3′. Reactions were overlaid with oil and cycled through the following profile: 94 °C for 7 min with hot start for initial denaturation, 4 cycles consisting in 40 °C 5 min, 72 °C 5 min, 94 °C 5 min, then 30 cycles consisting of 55 °C 1 min, 72 °C 2 min, 94 °C 1 min. Amplification products were analysed by electrophoresis in a 6% denaturing polyacrylamide gel for 5 h at 55 W. Autoradiographs were exposed for 2–6 days.

RESULTS

Restriction fragment length polymorphism

Hybridization of total DNA digested with restriction enzymes *Bst*E II, *Pvu* II or *Pst* I with an IS 900 probe gave at least 13 bands, ranging from less than 1 to 15 kb (Fig. 1). Among the 13 *M. paratuberculosis* strains studied, 3, 4 and 4 different RFLP profiles were distinguishable with enzymes *Bst*E II, *Pvu* II and *Pst* I, respectively (Table 1, Fig. 1). *Bst*E II profiles were similar in the 4 strains isolated from CD and in 6 of the 9 strains isolated from JD (profile **b**). Profiles of bovine isolates 74 and 76 (profile **a**) differed in the size of the fifth fragment (4·7 rather than 4·3 kb). Profile of the caprine isolate 78 (profile **c**) differed in the size of the sixth fragment: 3·3 kb rather than 3·5 kb.

Two different *Pvu* II profiles were observed among the 4 strains isolated from CD. The first, profile **e**, observed in strains 64 and 67, was also observed in 6 of the 9 strains isolated from JD (Fig. 1, lanes e and g; Table 1). The second, profile **d**, observed in strains 65 and 68, differed from profile **e** in the absence of the 1·6 kb band (Fig. 1). Profile **f**, observed in bovine isolate 76 and in caprine isolate 78, differed from profile **e** in the absence of the 2·1 kb band. Profile **h**, observed in bovine isolate 73, differed from profile **e** by the presence of an additional band of 2·6 kb.

Pst I profiles were similar in the 3 strains isolated from CD (profile **i**) and in 5 of the 9 strains isolated from JD (4 bovine strains and the caprine strain) (Table 1). Profile **j**, observed in bovine isolate 73, differed from profile **i** by the presence of an additional band of 1·8 kb. Profile **k**, observed in bovine isolates 75 and 77, differed from profile **i** by the presence of an additional band of 1·7 kb. Profile **l**, observed in bovine isolate 74, differed from profile **i** in the presence of an additional band of 4·7 kb (Fig. 1).

From these results, the 13 strains isolated from CD and JD could be differentiated into 7 groups. The fact that (i) observed variations are limited to a single restriction fragment, out of 13 or more fragments, visualized after hybridization with an IS 900 probe and that (ii) the changes observed with one enzyme are not paralleled with the two other enzymes, is more consistent with the alteration of specific restriction endonuclease sites rather than with differences related to the insertion sequence integration sites. Such a discriminative result is in part related to the choice of the studied strains isolated from JD, deliberately made to include strains with known high level of heterogeneity on previous RFLP studies [16]. Two distinct profiles were observed among the four strains isolated from CD. One was also the most common profile (profile **b**-e-**i**) found in strains isolated from JD. The second profile was not found in any of the nine strains isolated from JD.

Arbitrarily primed PCR

Various technical conditions were tested for random PCR. In our hands, reproducible amplification profiles were obtained with the AP-PCR procedure, but not with the RAPD procedure. AP-PCR procedure used differed from RAPD on three points: use of larger primers, amplification at more stringent temperature conditions after a few cycles of annealing at low temperature and analysis on polyacrylamide gels of labelled amplified DNA fragments [18–20]. Moreover, the quality of DNA appeared critical. We first tested 18 different AP-PCR primers with different GC contents (60–70%) and of different lengths (10-, 15-, and 20-mers). None of these primers succeeded in generating a sufficiently large number of fragments to provide a discriminative analysis. We then designed two 20-mer primers possessing one or two thymidine residues at their 3′ end, based upon the fact that this nucleotide, at this position, is known to support

Table 1. *RFLP analysis. Restriction endonuclease digestion of total DNA, followed by Southern blotting and hybridization with a IS 900 specific probe*

