

Detection Of Escherichia Coli DNA From Interstitial Cystitis Bladder Biopsies Provides Little Evidence Of A Causal Microbe

R Dixon, M Agarwal

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Abstract

The aetiology of interstitial cystitis in patients remains unknown and despite many investigations no single micro-organism has been implicated. We have studied the possible role of bacteria in interstitial cystitis by investigating PCR amplified DNA sequences from 33 individually well-documented interstitial cystitis patients. The frozen or archived bladder biopsy specimens have been examined for the presence of bacterial DNA by the amplification of 16S rRNA and subsequent RFLP of cloned DNA fragments. Bacterial DNA was detected from 21/33 (64%) of frozen or archived bladder biopsy samples. RFLP analysis of cloned DNA strongly resembled the fragment profile of Escherichia coli DNA in 10/33 (30%) of the IC biopsy samples overall or 10/21 (48%) of the samples that had yielded any DNA. Bacterial DNA was however identified in 4/5 (80%) of samples from non-interstitial cystitis bladder biopsy patients. The significance of the presence of E.coli DNA in 48% of interstitial cystitis bladder biopsies is clearly tempered by the finding of a high proportion of E.coli DNA in non-interstitial cystitis patients. The issues of appropriate control groups and adequate methods to ensure authentic DNA are discussed.

INTRODUCTION

The aetiology of interstitial cystitis (IC) in patients continues to elude us and despite intensive research, the possible causative agent of the disease and its ideal treatment remains unknown. Acute onset with exacerbations and remissions, a previous history of urinary tract infections, virtual confinement of the disease to female patients in which the short urethra allows easy access to bacteria, all strongly suggest an infective aetiology [1]. However, over the past three decades, numerous studies using traditional isolation techniques have failed to convincingly identify any bacterial, viral or protozoal agent(s) responsible for the disease. The requirement to recover organisms from patients by growing them on artificial media or identify extremely fastidious or 'non-culturable' organisms has limited progress in the investigation of IC. Advances in molecular-based diagnosis, however, have allowed a new strategic approach by which workers use nucleic acid-based methodology to exclude the presence of specific exogenous microbes as the aetiological cause. PCR amplification of bacterial DNA for the potential identification of specific causal pathogens in frozen IC biopsy specimens has been investigated previously. Using this approach, Hampson et al [2] excluded the specific presence of mycobacterial DNA in IC biopsy specimens and

Hukkanen et al [3] excluded the presence in IC specimens of the adenovirus or BK virus genome. Agarwal and Dixon [4,5] specifically excluded the bacterial pathogens Gardnerella vaginalis and Helicobacter pylori respectively and various other pathogens have also been excluded [6]. Escherichia coli remains a possible candidate in the pathogenesis of IC especially as data from Anderson et al [7] suggests that E coli in mice is internalized into bladder epithelial cells and persists for months. In some patients, onset of IC can correlate with evidence of UTI or urinary inflammation originating from episodes of E.coli infection. (J.W. Warren - personal communication).

Investigations into the aetiology of IC using patient groups has remained limited, often due to insufficient frozen biopsy specimens and to date, studies have been small and conclusions difficult to make. In the present study, we are attempting to detect DNA from any bacteria present not only from frozen biopsies but a larger number of material preserved in paraffin wax.

MATERIALS AND METHODS

Thirty-three bladder biopsy specimens (including 29 paraffin-embedded and 4 fresh frozen specimens) were collected from patients with a clinical diagnosis of IC

fulfilling (National Institute of Diabetes and Digestive and Kidney diseases - NIDDK) criteria [8] with some modifications (criteria based on cystometrograms were not strictly adhered to, as urodynamic studies were not performed in all the patients [9]). Five 'normal' bladder biopsy specimens from patients with previous bladder tumours, three biopsy specimens from patients with chronic cystitis and one from acute cystitis were included in the study as controls. One 'normal' bladder biopsy specimen was spiked with *E.coli*, and the other with *Gardnerella vaginalis*, and included in the study.

