

Role of IgG anti- β -lactoglobulin antibody in the diagnosis of cow's milk protein intolerance in India

Ujjal Poddar,¹ Priyanka Shukla,¹ Surender K Yachha,¹ Rakesh Aggarwal,¹ Narendra Krishnani²

Departments of ¹Gastroenterology and ²Pathology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India

Objective: Little is known about cow's milk protein intolerance (CMPI) in India. This study was aimed at finding CMPI cases and determining the role of IgG anti- β -lactoglobulin antibody in the diagnosis of this condition in India.

Methods: From June 2004 to December 2005, 30 children with presumptive diagnosis of CMPI, based on endoscopic rectal or duodenal biopsy showing excess eosinophils and response to milk withdrawal, were enrolled and studied prospectively. Definite diagnosis was made in 20 children on the basis of positive milk challenge. IgG anti- β -lactoglobulin antibodies were tested in children with CMPI before and after stopping milk and after milk challenge. Antibody levels were also studied in 27 age-matched disease controls and 50 healthy adults.

Results: The median age of 20 children (16 boys) with CMPI was 16.5 (6–36) months. Of them, 18 presented with diarrhea (12 bloody) and 2 had rectal bleeding. The presumptive diagnosis was most often (85%) based on colonic or rectal biopsy findings. Rectal biopsy was diagnostic in all 20 cases irrespective of the mode of presentation compared with duodenal biopsy which was diagnostic in 3 cases ($p < 0.0001$). There was no difference in antibody levels between cases and controls; the antibody level decreased significantly after milk withdrawal ($p < 0.005$), but did not rise significantly after milk re-challenge.

Conclusions: CMPI is a common cause of chronic diarrhea in children in northern India. Sigmoidoscopy and rectal biopsy help in establishing the diagnosis in most cases. IgG anti-lactoglobulin antibody test is not useful in diagnosing CMPI in the Indian setting.

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Cow's milk protein intolerance (CMPI) is a disorder of infancy, which is characterized by allergy against β -lactoglobulin, a protein contained in cow's milk. In Western countries, symptoms suggestive of CMPI are observed in 5%–15% of infants, and the prevalence of CMPI, as confirmed by double-blind placebo-controlled challenge, is as high as 2%–5%.¹ Infants with CMPI may present with gastrointestinal symptoms alone or in combination with skin and respiratory symptoms.² Common gastrointestinal symptoms include chronic diarrhea, vomiting, anemia, failure to thrive and sometimes, bleeding per rectum.³ Little data are available about this disease from Asia, and in particular India. In a study of chronic diarrhea with malabsorption among children from Chandigarh, 62 patients were <2 years of age; of them, 8 (13%) had CMPI.⁴

Diagnosis of CMPI is based either on repeated withdrawal of and challenge with cow's milk, or by demonstration of histological changes on repeated endoscopic biopsies. The gold standard in the diagnosis of CMPI have

been the 'Goldman criteria'.⁵ According to these, symptoms should subside following withdrawal of cow's milk and should recur within 48 hours of its reintroduction. Positive reaction to three such challenges, and each having similar onset, duration and clinical features will make the diagnosis definite. Re-challenge is a cumbersome process, based on clinical observation only and there is risk of serious reaction during repeated challenge. Most mothers are reluctant to submit their infants to three potentially hazardous challenges, especially after one positive challenge. Iyngkaran *et al*⁶ have suggested more objective diagnostic criteria, i.e. improvement in symptoms on withdrawal of cow's milk, normal or mildly abnormal intestinal histology 6–8 weeks after improvement in symptoms, and histologic relapse with or without clinical relapse after 24 hours of re-exposure to cow's milk. However, these criteria are invasive and require repeated endoscopic small intestinal biopsies. Thus, in Europe, tests for specific IgE antibodies against milk protein (detected using *in vivo* skin test or *in vitro* radioallergosorbent test

[RAST]), and for IgG anti- β -lactoglobulin antibody in serum have been used for diagnosing CMPI.^{7,8,9} Of these, detection of IgG anti- β -lactoglobulin antibody, which uses an enzyme immunoassay (EIA) format, has the advantage of being simple, and reported to have 88% sensitivity and 100% specificity in one study¹⁰ in diagnosing CMPI. In another study, involving 348 CMPI patients, the sensitivity of this test was 71%.⁷ However, this test has not been used in India.