Isolated from	Strain no. (ATCC/USDA)	Geographical origin	RFLP with restriction enzyme		
			*Bst*E II	*Pvu* II	*Pst* I
Crohn's disease	64 (19851)	USA	**b**	**e**	**i**
	65 (43015)	Rhode Island	**b**	**d**	**i**
	67 (43545)	USA	**b**	**e**	
	68 (49164)	Netherlands	**b**	**d**	**i**
Bovine	63 (19698)	USA	**b**	**e**	**i**
	71 (1001)	Pennsylvania	**b**	**e**	**i**
	72 (1003)	Ohio	**b**	**e**	**i**
	73 (1018)	New York	**b**	**h**	**j**
	74 (1021)	Tennessee	**b**	**e**	**l**
	75 (1036)	Virginia	**a**	**e**	**k**
	76 (1038)	North Dakota	**b**	**f**	**i**
	77 (1113)	Colorado	**a**	**e**	**k**
Caprine	78 (4138)	Argentina	**c**	**f**	**i**

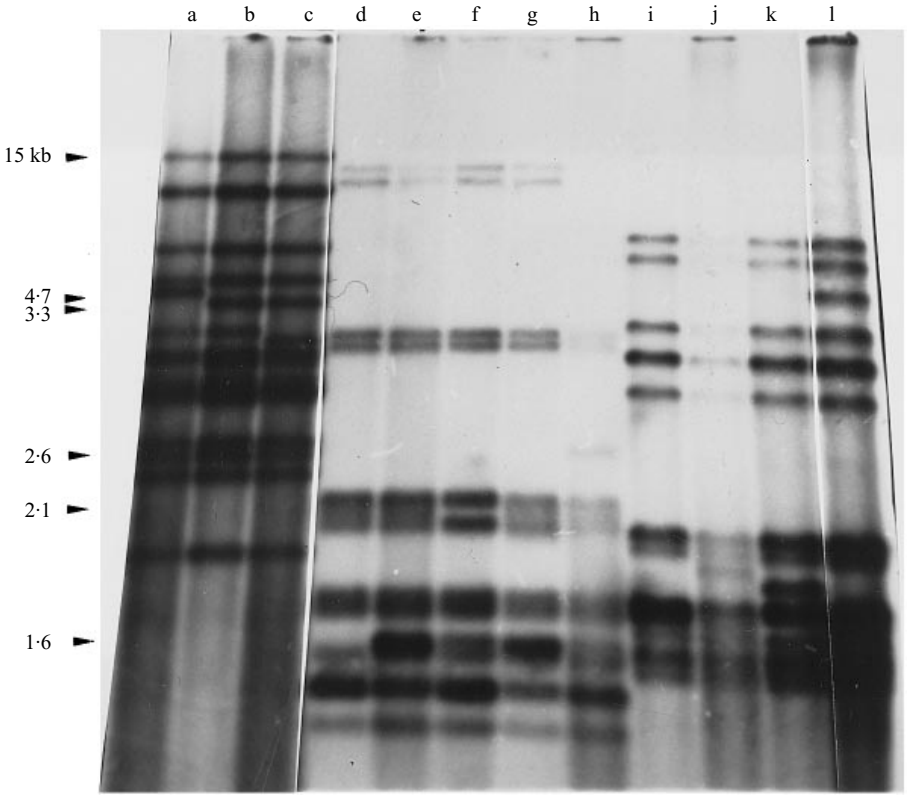

Fig. 1. RFLP analysis of IS 900 surrounding sequences . Lanes a–c: digestion with *Bst*E II. The respective profiles are **a**, **b**, **c** in Table 1. Lanes d–h: digestion with *Pvu* II. Respective profiles in table 1 are **d**, **e**, **f**, **h**. Profiles **e** and **g** are similar. Lanes i–1: digestion with *Pst* I. Respective profiles in Table 1 are **i**, **j**, **k**, **l**.

amplification even with mismatches with respect to the template sequence [21]. Indeed, these two primers were able to provide highly reproducible specific profiles generating more than 20 major DNA fragments. AP-PCR products, analysed on polyacrylamide gels in denaturing conditions, were similar for the 4 strains isolated from CD and for 8 of 11 strains isolated from JD. The two primers provided identical

https://doi.org/10.1017/S0950268897007486 Published online by Cambridge University Press

