

SCREENING AND MONITORING COELIAC DISEASE: MULTICENTRE TRIAL OF A NEW SERUM ANTIBODY TEST KIT

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SUMMARY

A multicentre trial was conducted to evaluate a new test for anti-gliadin antibodies (AGA) in serum (Coeliac Screening Kit, CSK, Medical Innovations Limited, Artarmon, NSW, Australia). The test showed excellent reproducibility for both anti-gliadin IgA and IgG detection. The average intraassay coefficient of variation (CV) was 3.0% for IgA and 2.4% for IgG (n=6), while the average interassay CV was 6.4% for IgA and 4.3% for IgG (n=3). By defining a positive test as both IgA and IgG elevated, a sensitivity of 93% in untreated coeliacs (n=75) was observed. The corresponding specificities in healthy adults (n=130) and healthy children (n=77) were >99% and 100% respectively, while in patients with other gastrointestinal disorders (disease controls) the specificity was 94% (n=129). The test was also useful in monitoring patients, with anti-gliadin IgA and IgG falling for up to a year after commencing a gluten-free diet (GFD) (12 adults). In some patients however, antibody levels did not reach the normal cutpoint after many months on a GFD, which may reflect the patients' poor adherence to their gluten free diet. The test was superior to the Pharmacia anti-gliadin ELISA, and should be useful as an aid to the diagnosis of coeliac disease, as well as in the follow-up of treated patients.

KEY WORDS Coeliac disease Anti-gliadin antibody Multicentre trial

INTRODUCTION

Coeliac disease (CD) is defined as a permanent intolerance to dietary gluten, resulting in small intestinal villous atrophy and consequent malabsorption. While the symptoms are usually unequivocal in childhood, those presenting in adults are diverse, ranging from diarrhoea and abdominal pain to fatigue or joint pain (Howdle and Losowsky, 1992). Traditionally, CD has been diagnosed by small intestinal biopsies (Walker-Smith et

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al.,1990). However, this procedure is inconvenient and time-consuming for both patients and medical staff. It is also expensive. Measurement of circulating IgA and IgG anti-gliadin antibodies (AGA) has been proposed as a simple and reliable alternative for the diagnosis of coeliac disease (Kapusinska *et al.*,1987; Burgin-Wolff *et al.*,1989; Unsworth *et al.*,1983; Volta *et al.*,1985; Gonczi *et al.*,1991; Hill *et al.*,1991). Apart from screening individuals with suspected coeliac disease, there is a need to screen first degree relatives, and patients with Diabetes Mellitus or selective IgA deficiency who are at increased risk of CD (Stokes *et al.*,1976; Maki *et al.*,1984; Gadd *et al.*,1992; Collin *et al.*,1992). Thus, a simple, non-invasive screening test such as the detection of AGA would simplify the identification of individuals needing biopsy to confirm CD, and ensure uncharacterised forms of the disease are not overlooked.

Recently, an ELISA for the detection of IgA and IgG AGA was described (Gonczi *et al.*, 1991). By combining the results of IgA and IgG determinations, the overall sensitivity was found to be 100%, with corresponding specificities of 96% in children and 99% in adults. The perfect sensitivity indicated that the test could be used for screening, and would eliminate a large number of unnecessary biopsies. In addition, AGA levels were significantly lower in coeliacs treated by a gluten free diet (GFD), and the test was proposed as a means of monitoring patients after treatment. This test has now been developed into a commercial assay (Coeliac Screening Kit, CSK, Medical Innovations Limited, Artarmon, Australia). In the present study, its performance was assessed in a multicentre trial representing laboratories in Australia and the United Kingdom.

