

Adenovirus 12 E1A gene detection by polymerase chain reaction in both the normal and coeliac duodenum

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Abstract

A 12 amino acid sequence from the adenovirus 12 E1B protein is homologous at the protein level with a similar 12-mer derived from the wheat protein A-gliadin. It has been suggested that exposure to Ad 12 could sensitise individuals to gliadins with resultant gluten sensitive enteropathy. In this study, the polymerase chain reaction (PCR) was used to analyse duodenal biopsy tissue from patients with coeliac disease for the presence of Ad 12. The sensitivity of the assay system was at least 1 in 10⁵ cells and specificity was confirmed both by probing with an internal oligonucleotide and by direct sequencing. Ad 12 sequences were detected in three of 17 patients with adult coeliac disease and in five of 16 adult controls with normal duodenal biopsies. Since exposure to the virus would be predicted to occur in infancy we also studied patients with childhood coeliac disease diagnosed at less than 1 year of age. Ad 12 was positive in three of 10 childhood coeliac patients and one of seven controls. In addition, we studied a cohort of patients who presented with a diarrhoeal illness and associated anti α gliadin antibodies in 1983. These patients had duodenal biopsies performed at this time. One of three patients with abnormal histology had detectable Ad 12 while two of 14 with normal findings were positive for Ad 12. Finally, the potential oncogenic nature of Ad 12 prompted examination of a group of patients with intestinal tumours. Ad 12 DNA was, however, in only two of 19 tumour samples tested. These data indicate that Ad 12 can be successfully detected using PCR on paraffin embedded tissue. Furthermore, Ad 12 was detected at a relatively high level in normal duodenum. The results do not, however, support the hypothesis that prior exposure to Ad 12 is implicated in the pathogenesis of coeliac disease.

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Coeliac disease is a gluten sensitive enteropathy characterised by intestinal villous atrophy in response to dietary gluten. The disease is HLA linked and is tightly associated with HLA antigens including HLA A1, B8, DR3, and DQ2.^{1 2} In addition, an association with HLA DP 3·1 and 4·2 haplotypes has been

described.³ Since these molecules are involved directly in the processing and presentation of antigen to T cells, it has been proposed that coeliac disease is a T cell mediated disease with antigenic recognition of linear segments on the gluten peptides.^{1 4}

The alcohol soluble fractions of gluten are called gliadins and include α , β , γ , ω gliadins on the basis of electrophoretic shift. All of these fractions are known to precipitate the activity of coeliac disease but it has been suggested that α gliadin is more potent both in inducing disease and in inducing an antibody response.⁵ A 12 amino acid sequence on the A-gliadin molecule (one of the α gliadins) was found to be highly homologous with a corresponding 12 amino acid sequence on the E1B sequence of the adenovirus 12 (Ad 12) molecule.⁴ Thus, it was postulated that 'molecular mimicry' might be involved in the pathogenesis of coeliac disease, with loss of oral tolerance resulting from infection during childhood with the adenovirus.⁴ Hence, dietary gliadin would subsequently be recognised as a foreign antigen and result in local damaging immune response. Antibodies to Ad 12 were found in a subgroup of patients with active coeliac disease in one study.⁶ Antibodies to the E1B protein were not, however, detected in other patient populations.⁷ In a previous study, adenovirus DNA from the E1B region was detected by the polymerase chain reaction (PCR) in a

TABLE 1 Results of polymerase chain reaction (PCR) amplification of adenovirus 12 (Ad 12) DNA from a cohort of children aged 1-4 years presenting with a diarrhoeal illness and elevated antibodies to α gliadin in 1983. Anti- α gliadin antibody levels are expressed as ELISA index (EI). A normal level of <1·5 was established from the mean +2 SD of indices of a cohort of 25 normal age matched children

Patient no	Biopsy findings	Anti- α gliadin (EI)	PCR for Ad 12
1	Flat mucosa	8·7	No
2	Normal	2·8	No
3	Normal	2·6	Yes
4	Flat mucosa	12·3	Yes
5	Normal	3·2	No
6	Normal	6·9	No
7	Normal	2·5	No
8	Normal	9·9	Yes
9	Normal	3·2	No
10	Normal	2·4	No
11	Increased IEL	2·7	No
12	Normal	1·8	No
13	Normal	3·2	No
14	Normal	1·8	No
15	Normal	1·9	No
16	Normal	2·0	No
17	Normal	2·0	No