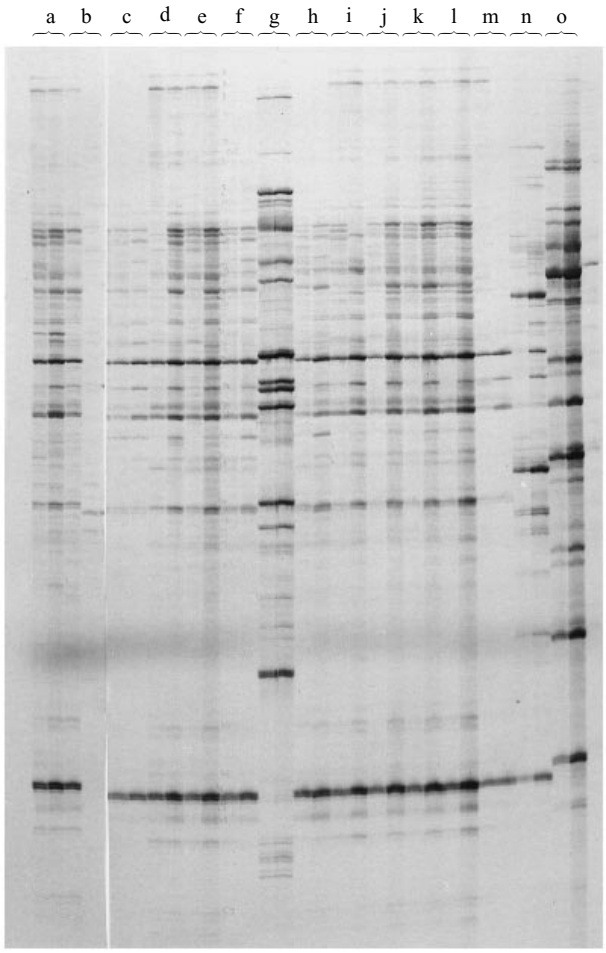

Fig. 2. AP-PCR analysis with primers P1. For each strain respectively 1 and 10 μl DNA samples have been used for PCR and both products have been loaded on the gel. Lanes a: strain 64; lanes b: strain 65; lanes c: strain 67; lanes d: strain 68; lanes e: strain 63; lanes f: strain 71; lanes g: strain 72; lanes h: strain 73; lanes i: strain 74; lanes j: strain 75; lanes k: strain 76; lanes l: strain 77; lanes m: strain 78; lanes n: strain 79; lanes o: strain 80. The last lane on the right corresponds to a mock PCR performed in the same conditions but with no DNA.

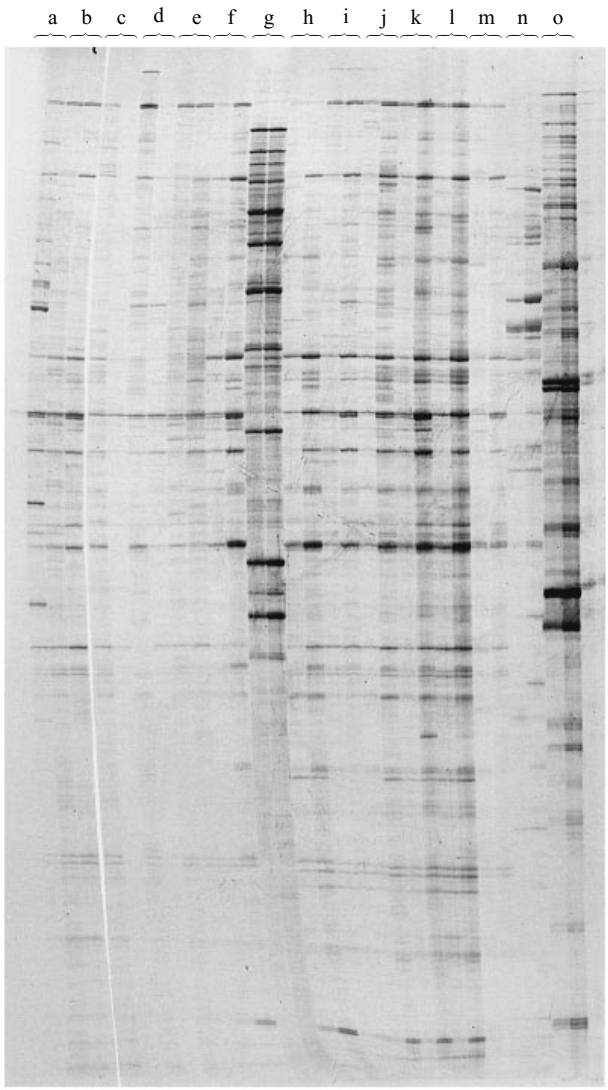

Fig. 3. AP-PCR analysis with primers P2. For each strain respectively 1 and 10 μl DNA samples have been used for PCR and both products have been loaded on the gel. Lanes a: strain 64; lanes b: strain 65; lanes c: strain 67; lanes d: strain 68; lanes e: strain 63; lanes f: strain 71; lanes g: strain 72; lanes h: strain 73; lanes i: strain 74; lanes j: strain 75; lanes k: strain 76; lanes l: strain 77; lanes m: strain 78; lanes n: strain 79; lanes o: strain 80. The last lane on the right corresponds to a mock PCR performed in same conditions but with no DNA.

results in terms of strain differentiation (Figs. 2, 3). The three JD strains (strains 72, 79, and 80) that were differentiated from the major group showed three AP-PCR profiles, each different from one another (lanes g, n and o for strains 72, 79 and 80, respectively in Figs. 2 and 3).