MATERIALS AND METHODS

Patients and Serum Samples

In seventy five patients a diagnosis of coeliac disease (CD) was made by intestinal biopsy, and blood was collected prior to the start of a gluten-free diet (GFD). Two hundred and seven healthy control (HC) donors were also included (130 adults and 77 children), as well as 129 patients suffering from various gastro-intestinal diseases (disease controls, DC). Conditions represented, included Crohn's disease, irritable bowel syndrome, diarrhoea, viral and bacterial gastro-intestinal infections, and iron and folate deficiencies. Nine untreated patients with dermatitis herpetiformis (UD) were included as a separate group since this condition is a feature of gluten sensitisation. Forty three treated coeliacs (TC; GFD for more than 1 month) and 18 treated patients with dermatitis herpetiformis (TD) were also assayed. Serum was also collected from 12 members of the Queensland Coeliac Society at diagnosis and throughout the course of their GFD. Blood was collected by venipuncture, allowed to clot, and the serum was collected by centrifugation and stored at -70°C until assay.

ELISA Procedure

The commercial assay procedure was identical to the research assay (Gonczi *et al.*,1991) except that an internal control serum was also included to permit adjustment for differences in assays performed by different laboratories (e.g. local temperature). Briefly, serum samples and internal control sera were diluted 1/50 (for IgA determination) or 1/100 (for IgG determination) in the diluent provided in the kit, and incubated in duplicate for 15 minutes at room temperature (RT) in a gliadin coated assay plate (100 µl/well). After washing with PBS-Tween buffer, 100 µl/well peroxidase-conjugated

sheep anti-human IgA or IgG at 1/1000 in diluent was added to each well for 15 minutes at RT. After washing as above, 100 µl/well ABTS-based substrate was added for 15 minutes at RT, and colour development was stopped by the addition of 50 µl/well 3% oxalic acid. Absorbance was measured at 405-414 nm in a microtitre plate reader.

Antibody concentration was determined using the following formula:

$$\text{Antibody (units/ml)} = (A/X) \times S$$

where A = known concentration of internal standard
 X = mean absorbance for the internal standard less mean background absorbance
 S = mean absorbance of test sample less mean background absorbance

As the assay was calibrated to give the same values as the research assay, the cutpoints used were as previously determined using 78 healthy children and 78 adults (Gonczy et al., 1991). That is: IgA = 25 and IgG = 46 in children; IgA = 34 and IgG = 42 in adults.

Sera from 21 UC, 24 TC, 6 UD, 16 TD, 9 DC, 26 HA, and 22 HC were also tested at The Prince of Wales Hospital or The University of Manchester using the Pharmacia Gluten IgA EIA (Pharmacia, Uppsala, Sweden) (Ascher et al., 1990). Briefly, after incubation of diluted serum for 1 hour at 37°C, the plate was washed and conjugate was added for 1 hour at 37°C. IgG values were also determined by substituting anti-human IgG-specific conjugate (Pharmacia, Uppsala, Sweden) in place of IgA-specific conjugate, as recommended by the manufacturer. After washing, substrate was added for 30 minutes at 37°C before the addition of stop solution. The plate was then read at 420 nm.

Analysis of Clinical Data

Clinical data were compared with serum antibody levels. The proportion of patients above the designated cutpoint was determined for all diagnostic classifications. Differences in sensitivity and specificity between different diagnostic groups were determined by Chi square analysis, while analysis of variance (ANOVA) and Duncan's multiple range test were used to determine any significant ($p < 0.05$) differences between the means of each diagnostic group. Pearson correlation analysis was performed after log transformation of data. The positive and negative predictive values (PPV and NPV) of the test were determined by dividing the number of true positive (TP) or true negative (TN) patients by the total number of positive (TP+FP) or negative patients (TN+FN). The efficiency of the test (i.e. the number of patients correctly classified as diseased or nondiseased) was determined by dividing (TP+TN) by (TP+FP+FN+TN).

RESULTS

1. Assay Performance

The average intra-assay variation was 3.0% for IgA and 2.4% for IgG (n=6), while the average inter-assay variation was 6.4% for IgA and 4.3% for IgG (n=3). These results indicate good reproducibility in this test.

2. Antibody Levels in Different Diagnostic Groups

The mean IgA and IgG concentrations in untreated coeliacs were significantly higher than in any other diagnostic group, including treated coeliacs and untreated patients with dermatitis herpetiformis (DH) (ANOVA + Duncan's Multiple Range Test, $p < 0.05$). In

addition, mean antibody concentrations in untreated patients with DH were significantly higher than those in the disease control (DC), healthy adult (HA), or healthy children (HC) groups (ANOVA + Duncan's Multiple Range Test, $p < 0.05$). However, levels in untreated DH were not significantly higher than those in treated DH patients (not shown).