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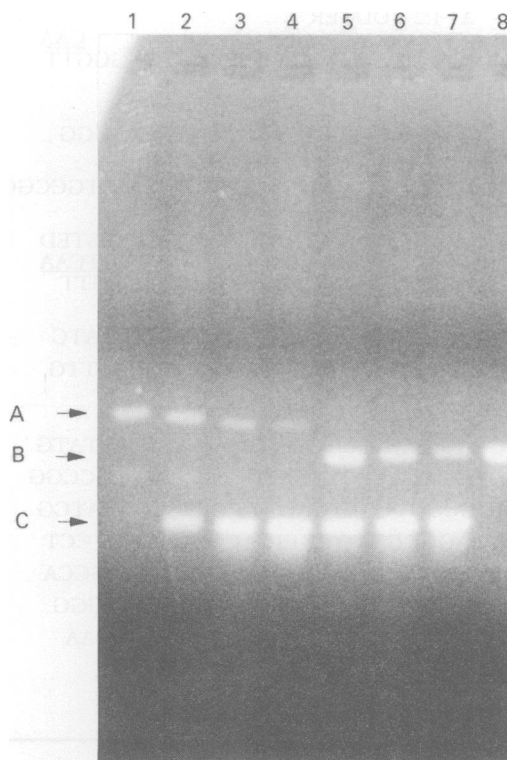


Figure 2: Sensitivity of the assay system.

Adenovirus 12 (Ad 12) positive cells were diluted in Ad 12 negative human neutrophils and prepared for PCR as described in the Methods section. They were amplified by first round PCR (A, lanes 1–4) or nested PCR (B, lanes 5–8). Amplification of primers from the rhodopsin gene (C) served as a positive control (lanes 2–7).

Lane 1: undiluted sample; lane 2: 10^{-1} dilution; lane 3: 10^{-2} dilution; lane 4: 10^{-3} dilution; lane 5: 10^{-1} dilution (nested PCR); lane 6: 10^{-3} dilution (nested PCR); lane 7: 10^{-5} dilution (nested PCR); lane 8 undiluted sample (nested PCR).

Model 391 Oligonucleotide Synthesizer PCR MATE (Applied Biosystems, Foster City, CA, USA). *Thermus aquaticus* DNA polymerase (Taq polymerase) was obtained from Perkin Elmer Cetus (Emeryville, CA, USA). *Alu 1*, *Pvu 2* and *Sau 3a* restriction enzymes and deoxynucleoside triphosphates (dNTPs) were obtained from Bethesda Research Laboratories (Bethesda, MD, USA).

PCR ON PARAFFIN FIXED SPECIMENS

Design of primers

The entire sequence could be amplified using primers A1.1 and A2.2 defining outer sequences of the region (Fig 1). This sequence was too large for amplification from paraffin fixed tissue, however, and hence primers A1.1 and A1.2 were used to give a fragment size of 286 base pairs (bp). Use of the nested primers (A1.1 nested and A1.2 nested) then gives an internal fragment of 149 base pairs (bp). Primers from the rhodopsin and anti-thrombin III loci were used to amplify these human sequences in the same tube as the Ad 12 amplification. This served as a positive control to ensure that reaction conditions would promote amplification in all cases.

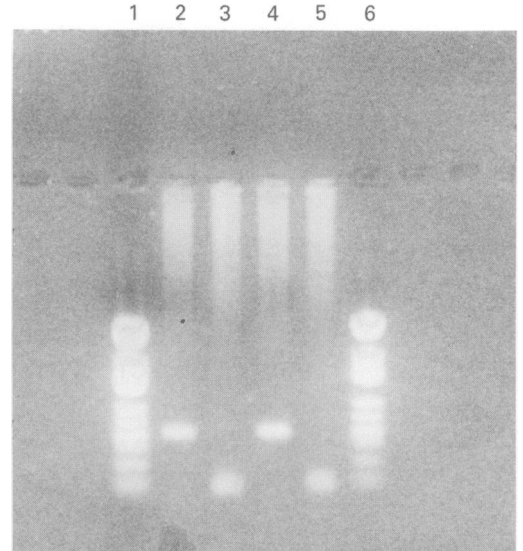


Figure 3: Restriction enzyme digestion of amplified DNA. Lane 1 marker DNA; lane 2 uncut DNA; lane 3 *Alu 1* cut DNA; lane 4 uncut DNA; lane 5 *Sau 3a* cut DNA; lane 6 marker DNA.