Five μm thick tissue sections were cut from the paraffin blocks. The first 5 sections from the blocks were discarded to ensure that the outer surface remained free of exterior contamination. A total of 5 thin sections were collected in an Eppendorf tube without de-waxing. The cutting knife was changed frequently and all surfaces coming into the contact with the specimen wiped clean with alcohol between each specimen and between first 5 discarded sections and the 5 sections used for DNA extraction. Pre-treatment of all specimens with lysozyme and lysostaphin preceded DNA extraction which was carried out by the methods described previously [5]. DNA was isolated from all specimens with the Easy-DNA™ genomic DNA isolation kit, version 1.0 (Invitrogen), using a protocol designed for the isolation of genomic DNA from cells, tissues or bacterial cells.

All specimens were amplified for the presence of a 97 bp sequence of the mitochondrial d loop region specific only to humans [10] with human placental DNA as a control [10]. Amplification reactions were performed in Eppendorf tubes kept on ice. 5 μl of 10 \times PCR buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, 0.1% [wt/vol.] gelatin [pH 8.3]), 8 μl of deoxynucleoside triphosphate mixture - 0.02 μmoles each of dATP [10 mM], dCTP [10 mM], dGTP [10 mM], and dTTP [10 mM], 50 pmol each of M1 and M2 primers (M1 - 5' CGC CCT TAC ACA AAA TGA CAT CAA 3' - i.d. 13232, M2 - 5' GTG TGG TTG GTT GAT GCC GA 3' - i.d. 13286, [10] distilled water and 2 μl of template DNA were added to the reaction mixture. These components were added together to give a final reaction volume of 50 μl and tubes were sealed with an overlay of 40 μl of autoclaved mineral oil. Taq DNA Polymerase 2.5 U of a 5000 units /ml stock solution (Pharmacia Biotech) was added just before starting PCR cycling as described previously [10].

2.1 Amplification of 16S rDNA. Extracted DNA from all specimens was used to amplify a 1156 base-pair product

specific for amplification of the 16S rRNA region. 50 pmol / μl of primer U1 - CGG TTA CCT TGT TAC GAC TT - bp 1491-1510, and U4 - CAG TGG GGA ATA TTG CAG AA - bp 354-373 [11] were added to the above reaction mixture. PCR reactions were performed in a thermocycler after denaturation at 95 C for 5 min and comprised 30 cycles (95 C for 1 min, 60 C for 1min and 72 C for 1min), further extension was completed with 72 C for 5min and products stored at 4 C. Amplified products from all PCR reactions were electrophoresed in agarose gels as described previously [4]. Restriction fragment length polymorphism (RFLP) analysis of the amplified DNA products using Hae III and other restriction enzymes used standard methodology [12]

2.2 Cloning Five positive bladder biopsy specimens from IC patients with reproducible and similar patterns on restriction enzyme analysis (REA) were selected for cloning (specimen nos. 4, 65, 69, 70, 76) by the direct cloning method for PCR products. Bands of agarose containing 16S rDNA amplicons were cut out of the gel, DNA extracted and ligated to plasmid pCR^{2.1} vector (Invitrogen - K2000-J10 and K2030-J10), linearized (2 μl of 25 ng/ μl in 10mM Tris-HCl, 1mM EDTA, pH 7.5) 1 μl of 10 \times ligation buffer and 1 μl of T₄ DNA ligase was used. The reaction mixture was incubated at 14 C overnight and then stored at -20 C. The One Shot™ competent cell kit containing SOC medium, β -mercaptoethanol (0.5 M), INV Φ ' cells and pUC18, supercoiled (10 ng/ μl in 5mM Tris-HCl, 0.5 mM EDTA) was used according to the manufacturers' instructions for transformation. Five white transformant colonies were obtained and grown in LB broth containing 50 $\mu\text{g/ml}$ ampicillin. Plasmids were isolated from the colonies grown in broth by miniprep method (Qiagen) according to the manufacturers' instructions. Pure plasmid DNA was obtained and analysed by the RFLP with HaeIII method as above.