The number of patients with elevated antibody levels are summarised in Table 1. Using the cutpoints suggested by the manufacturer, and taking both elevated IgA and IgG levels as positive, 93% of untreated coeliacs were detected in this test. This level of detection was significantly higher ($p < 0.001$) than in any other diagnostic group, including treated coeliacs (40%), untreated DH (56%) and treated DH (44%) patients. The number of patients detected in the untreated DH group (56%) was significantly higher ($p < 0.01$) than those in disease control (6%), and healthy control (<1%) groups. However, there was no significant difference between the number of patients detected in the untreated and treated DH groups.

The combined use of IgA and IgG led to a large improvement in specificity without a significant decrease in sensitivity. Sensitivity in the CD group was 92% with IgA alone and 93% with the IgA/IgG combination. In the DC group, 26% and 17% of patients had elevated IgA or IgG respectively, while only 6% of these patients had elevated levels of both antibodies. Similar results are also observed in the healthy control groups, where only 1/207 patients had elevated levels of both antibody classes. Furthermore, the additional use of IgG led to the identification of four IgA-deficient coeliacs (total IgA < 1.25 g/L).

Due to the improved specificity, the positive and negative predictive values and the efficiency of the test were also improved by using the IgA/IgG combination (Table 1). With IgA or IgG alone the PPVs were only 68% and 76% respectively, while the combined use of IgA and IgG gave an acceptable PPV of 90%. Furthermore, the proportion of patients correctly classified as diseased and nondiseased (efficiency) was increased from 81% and 87% with IgA or IgG alone to 94% with the IgA/IgG combination.

Eight of 129 disease control patients had elevated IgA and IgG levels: 1/1 with pernicious anaemia, 1/7 with Crohn's disease, 1/1 with a renal tumour, 1/9 with diarrhoea, 1/28 with a viral GIT infection, 1/1 with chronic pancreatitis, 1/16 with a folate deficiency, and 1/25 with an iron deficiency. However, in the first three patients above, CD was not excluded by jejunal biopsy. In addition, no particular control disease was consistently positive in the test, with each of eight false positive results being associated with a different disease. As observed previously, the combined use of IgA and IgG led to a significant increase in specificity relative to the use of IgA or IgG alone: 33/129 patients showed elevation of IgA alone and 22/129 showed elevation of IgG alone. Patients with ulcers ($n=2$), irritable bowel syndrome ($n=5$), giardiasis ($n=2$), bacterial GIT infections ($n=17$), malabsorption ($n=3$), steatorrhoea ($n=1$), chronic gastritis ($n=1$), ulcerative colitis ($n=2$), cystic fibrosis ($n=1$), rheumatoid arthritis ($n=5$) and Zollinger-Ellison Syndrome ($n=1$) showed no elevation for either IgA or IgG.

3. Monitoring Gliadin Antibody Levels during GFD

Antibody levels were monitored in 12 adult coeliacs after commencing their gluten-free diet (GFD) (Figure 1). In all cases, anti-gliadin IgA and IgG continued to fall for upto one year. However, these levels did not necessarily reach the normal cutpoint values recommended by the manufacturer.

Table 1. Performance Parameters of CSK

Group ^a	No. Elevated (%)			
	n	CSK-A	CSK-G	CSK(A+G)
UC ^a	75	69 (92%) ^c	71 (95%) ^d	70 (93%) ^b
TC	43	21 (49%) ^e	24 (56%) ^d	17 (40%) ^d
UD	9	7 (78%) ^d	7 (78%) ^d	5 (56%) ^d
TD	18	10 (56%) ^e	13 (72%) ^d	8 (44%) ^d
DC	129	33 (26%)	22 (17%)	8 (6%)
HA	130	20 (15%)	6 (5%)	1 (<1%)
HC	77	4 (5%)	11 (14%)	0 (0%)
PPV		68%	76%	90%
NPV		94%	96%	96%
Efficiency		81%	87%	94%

^a UC, untreated coeliacs (4 IgA deficient patients); TC; treated coeliacs; UD, untreated dermatitis herpetiformis; TD; treated dermatitis herpetiformis; DC; disease controls; HA; healthy adults; HC; healthy children; PPV, positive predictive value; NPV, negative predictive value.