This prospective study was aimed at finding CMPI cases and determining the role of IgG anti- β -lactoglobulin antibody in the diagnosis and follow-up of this condition in India.

Methods

From June 2004 to December 2005, consecutive children (< 3 years of age) admitted to pediatric gastroenterology unit with chronic diarrhea who had been consuming cow's/buffalo milk were included in this study. Children with chronic diarrhea exclusively fed on breast milk were excluded.

Initial (presumptive) diagnosis of CMPI was based on characteristic endoscopic biopsy features and clinical response to withdrawal of cow's milk. The endoscopic biopsy features suggestive of CMPI are; (i) shortening of villi (mild to moderate partial villous atrophy) with excessive eosinophilic infiltrate in the lamina propria in endoscopic duodenal biopsy;³ or (ii) focal eosinophilia (>6 eosinophils/HPF in the lamina propria of one or two crypt regions) or eosinophilic proctitis (focal eosinophilia + eosinophilic infiltrates in muscularis mucosa and/or eosinophilic crypt abscess) on rectal biopsy.^{11,12} In addition, sigmoidoscopic appearance of aphthous ulcers (small discrete ulcers with surrounding erythema and normal intervening mucosa)¹³ was taken as supportive feature of CMPI.

Definite diagnosis of CMPI was made when the presumptive diagnosis was followed by a positive rechallenge.¹⁴ Re-challenge was planned after 2–3 months of cow's milk-free diet, but in some cases it was delayed due to irregular follow-up. Re-challenge was done after admission to the hospital and under close supervision. After documenting normal histology (duodenal or rectal, whichever was initially abnormal), cow's milk was re-introduced at a dose of 10 mL/kg (0.3 g proteins/kg) and watched for one hour. If there was no symptom (diarrhea, vomiting, abdominal pain, skin rash or respiratory distress), milk was continued *ad libitum* for 48 hours and histological examination (rectal/duodenal depending upon the baseline abnormality) was repeated at that time. The challenge test was considered positive if histological

changes reappeared on re-exposure, irrespective of the occurrence of symptoms. Children with definite CMPI were advised milk-free diet for at least 1 year or till 3 years of age, whichever was later. After this, milk was re-introduced gradually, as in milk re-challenge.¹⁵ Informed consent was taken from the parent before each endoscopic procedure and challenge. This study was approved by the Institute's Ethical Committee.

Detection of IgG anti- β -lactoglobulin

Serum specimens were collected from children with CMPI, while they were receiving cow's milk, before challenge (off cow's milk), and during re-challenge. In addition, sera from 27 aged-matched children with gastrointestinal diseases other than CMPI, and 50 normal healthy blood donors were used as controls.

All the sera were tested for the presence of IgG anti- β -lactoglobulin using an in-house ELISA. For this assay, microtiter wells (Nalgen Nunc, Denmark) were coated with 100 μ L each of β -lactoglobulin protein (Sigma-Aldrich, St Louis, MO, USA) dissolved in the 0.5 M carbonate buffer, pH 9.6 at a concentration of 10 μ g/mL. The plate was incubated overnight at 4°C, washed four times with wash buffer (phosphate-buffered saline [PBS]; pH 7.2, 0.15 M and 0.05% Tween-20) and blocked with 200 μ L of 2% bovine serum albumin (Sisco, Mumbai, India) in PBS pH 7.2, 0.15 M at 37 °C for 2 hour. After washing, 100 μ L of test sera diluted 1:100 in PBS (pH 7.2, 0.15 M) were added to the wells and incubated at 37 °C for 1 hour. The wells were washed five times and 100 μ L of alkaline phosphatase-labeled goat anti-human IgG (1:5000 in 1% BSA; Sigma-Aldrich) was added to each well, followed by incubation at 37 °C for 1 hour. The wells were washed five times and incubated at room temperature for 15 min in dark after addition of 100 μ L of *p*-nitrophenyl phosphate (1.0 mg/mL in Tris buffer 0.2 M; Sigma Aldrich Co) to each well. The enzyme-substrate reactions were stopped by adding 50 μ L of stop solution (0.75 N NaOH) per well and absorbance was measured at 405 nm with reference wavelength of 620 nm.