RESULTS

All 33 specimens from individually documented IC patients produced the anticipated amplification products of 97 bp from the mammalian mitochondrial region (data not shown).

PCR-amplified sequences of bacterial 16S rRNA products resulted in 21 out of 33 (64 %) biopsy samples from IC patients producing the expected sized amplification band (1156bp). The bands obtained from each of the 21 biopsies were subjected to restriction fragment length polymorphism (RFLP) analysis and compared with RFLP obtained from reference *E.coli* and other bacterial species. Analysis of the

RFLP fragment profiles showed 10 of the 21 biopsy samples (48%) to be identical to the reference strain of E.coli when digested with several enzymes. A typical RFLP profile for some of the isolates and recombinants is shown in Fig 1. Other isolates (not shown) were typical of Pseudomonas aeruginosa and other members of the Enterobacteriaceae.

Bacterial DNA was detected in 21/33 (64%) of bladder biopsy samples from well-documented IC patients and 10 of these strongly suggested E.coli DNA. However, 4/5 (80%) of 'normal' or non-IC bladder biopsies also showed the presence of bacterial DNA.

Figure 1

Figure 1

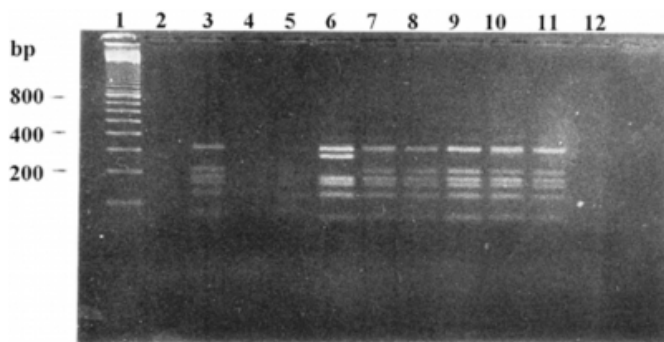


Figure Legend

Typical gel of restriction analysis of PCR products cut with Hae III:

- lane 1 100bp ladder;
- lane 2 negative control (no DNA added);
- lane 3 sample 20 - IC patient DNA
- lane 4 sample 24 – non-IC control
- lane 5 sample 58 - non-IC control
- lane 6 sample 34 - non-IC control UTI
- lane 7 sample 69 - IC patient (cloned DNA)
- lane 8 sample 76 - IC patient (cloned DNA)
- lane 9 sample 11 - non-IC patient spiked with E.coli
- lane 10 sample 63 - IC patient DNA
- lane 11 sample 4 - IC patient (cloned DNA)
- lane 12 sample 22 - non-IC patient specimen

DISCUSSION

Over the past 25 years the perception of IC has changed from a psychosomatic disorder to one as a distinct disease entity. Significant advances have been made by the application of molecular biology to the detection of microbes involved in the pathogenesis of IC and whilst pre-molecular approaches focused on culture, serologic and microscopic methods for detection of microbes, the molecular approach allows the direct detection of both prokaryotic and eukaryotic DNA. Amplification of human-specific sequences (97bp product) of mitochondrial DNA from clinical biopsies in the present study, served to provide an estimate of the general state of DNA preservation in the specimens, test for absence of PCR inhibitors as well as control for PCR-negative amplifications of bacterial 16S rDNA.[10].

In the present study, bacterial DNA could be detected by amplification in 21/33 (64%) of bladder biopsy samples from IC patients and cloned DNA from at least 5 of these confirmed that a profile identical to Escherichia coli was obtained. However, in the present study, 4/5 (80%) of 'normal' or non-IC bladder biopsies were also DNA positive. The significance of E. coli DNA in a high proportion of 'normal' bladder biopsies is problematic since it raises the difficulty of finding a suitable negative control group. The so-called 'normal' bladder samples are from patients with underlying bladder oncology – the control samples were biopsy samples from patients with infected bladders and chronic cystitis. It proved ethically unacceptable to obtain normal bladder biopsy samples from regular healthy individuals hence our 'normal' biopsy samples were from patients with a history of bladder tumour, biopsies being generally performed to exclude tumour recurrence.. The very reason that biopsies were performed indicates that these samples were not from epithelium with a normal-looking appearance. Repeated cystoscopies and previous tumour resections might suggest that these patients would probably have had prior clinical or sub-clinical urinary infections and therefore not surprisingly, a large number of these 'normal' control specimens, in the present study, were found to harbour bacterial DNA.