^b Significantly higher than all other diagnostic groups (χ^2 , $p < 0.001$)

^c Significantly higher than all other diagnostic groups (χ^2 , $0.001 < p < 0.01$)

^d Significantly higher than all control groups but not all other diagnostic groups (χ^2 , $p < 0.001$)

^e Significantly higher than all control groups but not all other diagnostic groups (χ^2 , $0.001 < p < 0.01$)

4. Comparison of Medical Innovations and Pharmacia Tests.

CSK was also compared to the Pharmacia AGA test (Table 2). Results from the two tests showed highly significant correlations (Pearson's $r = 0.87$ for IgA and 0.70 for IgG, $p < 0.0001$) and the mean IgA and IgG values in untreated coeliacs were significantly higher ($p < 0.05$) than in any other diagnostic group using either assay (ANOVA + Duncan's multiple range test, $p < 0.05$, not shown). Using only the IgA result, CSK detected more patients than the Pharmacia test (Table 2). Three IgA deficient patients could not be diagnosed through the use of IgA alone. Corresponding specificities in

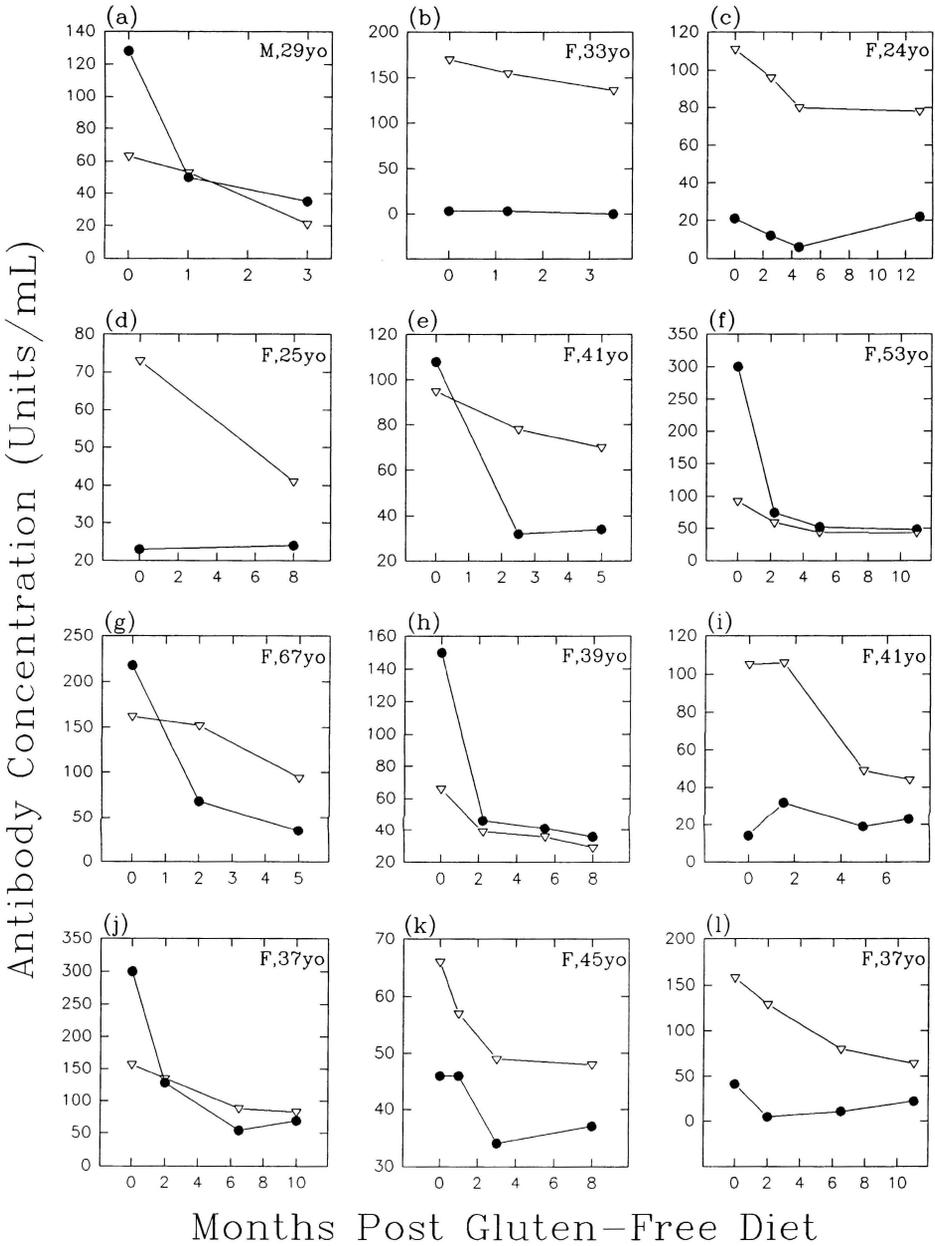


Figure 1. Monitoring patients with CSK IgA (●) and IgG (○) levels in adults diagnosed with coeliac disease. Patients b, c and i are IgA deficient. Age and gender are also shown.

Table 2. Comparison of Medical Innovations CSK and Pharmacia Tests

A. Assay Methods									
	MIL			Pharmacia					
Sera	15 min, RT			60 min, 37°C					
Conjugate	15 min, RT			60 min, 37°C					
Substrate	15 min, RT			30 min, 37°C					
Total	45 min, RT			150 min, 37°C					

B. Performance Parameters									
