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Deoxyribonucleic acid amplification and hybridisation in Crohn's disease using a chlamydial plasmid probe

B H McGarity, D A F Robertson, I N Clarke, R Wright

Abstract

The possibility that Crohn's disease is caused by infection with *Chlamydia trachomatis* was examined by probing for chlamydial plasmid deoxyribonucleic acid (DNA) in DNA extracts from Crohn's disease tissue and by means of a serological study. Gut DNA extracts were obtained from 10 patients with Crohn's disease and four control subjects and were probed with a chlamydial plasmid probe after Southern blotting. The polymerase chain reaction was also used to amplify any chlamydial plasmid DNA present in tissue DNA extracts, before Southern blotting and probing. Chlamydial proctitis control specimens were not available: gut DNA extracts mixed with traces of chlamydia plasmid served as positive controls. Using these techniques, no chlamydial plasmid DNA sequences were found in Crohn's disease tissue. An enzyme linked immunosorbent assay for *C trachomatis* LI was performed on 48 patients with Crohn's disease and 48 control subjects. Seropositivity was present in 14·6% of patients and 29% of control subjects and was not statistically significant (p>0·05). The failure to show chlamydial DNA and the lack of serological response to chlamydia make *C trachomatis* infection a very unlikely factor in the pathogenesis of Crohn's disease.

The cause of Crohn's disease is unknown. An infectious cause has long been suspected and remains an attractive hypothesis, although much research work has failed to definitely implicate any one organism. Chronic infection of the gastrointestinal tract with *Chlamydia trachomatis* has been suggested as a cause of Crohn's disease because *C trachomatis* exhibits tropism for the gut and can cause proctitis.[1-3] The proctitis caused by lymphogranuloma venereum strains of *C trachomatis* may be complicated by perianal disease and may be indistinguishable histologically from Crohn's disease: fistulaes and granulomas occur frequently in both conditions.[4] Infection with *C trachomatis* is often persistent and occult, and gastrointestinal carriage occurs in patients with ocular and genitourinary infection.[5] *C trachomatis*, then, has several features that make it a possible aetiological agent in Crohn's disease.

Attempts to isolate chlamydia from the stool and tissues of patients with Crohn's disease and immunocytochemical staining of tissue from these patients have been unsuccessful in showing chlamydial infection.[6] Serological studies by Schuller *et al* showed raised titres against the lymphogranuloma venereum serovars in patients with Crohn's disease,[7] but other workers have been unable to repeat this finding.[5 8-11] Latent or low levels of infection may, however, make it difficult to detect *C trachomatis* by these techniques, and a lasting serological response to infection does not always occur.[12] Recent advances in the molecular biology of *C trachomatis* make it possible to apply new, more sensitive techniques to chlamydial detection.

C trachomatis is an obligate intracellular organism dependent on host adenosine triphosphate (ATP) for survival. The spore like 'elementary body' is responsible for cell to cell transmission, while the intracellular 'reticulate body' is the metabolically active and reproductive form.[13] The genome consists of a single 1×10^6 base pair chromosome and up to 10 copies of a $7{\cdot}5\times10^3$ base pair plasmid.[14] The presence of multiple plasmid copies makes the plasmid an ideal target for a deoxyribonucleic acid (DNA) probe.[15] The function of the plasmid is unknown but its conservation across different serovars of *C trachomatis* suggests a function vital to survival.[16] *C trachomatis* plasmid probes from one serovar will readily hybridise to plasmids from other *C trachomatis* serovars because of DNA sequence homology.[17] The use of *C trachomatis* plasmid probes has been described in several studies of urogenital infection.[15 18 19] DNA hybridisation, using a plasmid probe to detect infection in clinical specimens, has been shown to have a sensitivity and specificity approaching that of culture.[19] As the DNA sequence of *C trachomatis* plasmids from serovars L1, L2, and B is known,[20-22] DNA amplification can be readily applied to amplify portions of the plasmid in clinical specimens.