Statistical analysis

The results are presented as median (range). Intergroup comparisons were done using Mann–Whitney U test, intragroup comparisons were done using Wilcoxon signed rank test and categorical variables were compared using Fisher exact test. Values of $p < 0.05$ were considered significant.

Results

The flow chart shows the method of recruitment of CMPI subjects (Figure). Of the 30 presumptive cases of CMPI, definite diagnosis was made in 20 on the basis of milk

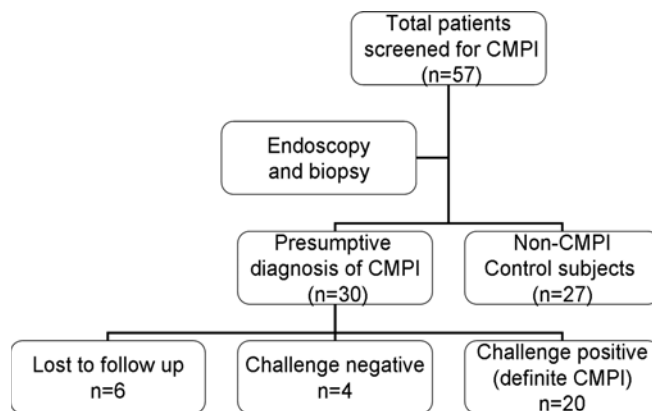


Figure: Flow chart showing patient selection in the study

challenge. The median age of these 20 children (16 boys) was 16.5 (range 6–36) months. The median age of control children was 24 (range 5–42; n=27; 19 boys) months. The median duration of symptoms in children with CMPI was 5.25 (range 1–18) months. The median age of introduction of cow's milk was 3 months (range 0–7 months); 5 children had been on cow's milk since birth. Diarrhea was the presenting symptom in 18 children (90%); 10 had bloody diarrhea, 6 had watery diarrhea and 2 had alternative bloody and watery diarrhea. Both the children who did not have diarrhea at presentation, presented with rectal bleeding. Symptoms subsided in all children on milk withdrawal. All children in control group were on cow's milk at the time of enrolment.

In the control group 13 children had diarrhea and the etiology of diarrhea in them were: celiac disease 4, non-specific diarrhea 6, Crohn's disease 3. The remaining 14 did not have diarrhea and their diagnoses were extrahepatic portal venous obstruction 3, juvenile rectal polyp 2, functional dyspepsia 3, resistant rickets 2 and nutritional anemia, functional constipation, Meckel diverticulum and Wiskott–Aldrich syndrome in one child each. There was no suggestion of CMPI in the rectal and/or duodenal biopsy of all control subjects. CMPI was diagnosed in 18 of 31 children with chronic diarrhea (58%). The chance

of diagnosing CMPI was significantly more in children with bloody diarrhea [12 of 14 (86%)] than with watery diarrhea [6 of 17 (35%)] ($p<0.009$) in this age group.

The accuracy of sigmoidoscopy, duodenoscopy, rectal biopsy and duodenal biopsy in diagnosing CMPI is given in the Table. Accuracy of sigmoidoscopy and rectal biopsy were significantly higher than that of duodenoscopy and duodenal biopsy in diagnosing CMPI ($p<0.0001$). Anti-endomysial antibody (EMA) had been tested in 15 cases and was negative in all. Anemia (hemoglobin <11 g/dL) was present in 17 (85%) cases and mean (SD) hemoglobin level was 8.4 (1.6) g/dL.