Group ^a (n)	Percentage Patients Elevated								
	CSK			Pharmacia>10			Pharmacia>20		
	A	G	A+G	A	G	A+G	A	G	A+G
UC (21)	81	90	90	76	90	86	67	81	71
TC (24)	42	50	25	38	54	29	29	29	25
UD (6)	83	83	67	83	67	67	33	50	33
TD (16)	56	75	50	44	63	38	38	13	13
DC (9)	44	44	33	44	67	22	33	33	22
HA (26)	19	0	0	42	46	19	4	8	0
HC (22)	0	9	0	9	55	5	0	36	0
PPV (%)	81	83	86	80	76	90	82	85	88
NPV (%)	56	71	75	50	60	70	46	60	54
Eff (%)	73	80	83	70	73	83	67	77	73

^aUC, untreated coeliacs (3 IgA deficient); TC, treated coeliacs; UD, untreated dermatitis herpetiformis; TD, treated dermatitis herpetiformis; DC, disease controls; HA, healthy adults; HC, healthy children; PPV, positive predictive value; NPV, negative predictive value; Eff, efficiency.

healthy control and disease control patients were 90% and 56% (CSK), 63% and 56% (Pharmacia >10 units/ml), and 98% and 67% (Pharmacia > 20 units/ml). There was little difference in the positive predictive values (PPV) when the different tests were used, while CSK gave a slightly better negative predictive value (NPV) and efficiency. When both IgA and IgG were used, sensitivities were 90% (CSK), 86% (Pharmacia > 10 units/ml), and 71% (Pharmacia > 20 units/ml), while specificities in the control groups were 100% and 67% (CSK), 87% and 78% (Pharmacia > 10 units/ml), and 100% and 78% (Pharmacia > 20 units/ml). There was little difference in PPV, NPV, and efficiency when CSK and Pharmacia > 10 units/ml were compared. However, the Pharmacia test showed a lower NPV and efficiency when a cutpoint of 20 units/ml was used (Table 2).