Each positive sample was tested with *Alu 1*, *Sau 3a* and *Pvu 2* (not shown) to confirm the identity of the amplified product.

Preparation of genetic material

A number of sections (3 mm) were cut from each block using a new knife for each block to avoid cross contamination. Samples were dewaxed using mixed xylenes and DNA was extracted using a modified proteinase K/SDS method as previously described.¹² Samples were prepared in a separate area from DNA amplification as a precaution against contamination.

PCR

All amplifications were performed in a 50 μ l reaction volume using conditions already described.¹³ For nested PCR, excess primers and dNTPs were removed from first round PCR by centrifugation through spin columns. Some 2 μ l of the initial amplification product were then subjected to second round amplification using internal primers (Fig 1). In order to examine the sensitivity of the assay system, serial dilution of BMK cells in Ad 12 negative human neutrophils was performed. Cells were then pelleted, formalin fixed, and paraffin embedded. Sections through these cell blocks were then dewaxed, DNA extracted, and subjected to 30 cycles of amplification. For all amplifications, the procedures recommended by Kwok and Higuchi¹⁴ were followed to avoid contamination.

Restriction enzyme digestion

The *Alu 1* enzyme is predicted to cut the amplified sequence in two fragments of known molecular weight, 67 bp and 82 bp and allowed confirmation of the identity of the amplified product. In addition, we also performed restriction digests with *Pvu 11* (which overlaps the *Alu 1* site) and *Sau 111a*, which yields fragments of 71 and 78 bp. All

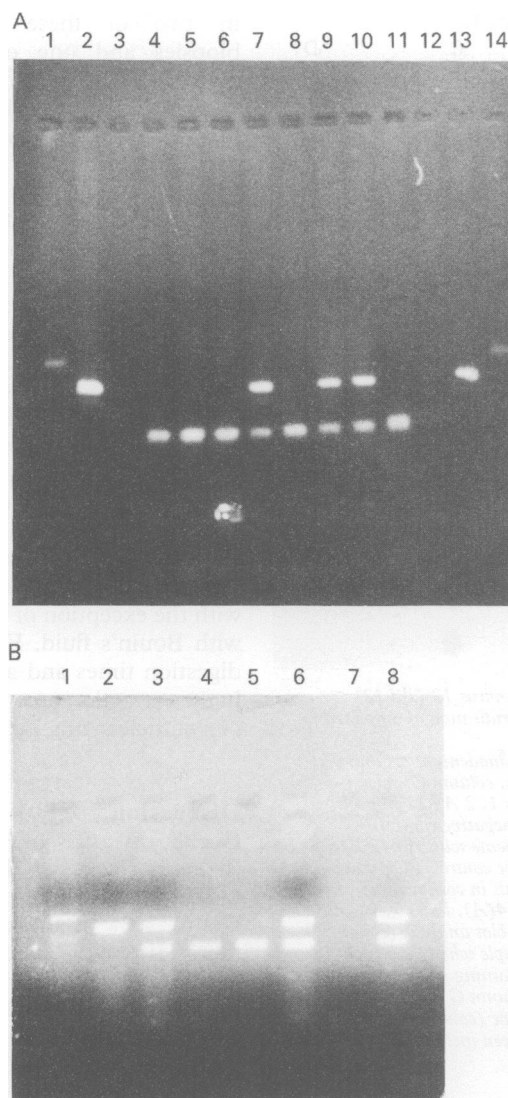


Figure 4: (A) Subset of coeliac patient samples amplified to detect adenovirus 12 (Ad 12) DNA sequences.