Milk challenge was positive in 20 of 24 children (83%), 5 were lost to follow-up before challenge, parents of one child refused consent. The challenge was done 2–3 months after introduction of milk-free diet in 17 children, after 4 to 6 months in 3 children and after 12 months in 4 children. The challenge was positive (histological relapse with or without symptomatic relapse) in 19 of 20 children (95%) when it was done early (within 6 months), whereas it was negative in 3 of 4 children (75%) when it was done late (after 12 months of milk-free diet). During challenge, baseline sigmoidoscopy/duodenoscopy was normal in all 24 children; of these, 6 (25%) showed reappearance of aphthous ulcers on sigmoidoscopy. Symptoms reappeared in 8 (33%) children on milk exposure and the remaining 16 were asymptomatic.

There was no difference in optical density (OD) values of IgG anti- β -lactoglobulin antibody observed in ELISA between 20 CMPI cases and 27 non-CMPI diseased controls. The median OD values (95% CI) in three groups were 0.3 (95% CI: 0.12–0.48) in cases, 0.32 (95% CI: 0.21–0.42) in disease controls and 0.17 (95% CI: 0.162–0.178) in 50 healthy controls. The cases with CMPI and children studied as disease controls had higher OD values than healthy adults ($p<0.001$). In 20 CMPI cases, the median OD value of IgG anti- β -lactoglobulin antibody decreased significantly from those at the time of initial diagnosis (while on milk) to the second sample (after milk discontinuation; [0.3 with 95% CI: 0.12–0.48 vs. 0.2 with 95% CI: 0.13–0.27, $p<0.005$]). However, there was no change in the median OD values between second and third sample during milk rechallenge (0.2 with 95% CI: 0.13–0.27 vs. 0.18 with 95% CI: 0.12–0.24, $p=ns$), or as time after milk discontinuation increased from 2–3 months to >12 months (0.2 with 95% CI: 0.13–0.27 vs. 0.16 with 95% CI: 0.02–0.3, $p=ns$). These findings suggested that anti- β -lactoglobulin antibody levels depended on milk exposure, irrespective of the disease state. Children with milk exposure (CMPI and disease controls) had higher level of antibody than adults.

Table. Performance of endoscopy and biopsy in diagnosing CMPI

Investigation	Performed (n)	Positive results (n)	Accuracy (%)
Sigmoidoscopy*	20	17	85
Duodenoscopy	12	0	0
Rectal biopsy**	20	20	100
Duodenal biopsy	12	3	25

Accuracy: Accurately diagnosed cases/ performed cases $\times$ 100

* $p<0.0001$ between sigmoidoscopy and duodenoscopy

** $p<0.0001$ between rectal biopsy and duodenal biopsy

On follow-up (17.7 [9.3] months), milk was restarted in 15 (75%) children after a median follow up of 15 (6–22) months, 5 (25%) were still on milk-free diet after a median follow up of 5 (3–9) months. Symptoms subsided in all the children on stopping milk and their weight increased from median 80 (range 63–99)% of expected weight to 87.5 (73–103)% of expected weight ($p=0.001$). Milk was re-started in 15 children after second challenge. The second challenge was negative in 13 and positive (histological relapse) in 2. However, all 15 were asymptomatic on milk for a median follow up of 5 (2–15) months.

Discussion

It has been believed that CMPI is uncommon in India. However, this belief may be due to lack of awareness about this entity and lack of diagnostic facilities (endoscopic biopsies and serology). Since cow's/buffalo milk is used as frequently in India as in the West, CMPI is expected to be in India as well. Our study shows that CMPI is a common cause of chronic diarrhea in children <3 years of age in India. Similar data have not been published from India earlier.