DNA hybridisation techniques have previously been used to search for underlying infection in Crohn's disease using probes specific for mycobacteria,[23 24] adenovirus,[25] cytomegalovirus,[26] and *Pseudomonas maltophilia*.[27] No DNA from these organisms was found in tissue from patients with Crohn's disease. We have used DNA hybridisation[28] to search for the plasmid of *C trachomatis* in tissue from Crohn's disease patients. We have used the polymerase chain reaction[29] to amplify a portion of the plasmid to improve the detection threshold. In addition, we have carried out a serological survey on a large group of patients with Crohn's disease.

Department of Medicine II and Department of Microbiology, University of Southampton
B H McGarity
D A F Robertson
I N Clarke
R Wright

Correspondence to:
Dr D A F Robertson, Department of Medicine, Royal United Hospital, Combe Park, Bath BA1 3NG.

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Methods

PATIENTS

Tissue samples from patients with Crohn's

Crohn's disease (CD) and control patient characteristics

Patient	Sex	Age (yrs)	Tissue examined	Disease	Activity
1	M	27	Terminal ileum*	CD	+
2	F	27	Ileum*	CD	+
3	F	38	Terminal ileum*	CD	+
4	M	76	Appendix*‡	CD	−
5	M	22	Rectum†	CD	+
6	F	69	Colon†	CD	+
7	M	21	Rectum†	CD	+
8	F	77	Colon†	CD	+
9	M	22	Colon†	UC	+
10	M	68	Colon†	CD	+
11	F	23	Colon*	CD	+
12	F	25	Colon†	UC	+
13	F	33	Rectum†	UC	+
14	M	57	Colon*	Cancer§	N/A

*Resection tissue; †mucosal biopsy; ‡normal appendix removed at laparotomy; §tissue obtained 6cm away from tumour.
UC=ulcerative colitis.

disease undergoing surgical resection or mucosal biopsy at sigmoidoscopy or colonoscopy were obtained between June and December 1988 at the Southampton General Hospital. Patients with Crohn's disease were diagnosed on the basis of radiological, endoscopic, and histological studies.[30] There were 10 patients with Crohn's disease and four controls – three with ulcerative colitis and one with colonic cancer. The characteristics of individual patients and controls, with the type of tissue obtained are listed in the Table. Full thickness pieces of resection tissue and mucosal biopsy samples were taken from areas of macroscopic involvement, snap frozen, and stored in liquid nitrogen.

Sera were collected from 48 consecutive patients with Crohn's disease attending a gastroenterology clinic at this hospital between June and August 1988. The mean age of patients was 47·3 years. There were 20 men and 28 women. Control patients consisted of 48 patients with non-gastroenterological disease including 15 with osteoarthritis, 11 with Paget's disease, 10 with a variety of neurological diseases, and 12 healthy subjects. The mean age of control subjects was 57·2 years and there were 19 men and 29 women.

EXTRACTION OF TISSUE DNA

Resection tissue (0·5–1·0 g) was ground with pestle under liquid nitrogen. The smaller mucosal biopsy samples did not require grinding or homogenisation. Samples were deproteinised by digestion with proteinase K 1 mg/ml in 10 mmol/l Tris-Cl, 1 mmol/l disodium ethylene diamine tetra acetate (EDTA), and 1% sodium dodecyl sulphate (SDS) at 50°C for three hours. After phenol/chloroform extraction, samples were concentrated using n-butanol before cleaning with water saturated ether. The concentration of high molecular weight DNA was estimated by staining with ethidium bromide after agarose gel electrophoresis.[28]

DNA PROBE

The probe used was the complete 7·5 kilobase pair chlamydial plasmid designated pLGV440 from *C trachomatis* serovar L1/440/LN. This had previously been cloned in *Escherichia coli* JM83 as a recombinant plasmid designated pCTL12a. Plasmid pLGV440 was released from pCTL12a by digestion with restriction endonuclease *Pst 1*, separated from the vector plasmid by gel electrophoresis, and extracted from the gel by melting and glass milk absorption of DNA (Geneclean, Bio 101 Inc, La Jolla, CA). The probe was labelled with (α-^{32}P) deoxycytosine triphosphate by nick translation (Amersham plc, Amersham, UK) to obtain a specific activity of 5×10^7 dpm/μg DNA. Probes were denatured by heating to 100°C for five minutes and rapid cooling on ice.