DISCUSSION

To test if the strains of *M. paratuberculosis* involved in the pathogenesis of CD belonged to a restricted group of specific clones, we explored the level of DNA heterogeneity among strains isolated from CD as compared to strains isolated from JD.

Previous studies performed on rather small numbers of *M. paratuberculosis* strains, using DNA–DNA hybridization, restriction endonuclease digestion and pulsed field gel electrophoresis, identified a high level of homogeneity at the genomic level within the subspecies, consistent with a clonal structure of the population [14, 16, 22–24]. However, use of the IS 900 insertion sequence as a probe in the analysis of RFLP or PFGE allowed the classification of 50 *M. paratuberculosis* strains isolated from bovine faeces,

https://doi.org/10.1017/S0950268897007486 Published online by Cambridge University Press

sheep and goats into two groups [14]. Most of these studies were carried out on animal strains. Only a few of them included one or two human strains which were found to fall into the bovine group.

Because of their ability to analyse simultaneously multiple loci, spread over the genome, randomly primed PCR procedures are highly discriminative for bacterial population analysis [17–19]. Also, the difficulties of growing some *M. paratuberculosis* strains were so restricting that growth was inadequate for RFLP studies. This argues for the use of PCR-based procedures to compare strains of this subspecies. Using such a procedure, we found similar profiles for the 4 strains isolated from CD and the major group of strains isolated from bovine JD. Among the JD strains we studied, only 3 were differentiated from the 11 strains.

Results obtained with the RFLP assay around and within the IS 900 sequences revealed only limited diversity and thus underline the strong genetic homology among all strains studied. It could be argued that such data could be related to the choice of the probe used in the study. Indeed, variation in the patterns of IS 900-containing restriction fragments is expected to reflect two types of polymorphisms. The first corresponds to variation in the number and precise localization of integration sites, which are site specific for IS 900. The second type of polymorphism corresponds to classical RFLPs in sequences flanking the insertion sites. As the variations observed were not consistently found with all enzymes, they are more likely to reflect RFLPs in flanking sequences. Since sequences flanking the insertion sites are by nature recombination sites, and thus susceptible *per se* to genetic variation, we could hypothesize that such variations might reflect events posterior or secondary to the integration of the IS 900 sequence, rather than clonal diversity. This might explain the high level of discrimination achieved by RFLP obtained with IS 900 as probe.

Results obtained in RFLP and AP-PCR studies outline the restricted genetic heterogeneity among strains isolated from CD and the major group of strains isolated from JD, and evoke the same clonal origin. Investigation of a large population of *M. paratuberculosis* has to be continued, including strains isolated from JD, in order to bring further precision to the population structure of strains isolated from CD. As significant arguments exist for the implication of some mycobacteria in the pathogenesis of some cases of CD, i.e. high level of antibodies against mycobacterial heat shock proteins (50% of CD for Hsp 65) [25], overexpression of cytokines (IL1, IL6, TNF) implicated in immunity to mycobacteria [26–28], of particular interest is the finding of restricted genetic heterogeneity of *M. paratuberculosis* strains isolated from CD, when compared to strains of this subspecies usually isolated in JD. Such a result could imply the involvement of specific virulence factors and/or specific host–pathogen interactions in the pathogenesis of CD.

ACKNOWLEDGEMENTS

We would like to thank Dr Marie-Françoise Thorel of the École Nationale Vétérinaire de Maison Alfort for the kind gift of two JD strains.

REFERENCES

1. Yoshimura HH, Graham DY. Nucleic acid hybridization studies of mycobactin-dependent mycobacteria. J Clin Microbiol 1988; **26**: 1309–12.
2. Thorel MF, Krichevsky M, Levy-Frébault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int Syst Bacteriol 1990; **40**: 254–60.
3. Merkal RS. Proposal of ATCC 19698 as the neotype strain of *Mycobacterium paratuberculosis* Bergey et al. 1923. Int J Syst Bacteriol 1979; **29**: 263–4.
4. Green EP, Tizard MLV, Moss MT, et al. Sequence and characteristics of IS 900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucl Acids Res 1989; **17**: 9063–73.
5. Chiodini RJ, Van Kruiningen HJ, Merkal RS, Thayer WR, Coutu JR, Coutu JA. Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease. J Clin Microbiol 1984; **20**: 966–71.
6. Graham YD, Markesich DC, Yoshimura HH. Mycobacteria and inflammatory bowel disease. Results of culture. Gastroenterology 1987; **92**: 436–42.
7. McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. J Clin Microbiol 1987; **25**: 796–801.
8. Yoshimura HH, Graham YD, Estes MK, Merkal RS. Investigation of association of mycobacteria with inflammatory bowel disease by nucleic acid hybridization. J Clin Microbiol 1987; **25**: 45–51.
9. Wall S, Kunze ZM, Saboor S, et al. Identification of spheroplast-like agents isolated from tissues of patients