DISCUSSION

The CSK test detected the vast majority of patients with CD, and combined use of IgA and IgG led to improved differentiation between CD and control groups. Sensitivity was

not affected significantly, but specificity was improved dramatically, with only 6% of disease control patients having elevation of both IgA and IgG. Elevated AGA in patients without coeliac disease has been reported by other groups (Lindh *et al.*,1992; Storm,1990; Rostoker *et al.*,1990; Michaelsson and Gerden,1990), but false positivity was not associated with any specific diagnosis among disease controls. The present results are in close agreement with those reported for the research ELISA (Gonczi *et al.*,1991). Poor specificities using IgA or IgG alone have also been reported by other groups (Unsworth *et al.*,1983; Hill *et al.*,1991; Juto *et al.*,1985; Kelly *et al.*,1987), and an improved performance with the IgA/IgG combination has also been demonstrated (Burgin-Wolff *et al.*,1989; Tucker *et al.*,1988; Kilander *et al.*,1983).

The sensitivity of the test in untreated patients with DH (56%) was not as high as with CD (93%), which was surprising since DH patients are gluten-sensitised. However, the discrepancy is probably related to the fact that skin, and not the intestine, is the primary organ affected in many of these patients. Indeed, some patients diagnosed with DH show normal intestinal histology and may not produce gut antibodies to gluten. Similar detection levels in patients with DH have been reported previously (Vainio *et al.*,1983; Volta *et al.*,1984; Barnes and Lewis-Jones,1989).

The ELISA is also useful in monitoring a patient's adherence and response to a gluten-free diet (GFD). Antibody levels in untreated coeliacs were significantly higher than those in treated coeliacs, in agreement with the results of other studies (Burgin-Wolff *et al.*,1989; Gonczi *et al.*,1991; Juto *et al.*,1985; Valetta and Mastella,1990). In addition, when serial serum samples were assayed, anti-gliadin IgA and IgG fell for up to a year after commencing the GFD. In some patients however, antibody levels did not reach the normal cutpoint after many months on a GFD, which may reflect the patients' poor adherence to their gluten free diet, or some failure of the humoral immune system to retune itself. Indeed, it has been proposed that complete recovery of the intestine after commencing the GFD can take up to two years (Grefte *et al.*,1988). In contrast to CD, antibody levels in treated DH were not significantly lower than those in untreated DH, which may be a reflection of different treatments used in the two diseases, a slower recovery of the skin relative to the intestine, or a difference in the site of antibody production in the two diseases. However, serial samples from the same DH patients need to be tested to evaluate the use of this test in monitoring these patients.

CSK was also compared to the Pharmacia AGA test. CSK is quicker to perform than the Pharmacia test (45 minutes compared with 150 minutes), and does not require special incubation conditions (room temperature rather than 37°C). At the time of testing, the Pharmacia test only detected IgA antibodies. However, following the instructions of the manufacturer, IgG was also determined by substituting Pharmacia anti-IgG conjugate in place of the IgA conjugate. Two cutpoints (10 and 20 units/ml) were evaluated in the Pharmacia test, as each had been suggested by the manufacturer. There was little difference between CSK and the Pharmacia test when either IgA alone or the IgA/IgG combination were used. The Pharmacia test showed a lower sensitivity in CD at either cutpoint, though the specificity was slightly better than CSK when a cutpoint of 20 units/ml was used. Furthermore, there was little difference in PPV, NPV, and efficiency when CSK was compared to Pharmacia (cutpoint 10 units/ml), but the NPV and efficiency were lower when a cutpoint of 20 units/ml was used in the Pharmacia test. Differences observed using the two tests are likely to be due to the different preparations of gliadin used. The CSK uses a total gliadin preparation while the Pharmacia test uses an alpha

gliadin antigen (Gonczi *et al.*, 1991; Ascher *et al.*, 1990). Since some coeliacs only have antibodies to gliadins other than alpha gliadin (Skerritt *et al.*, 1987), fewer patients with coeliac disease would be expected to be detected in the Pharmacia test.

The CSK test evaluated in the present study appears to have advantages for the diagnosis of coeliac disease compared to other published methods of detecting AGA. It is rapid, simple, and reliable, and relies on equipment which should be available in most laboratories. The test should prove useful as an aid to intestinal biopsy, particularly since some coeliac patients undergo more than one biopsy before a positive diagnosis, and an increase in antibody levels may occur before intestinal change. Furthermore, the test is a useful tool for monitoring a patient's compliance with treatment, and may reduce the need for confirmatory repeat biopsies.

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