Lanes 1, 14: control Ad 12 cell line (1:10 000 dilution) amplified with outer primers; lanes 2, 13: nested PCR of control dilution; lanes 3, 12: negative control; lanes 4–11: coeliac patients coamplified with Ad 12 (nested) and rhodopsin primers. Lanes 7, 9, and 10 show a positive signal which was confirmed by oligonucleotide hybridisation and restriction enzyme digestion.

(B) Subset of control duodenum samples amplified to detect Ad 12 E1a DNA sequences.

Lane 1: control Ad 12 E1a cell line DNA cell line DNA (1:10 000 dilution) amplified with outer primers; lane 2: nested PCR of 1:10 000 control dilution; lane 7: negative control; lanes 3–6, 8 control duodenum amplified with Ad 12 (nested) and rhodopsin primers. Lanes 3, 6, and 8 showed a positive signal which was confirmed by oligonucleotide hybridisation and restriction enzyme digestion.

restriction digests were performed using the manufacturers' instructions.

Oligonucleotide hybridisation

An internal oligonucleotide was designed of the sequence 5' CGC GTT TAT TGT TCT GTC AGC TGA 3' (position 391–415) to be used as a probe. This oligonucleotide was labelled at the 5' end with a radioactive deoxycytosine triphosphate using polynucleotide kinase. Amplified material was denatured and transferred to nylon membranes using a Hybridot manifold (BRL).

TABLE II Adenovirus 12 (Ad 12) positivity as judged by polymerase chain reaction in different patient groups

Diagnosis	No studied	Ad 12+ve	NR*
Adult coeliac disease	17	3	3
Normal duodenum	16	5	–
Childhood coeliac disease	10	3	–
Normal duodenum (<1 y)	7	1	–

NR* – No results were achieved in these samples as all had been fixed in Bouin's medium which we found refractory to DNA analysis.

Blots were probed with the 24 mer internal oligonucleotide, washed under high stringency and subjected to autoradiography for eight to 24 hours.

ANTI-GLIADIN ANTIBODY DETECTION

Antibodies to α gliadin were measured using an ELISA method as previously described.¹⁵

Results

SENSITIVITY OF ASSAY

PCR using synthetic oligonucleotides specific for Ad 12 E1A sequences was capable of detecting the presence of E1A sequences in paraffin embedded sections of BMK cells containing the integrated E1 region. No other adenovirus sequences were identified in this cell line. The sensitivity of identification was such that 1:10⁶ cells could be identified by PCR using this technique with nested primers (Fig 2). The specificity of the amplification was confirmed by cleavage of the amplified fragment using the restriction enzyme *Alu 1* into two fragments of 67 bp and 82 bp, as predicted from the location of the *Alu 1* site in the nucleotide sequence of the published Ad 12 sequence (Figs 1 and 3). In addition, the restriction enzymes *Sau 111a* (Fig 3) and *Pvu 11* (data not shown) gave diagnostic DNA fragments when the amplification product was digested with either of them. Thus, this technique was sensitive and specific for Ad 12 amplification in formalin fixed, paraffin embedded tissue.

AD 12 DETECTION IN ADULT COELIAC PATIENTS

Adenovirus sequences were detected by PCR in duodenal or jejunal sections of three of 17 patients with adult coeliac disease (Table II). In all cases these sequences were detected using Ad 12 specific primers. An example of the results achieved is shown in Figure 4(A). However, Ad 12 sequences were also detected in five of 16 patients with normal duodenal biopsy tissues (Table II, Fig 4(B)). These sequences were confirmed as Ad 12 both by restriction enzyme digestion as above and also by direct sequencing in one case (data not shown). No other adenoviral sequences were detected. The DNA sequence obtained correlated directly with the published Ad 12 sequence. In addition, we examined biopsy material from other sites, mainly from the