Other investigators have used amplified 16S rDNA analysis as a tool for the potential identification of bacterial pathogens in IC biopsy specimens. Our findings are consistent with the molecular study of Keay et al [13] who investigated 6 IC patients and 6 controls by amplification of

16S rRNA gene from bladder biopsies. All of the specimens and controls were positive and sequence data suggested several different genera of bacteria. Only 2/6 IC samples and 4/6 control samples showed similarity to *E. coli* DNA. Viable bacteria were not cultured from biopsy specimens or urine obtained at cystoscopy with these patients. Keay and co-workers argued that all or most of the women participants with or without IC, probably had non-culturable or non-viable bacteria on or in the bladder wall and hypothesised that contamination at the time of collection of the specimens was the most likely source [13]. Additionally, the choice of a DNA extraction method (proteinase K) used in their study is known to fail to extract DNA from some Gram-positive bacteria and therefore their identification might have been missed for technical reasons. Haarala et al [14] studied bladder biopsies and sterile urine obtained by suprapubic puncture from 11 IC patients, 4 patients with other urological problems and 5 healthy individuals were included as controls. DNA was extracted by the proteinase K method and then subjected to PCR using the 16S rRNA gene amplification target for the detection of bacterial DNA. All the IC samples were negative for the presence of bacterial DNA and out of 9 non-IC patient controls, only 1 was positive for bacterial DNA. Domingue et al [15] demonstrated 16S rRNA amplicons in bladder biopsies from 29% of patients with IC. In their study, 14 IC patients and 15 control patients with different urological problems were included. Their results show that 4 of 14 IC patients (approximately 29%) yielded bacterial DNA, whilst no bacterial DNA was found in control samples. Further analysis of the amplified product in positive samples showed that they were mainly Gram-negative bacteria, 2 were similar to *E. coli* and 2 were similar to *Pseudomonas* spp. Heritz et al [16] also using amplification of 16S rRNA bacterial DNA in bladder biopsy samples reported positive results in 16/30 (53%) IC patients and 9/15 (60%) of control patients. Urine samples from 12/26 (46%) IC patients and 5/12 (42%) control patients also yielded bacterial DNA. Three positive IC bladder biopsy specimens and three positive specimens from the control group were cloned and analysed but only one IC sample clone showed homology to *E. coli*.

The interpretation of results on IC biopsy samples from frozen and archival samples in our present study is difficult for two major reasons: Firstly in the light of results from 'normal' controls. We failed to find alternative controls to address this problem and to validate the presence of bacterial

DNA in the IC group of patients. Secondly, the other major issue was the authenticity of DNA from archival tissue. Most biopsies are probably unavoidably contaminated by bacteria and DNA at the point of collection and although it is possible to ensure the sterility of forceps we were unable to ensure that amplifiable DNA was avoided [17].

CONCLUSIONS

The possible role of bacteria in the pathogenesis of IC has been investigated by DNA -based techniques in the present study. Bladder biopsies from both IC and control patients appeared to reveal putative *E. coli* DNA. The significance of the presence of bacterial DNA recovered from a high proportion of 'normal' bladder biopsies is equivocal since these so called 'normal' samples are from patients with underlying bladder oncology. The use of paraffin wax sections of biopsy specimens has extended the scope of these studies allowing more archived samples to be investigated but further advances in this field may depend on other molecular markers of bacterial infection such as the presence of inflammatory markers - biomarkers of disease less prone to misinterpretation than contamination.

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Author Information

Ronald A. Dixon

Department of Forensic and Biomedical Sciences, University of Lincoln

Meena Agarwal

Department of Urology, University Hospital of Wales