HYBRIDISATION ANALYSIS

High molecular weight tissue DNA extracts were digested with *Pst 1* and 5-10 μg of DNA electrophoresed in 0·7% agarose gel using TAE buffer (40 mmol/l Tris-Cl, 2 mmol/l EDTA and glacial acetic acid to pH 8·0). Gels were stained with ethidium bromide 1 μg/ml and photographed on an ultraviolet transilluminator. DNA was denatured by soaking the gel twice in 0·5 mol/l NaOH, 1·5 mol/l NaCl for 30 minutes and neutralisation in 1·5 mol/l NaCl, 0·5 mol/l Tris-Cl (pH 8·0) for one hour. DNA was transferred to a nylon membrane (Hybond-N, Amersham plc) by Southern blotting using 20×SSC as transfer buffer (3 mol/l NaCl with 0·3 mol/l trisodium citrate, pH 7). After overnight transfer membranes were washed in 6×SSC for five minutes and oven baked at 80°C for two hours.

Prehybridisation was performed in 6×SSC, 0·5% SDS, 5×Denhardt's solution and 100 μg/ml denatured salmon sperm DNA at 65°C for three to six hours. Probe was added and hybridisation carried out overnight at 65°C. After washing, membranes were exposed to Kodak XAR film at −70°C for periods up to two weeks before developing.

DNA AMPLIFICATION

Using the known plasmid sequence of *C trachomatis* L1,[20] two 25 base deoxy-oligonucleotides (see Fig 1) were synthesised corresponding to bases 5706–5730 and complementary to bases 6176–6200 in an Applied Biosystems model 381A DNA synthesiser (Applied Biosystems Inc, Foster City, CA). These oligonucleotides were designed to serve as primers in the polymerase chain reaction to amplify the plasmid from bases 5706 to 6200. Within this sequence at sites 5737 and 6153 were *Eco RI* restriction endonuclease sites, used to verify that the amplified sequence originated in the chlamydial plasmid. Using the known plasmid sequence of *C trachomatis* L2,[21] amplification of nucleotides 5309 to 5779 should occur; using the known sequence of *C trachomatis* serovar B,[22] amplification of nucleotides 5701 to 6196 should occur using the primers described.

Amplification of DNA was performed using the polymerase chain reaction 'Gene Amp' DNA amplification kit (Perkin-Elmer-Cetus Corp, Norwalk, CT). Briefly 5–10 μg of tissue DNA together with 1·8 μmol/l of each primer in 100 μl of *Taq polymerase* buffer and deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate and thymidine

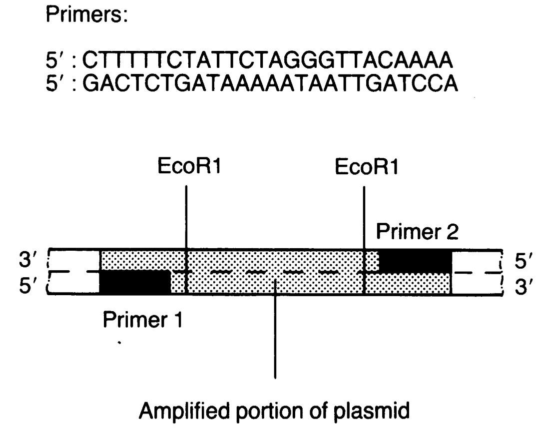

Figure 1: DNA amplification primers: primer 1 corresponding to bases 5706–5730, and primer 2 complementary to bases 6176–6200 of the Chlamydia trachomatis *L1 plasmid sequence (reference 20), with a schematic illustration of the amplified portion of the plasmid.*

triphosphate (0·2 mmol/l each). Samples were heated to 94°C for five minutes to denature DNA and destroy proteases before addition of 5 U of *Taq polymerase*. Samples were then heated to 37°C for two minutes to allow primer annealing, and to 72°C for three minutes to allow extension. At the start of following cycles two minutes was used to denature DNA, and the cycle repeated 25 times with the final extension segment increased to seven minutes. Aliquots of amplified DNA were than cut with *Eco RI* restriction endonuclease, electrophoresed, southern blotted, and hybridisation was performed as above.