with Crohn's disease and control tissues by polymerase chain reaction. J Clin Microbiol 1993; **31**: 1241–5.
10. Moss MT, Sanderson JD, Tizard MLV, et al. Polymerase chain reaction detection of *Mycobacterium paratuberculosis*, and *Mycobacterium avium* subsp. *silvaticum* in long term cultures from Crohn's disease and control tissues. Gut 1992; **33**: 1209–13.
11. Sanderson JD, Moss MT, Tizard MLV, Hermon-Taylor J. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Gut 1992; **33**; 890–6.
12. Dell'Isola B, Poyart C, Goulet O, et al. Detection of *Mycobacterium paratuberculosis* by polymerase chain reaction in children with Crohn's disease. J Infect Dis 1994; **169**: 449–51.
13. Fidler HM, Thurrell W, Johnson NMc, Rook GAW, McFadden JJ. Specific detection of *Mycobacterium paratuberculosis*, DNA associated with granulomatous tissue in Crohn's disease. Gut 1994; **35**: 506–10.
14. Collins DM, Gabric DM, de Lisle GW. Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. J Clin Microbiol 1990; **28**: 1591–6.
15. Cavé H, Bingen E, Elion J, Denamur E. Differentiation of *Escherichia coli* strains using randomly amplified polymorphic DNA analysis. Res Microbiol 1994; **145**: 141–50.
16. Whipple D, Kapke P, Vary C. Identification of restriction fragment length polymorphisms in DNA from *Mycobacterium paratuberculosis*. J Clin Microbiol 1990; **28**: 2561–4.
17. Bose M, Chander A, Das RH. A rapid and gentle method for the isolation of genomic DNA from mycobacteria. Nucl Acids Res 1993; **21**: 2529–30.
18. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucl Acids Res 1990; **18**: 7213–8.
19. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 1990; **18**: 6531–5.
20. Welsh J, Petersen C, McClelland. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucl Acids Res 1991; **189**: 303–6.
21. Kwok S, Kellogg DE, McKinney N, et al. Effect of primer–template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus type 1 model studies. Nucl Acids Res 1990; **18**: 999–1005.
22. Whipple DL, Lefebvre RB, Andrews RE, Thiermann JR, Thiermann AB. Isolation and analysis of restriction endonuclease digestive patterns of chromosomal DNA from *Mycobacterium paratuberculosis* and other mycobacterium species. J Clin Microbiol 1987; **25**: 1511–5.
23. McFadden JJ, Butcher PD, Thompson J, Chiodini R, Hermon-Taylor J. The use of DNA probes identifying restriction-fragment-length polymorphisms to examine the *Mycobacterium avium* complex. Mol Microbiol 1987; **1**: 283–91.
24. Levy-Frébault VV, Thorel MF, Varnerot A, Gicquel B. DNA polymorphism in *Mycobacterium paratuberculosis*, 'Wood pigeon mycobacteria', and related mycobacteria analyzed by field inversion gel electrophoresis. J Clin Microbiol 1989; **27**: 2823–6.
25. Elsaghier A, Prantera C, Bothamley G, Wilkins E, Jindal S, Ivanyi J. Disease association of antibodies to human and mycobacterial hsp 70 and hsp 60 stress proteins. Clin Exp Immunol 1992; **89**: 305–9.
26. Cappello M, Keshav S, Prince C, Jewell DP, Gordon S. Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridization. Gut 1992; **33**: 1214–9.
27. Retzlaff C, Yomamoto Y, Hoffman PS, Friedman H, Klein TW. Bacterial heat shock proteins directly induce cytokine mRNA and interleukine-1 secretion in macrophage cultures. Infect Imm 1994; **62**: 5689–93.
28. Gilberts ECA, Greenstein AJ, Katsel P, Harpaz N, Greenstein RJ. Molecular evidence for two forms of Crohn's disease. Proc Nat Acad Sci USA 1994; **91**: 12721–4.