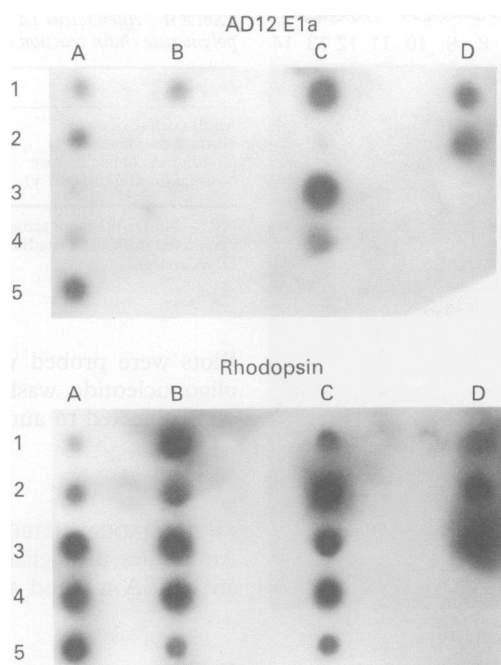


Figure 5: Confirmation of adenovirus 12 (Ad 12) positivity by oligonucleotide hybridisation of amplified DNA in a subset of samples.

Column A: rows 1–5 control duodenum; column B: rows 1–5 other tissue controls; column C: rows 1–5 coeliac samples; column D: rows 1, 2 Ad 12 controls; row 3 rhodopsin control; row 4 negative control.

Dot blots were probed in duplicate with Ad 12 E1a probe and rhodopsin probe as a positive control. PCR analysis had revealed three positive signals in coeliac patients by ethidium bromide staining (Fig 4(A), lanes 7, 9, and 10). These all proved positive on dot blot analysis (column C rows 1, 3, and 4). A fourth sample which had been negative by ethidium bromide staining proved faintly positive by dot blot analysis (column C row 2) but a subsequent biopsy proved negative (column C row 5). Therefore, this sample has not been included as a positive result in the final analysis.

gastrointestinal tract. All but one of these samples was negative. It is notable, however, that two patients who had Ad 12 sequences detected in the duodenum did not have these sequences present in concomitant gastric biopsy specimens. Ad 12 positive cells were confirmed in all cases by oligonucleotide hybridisation of amplified DNA (Fig 5).

AD 12 DETECTION IN CHILDHOOD COELIAC PATIENTS

Ad 12 was detected in duodenal biopsy specimens from three of 10 coeliac patients diagnosed within the first year of life. Ad 12 was also detected in one of seven normal duodenal biopsies performed during the first year of life (Table II).

AD 12 DETECTION IN CHILDREN WITH ANTIBODIES TO α GLIADIN

All of these children with antibodies to a gliadin (Table I) were subsequently followed up and had at least one duodenal biopsy. Two patients were found to have villous atrophy consistent with coeliac disease and a further case had a significant increase in the intraepithelial lymphocyte count. PCR amplification detected Ad 12 sequences

in two of these children with normal biopsies and one of three with abnormal biopsies (Table I). There was no correlation between the anti- α gliadin antibody titres and the presence or absence of Ad 12 DNA.

AD 12 DETECTION IN PATIENTS WITH INTESTINAL ADENOCARCINOMAS

Since Ad 12 has been reported to be oncogenic, we also examined material from a range of intestinal adenocarcinomas for Ad 12 DNA. Briefly, Ad 12 DNA was detected in none of nine colorectal carcinoma samples, none of four ampullary carcinoma samples and in two of five duodenal carcinoma samples tested. Thus, in total two of 19 tumour samples tested were positive for Ad 12 DNA.

DNA extraction and PCR amplification was achieved from all samples in the study with the exception of those that had been fixed with Bouin's fluid. Extension of proteinase k digestion times and a variety of modifications in the extraction protocol did not yield material of a sufficient quality for routine PCR.

Discussion

Coeliac disease is an HLA linked disease which is tightly linked to HLA DQ2 and, possibly, independently linked to HLA DP sequences. Up to 20% of the normal population carry these HLA types.¹ Furthermore, concordance in identical twins is only approximately 75%.¹⁶ These data have led to the suggestion that either a second gene or alternatively an environmental factor may be responsible for the full expression of the disease.⁴ Further support for the concept of an additional environmental factor comes from data indicating a reduction of incidence of coeliac disease in childhood in regions of high prevalence and it has been suggested that an infective agent such as a virus with changing virulence may be implicated.¹⁷ Sequence analysis of the A-gliadin molecule and of the Ad 12 E1B sequence have shown that there is significant sequence homology between a 12 amino acid sequence on Ad 12 and the A-gliadin molecule.⁴ This has led to the suggestion that Adenovirus 12 may have a role in the pathogenesis of coeliac disease by molecular mimicry. The region of homology is 12 amino acids long, approximately the same size as epitopes recognised by T cells. Furthermore, antibodies to the A-gliadin sequence have been shown to cross react with viral antigens.