To confirm that amplification of plasmid DNA would occur across different serovars of *C trachomatis*, DNA extracts from serovars A, B, C, D, E, G, H, LGV3, were amplified, as above. DNA from *Chlamydia psittaci* strain EAE (which has no plasmid) and *Neisseria meningitidis* served as controls.

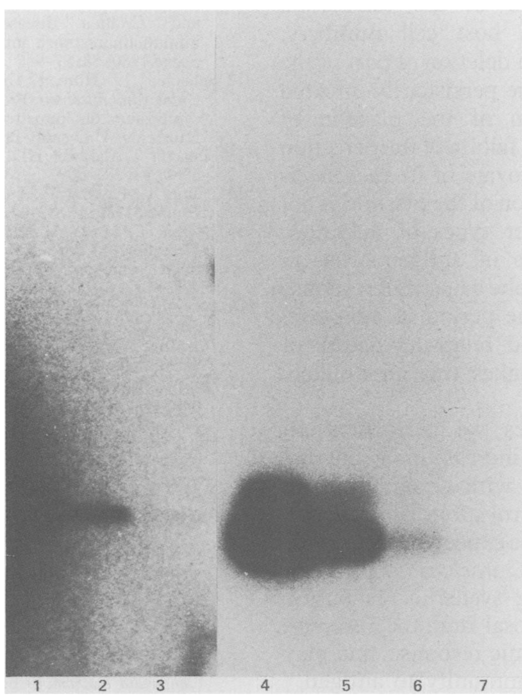

Figure 2: Sensitivity of hybridisation assay using probe PLGV440. Lanes 1, 2, 3: Southern blot of 5–10 μg of human gut DNA with 50 pg, 5 pg, and 0·5 pg of added chlamydial plasmid DNA. Lanes 4, 5, 6, 7: Southern blot of 5–10 μg of human gut DNA (patient 1) with 5 pg, 0·5 pg, 50 fg, and 5 fg of added chlamydial plasmid DNA, amplified with chlamydia *specific primers.*

SEROLOGY

Sera obtained from patients was stored at −20°C till chlamydial antibodies were determined using a conventional enzyme linked immunosorbent (ELISA) technique for immunoglobulin G, using whole *C trachomatis* L1 as antigen because of its reactivity to a wide range of chlamydial antibodies.[31] The properties of these antigens have been described elsewhere.[32] Duplicate 1/100 dilutions of sera were used. Statistical analysis was by use of χ^2 tables.

Results

SENSITIVITY OF THE HYBRIDISATION ASSAY

Under the conditions described above we could routinely detect 0·5 pg of chlamydial plasmid pLGV440 DNA against a background of 5–10 μg of human gut DNA extract (see Fig 2). This is equivalent to 6×10^4 plasmids. If there are 10 plasmid copies per *chlamydia*, and assuming more than 10 *chlamydiae* per infected cell, then each infected cell contains at least 100 plasmid copies. A sensitivity of 6×10^4 plasmids is equivalent to that of 600 infected cells. If the molecular weight ratio of the human genome is 3×10^{12}, and 5 μg of human DNA was analysed, the detection sensitivity is 1 infected cell per 10^4 cells. There was no cross hybridisation of the probe with eukaryotic DNA.

After DNA amplification in a background of 5–10 μg of human gut DNA extract, 5 fg of plasmid DNA was detectable or 600 plasmids (see Fig 2). This is the plasmid DNA contained in 6 infected cells or 1 infected cell per 10^6 cells.

Amplification of plasmid DNA occurred for all *C trachomatis* serovars tested (Fig 2). Amplification of serovars A, B, C, D, E, G, H and L2 was achieved.

ANALYSIS OF TISSUE DNA FROM PATIENTS WITH CROHN'S DISEASE

DNA was obtained from surgical resection tissue or mucosal biopsy samples from 10 patients with CD, three patients with ulcerative colitis, and one patient with colonic cancer. After Southern blotting and hybridisation of tissue DNA extracts with the chlamydial plasmid probe pLGV440 no DNA hybridisation was evident.

After amplification of tissue DNA for a portion of the chlamydial plasmid using the polymerase chain reaction, we were unable to detect the presence of any amplified sequence by hybridisation.

CHLAMYDIAL SEROLOGY

Using an ELISA technique we found antibodies to chlamydia in 14·6% (7 of 8) patients with Crohn's disease and 29% (14 of 48) control patients. There was no significant difference between the groups ($\chi^2=2·986$, $p>0·05$).

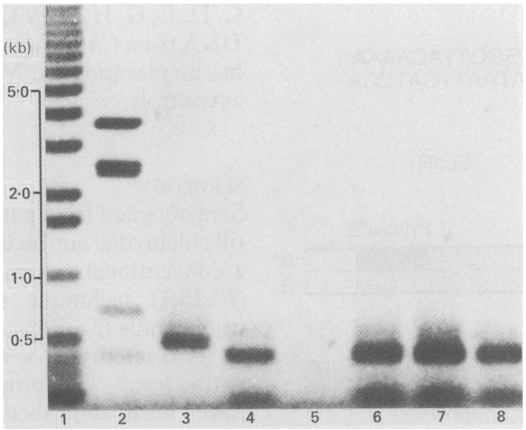

Figure 3: DNA amplification of different Chlamydia trachomatis *serovars: ethidium bromide staining after gel electrophoresis: Lane 1: DNA size ladder; 2:* Eco R1 *digest of pCTL12a; 3: amplified pCTL12a without* Eco R1 *digestion; 4–8: amplification and* Eco R1 *digestion of 100 pg DNA from 4:* C trachomatis *serovar C; 5:* Chlamydia psittaci *EAE; 6:* C trachomatis *serovar E; 7: C; 5:* Chlamydia psittaci *EAE; 6:* C trachomatis *serovar E; 7:* C trachomatis *B TW5; 8:* C trachomatis *serovar H. Note:* C psittaci *EAE does not amplify as it has no plasmid.*

Discussion

We were unable to show chlamydial plasmid DNA in extracts of DNA from diseased gut tissue from patients with Crohn's disease. In view of the sensitivity of the technique, the most likely explanation for our findings is that *C trachomatis* is not present in the gut of patients with Crohn's disease. There are, however, several other possible explanations for a failure to detect the organism. Infection may be present at such a low level that it falls below the sensitivity of the test: but even if some cell lines or gut associated lymphoid tissue only were infected this is unlikely in view of the calculation of sensitivity based on host cell numbers. Incomplete infection with deletion of part of the genome can occur in some persistently infected cell lines,[33] and deletion of the plasmid of *C trachomatis* would cause failure of the detection system. However, all serovars of *C trachomatis* carry a plasmid and deletion of the plasmid is not known to occur in other types of infection. Alternatively, persistence of antigen after an initial infection may provoke a host cell response that continues beyond the period of infection. The length of illness and relapsing nature of Crohn's disease again makes this an unlikely mechanism.

The serological studies we performed on patients failed to show an increase in chlamydial seropositivity in patients with Crohn's disease when compared with control subjects. Although high antibody titres may be expected in a chronic infection, infection with *C trachomatis* does not always provoke a lasting systemic response.[12] Furthermore, a gut mucosal immune response may exist without a systemic response, and may even suppress systemic immunity to antigen.[34] We did not assess coproantibody values in this study. Previous studies agree with our findings, with the exception of the study by Schuller *et al*, who found high levels of seropositivity to lymphogranuloma-venereum serovars in patients with Crohn's disease compared with controls. The lack of cross reactivity with non-lymphogranuloma-venereum serovars, the rarity of this infection in Europe and the inability of other workers to confirm this finding suggests that Schuller's findings may not be valid.

Our findings suggest that *C trachomatis* is not the cause of Crohn's disease. The use of new molecular biology techniques such as DNA amplification opens exciting new prospects in the search for an obscure microbiological cause for Crohn's disease.

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