The suggestion that Ad 12 might be implicated in the pathogenesis of coeliac disease was supported by a study in which a cohort of coeliac individuals had significantly increased titres of antibody reactive against the Ad 12 E1A protein relative to controls.⁶ Subsequent studies failed to show antibodies to E1B in patients with coeliac disease.⁷ Furthermore, a study using the PCR failed to show Ad 12 E1B sequences in coeliac patients.⁸ In the current study, we have performed amplification using the PCR and E1A primers to detect Ad 12

sequences in archival material. The E1A sequence was chosen since this sequence may integrate into the host genome and might potentially serve as a marker not only for current infection but also for prior infection. We have shown that the assay is sensitive and specific using paraffin fixed material. Furthermore, Ad 12 E1A sequences are detectable in approximately 25% of adult biopsy specimens. Our data are in broad agreement with those reported in previous studies using fresh biopsy tissues. However, we did not see any specific association of the detection of E1A sequences with coeliac disease. Furthermore, there did not seem to be an individual clinical syndrome associated with the detection of Ad 12 sequences.

Since the concept of molecular mimicry could also include the possibility that infection during childhood with subsequent eradication of viral antigen might permit recognition of the gliadin peptide in later life, we also examined the duodenal biopsy specimens of patients with coeliac disease detected during the first year of life, and frequently during the first 6 months. In these cases, the incidence of Ad 12 detection did not differ significantly from the adult incidence of Ad 12. Nor did Ad 12 positivity correlate significantly with anti- α gliadin antibody detection in infants with diarrhoeal illness. These data suggest that Ad 12 is not associated with the development of coeliac disease. It is still possible that infection in very early infancy could result in disease. However, given the very high rate of detection of this sequence, it suggests a relatively high prevalence of the virus. The prevalence seems to be similar in both adults and children suggesting that infection occurs at a very young age.

In view of the documented high prevalence of E1A sequences, we were concerned that this oncogenic virus might be associated with tumour development in the gastrointestinal tract. Patients with coeliac disease have an increased frequency of adenocarcinoma of the gastrointestinal tract.¹⁸ E1A sequences are known to affect cellular proliferation by a number of means including interaction with tumour suppressor genes and interactions with cyclin.^{19,20} While E1A sequences are known to transform cells in vitro,²¹ and Ad 12 E1A sequences are associated with the development of gastrointestinal carcinoma in transgenic mice,⁹ there is as yet no evidence of adenovirus involvement in the development of human cancers. In this study we have not observed any association between the presence of Ad 12 E1A DNA and the development of carcinoma of the gastrointestinal tract in humans.

PCR of paraffin embedded material is a robust technique and has been useful in the assessment of archival material.^{12,22} The lack of amplification seen in this study from any material that had been previously fixed with Bouin's fluid makes us recommend that this fixative is not used in material that may subsequently be needed for DNA analysis. A recent study has identified Ad 12 in both adult and childhood duodenal biopsy tissues.²³

The current study differs from both that study and the other study previously described⁸ in that E1A DNA sequences were studied. Secondly, the assay system was adapted for high sensitivity with paraffin embedded tissue. Furthermore, we have confirmed: (i) that Ad 12 is not present in newly diagnosed coeliac patients less than 1 year of age and (ii) that anti-gliadin antibodies may be detected in infantile diarrhoeal illnesses which are not associated with adenovirus 12. These data indicate that cross reactivity to Ad 12 is not implicated in anti-gliadin antibody generation in children and secondly that Ad 12 is not implicated in the continuing pathogenesis of infantile coeliac disease. One cannot, however, entirely exclude the possibility that brief infection of the neonate without integration might serve as an initiation event for coeliac disease. Since Ad 12 positivity is not uncommon in our patient population, in agreement with previous serological and molecular studies,^{6,8,23} this makes it unlikely that Ad 12 is implicated in the pathogenesis of coeliac disease.

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