The clinical presentation of CMPI cases in our study has opened an important issue in approaching diarrhea in young children. Diarrhea is the commonest presenting symptoms of CMPI, was present in 90%, and large bowel type diarrhea was present in two-third of our patients. In infants (≤ 12 months) with bloody diarrhea of >2 weeks' duration, it is more often CMPI than anything else; these children are frequently diagnosed to have inflammatory bowel disease (IBD) and put on anti-inflammatory and immunomodulatory therapy. It is important to recognize this entity, as prognosis is excellent if diagnosed early. The clue to diagnosis is age of onset of symptoms (IBD is less common in first two years of life), sigmoidoscopic appearance (aphthous ulcers) and rectal biopsy showing plenty of eosinophils without much change in crypt architecture (odd for IBD).^{12,16}

Two types of anti- β -lactoglobulin antibodies, IgG and IgE are known to develop in this condition. It has been shown that IgE anti-lactoglobulin antibody is present in around 60% of patients of CMPI and these patients also have respiratory and/or skin symptoms.¹⁷ However, detection of IgE anti-lactoglobulin antibody is cumbersome (fluorescence enzyme immunoassay or FEIA). On the other hand, assay for IgG anti-lactoglobulin is simpler (ELISA). It has been shown that on cow's milk withdrawal there is rapid reversal of histopathological changes and on re-exposure to milk there is rapid deterioration in 24–48 hours.³ However, the response of these antibodies to

milk withdrawal and re-exposure, is not known. We hypothesized that IgG anti-lactoglobulin antibody levels would decline with clinical response on cow's milk withdrawal, and again rise on milk re-challenge. However, we failed to find any role for IgG anti- β -lactoglobulin antibody in the diagnosis and management of CMPI.

Since a commercial kit was not available, we developed an in-house ELISA. This ELISA test showed that the level of IgG anti-lactoglobulin antibody depended on milk exposure, but not on the presence or absence of milk allergy. Thus, the level of this antibody depends on milk exposure irrespective of the disease state. Children with milk exposure (CMPI and diseased controls) had higher level of antibody than adults (likely to be on less amount of milk). In CMPI patients, antibody level decreased on milk withdrawal irrespective of the duration of abstinence from milk (2–3 vs. 12 months). Probably the level of antibody did not rise after re-exposure during challenge, as the duration of milk exposure was short (2–3 days only). It may be useful to study changes in IgE anti-lactoglobulin antibody on stopping milk and milk re-challenge. The main drawback of our study is that our in-house ELISA could not perform as effectively as has been reported in the literature.

In conclusion, CMPI is one of the common causes of chronic diarrhea in young north Indian children. Sigmoidoscopy and rectal biopsy help in establishing diagnosis in most of the cases. IgG anti- β -lactoglobulin antibody is not useful in diagnosing CMPI in the Indian setting.

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- Correspondence to:** Dr Poddar, Associate Professor, Department of Pediatric Gastroenterology, SGPGIMS, Lucknow 226014, India. Fax: 91 (522) 266 8017 E-mail: ujjal@sgpgi.ac.in
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Image

Phrygian cap in magnetic resonance cholangiogram

A 30-year-old man presented with progressive jaundice and pruritus of 3 months' duration. He had a history of recurrent fever with chills and abdominal pain. Physical examination showed icterus and mild hepatomegaly. The gallbladder was not palpable. Liver function tests showed total bilirubin 36 mg/dL, conjugated bilirubin 19 mg/dL and serum alkaline phosphatase 415 u/L. USG abdomen showed gross dilatation of the common bile duct, common hepatic duct and intrahepatic biliary radicals and distended gallbladder. Magnetic resonance cholangiography showed calculus in the distal common bile duct and a Phrygian cap deformity of the gallbladder (Figure). The patient underwent ERCP and stone extraction and improved well.

A Phrygian cap is a congenital anomaly characterized by kinking between the body and fundus of the gall bladder. This anomaly can sometimes cause diagnostic confusion in routine investigations.¹ Only a single case report has demonstrated a Phrygian cap in magnetic resonance cholangiogram.²

Sebastian Saji, Jose Tony,
K Sunil Kumar, Varghese Thomas
Department of Gastroenterology, Calicut Medical
College, Kerala, India



Figure: Magnetic resonance cholangiogram showing a Phrygian cap

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Correspondence to: Professor Thomas, Head, Department of Gastroenterology, Medical College Hospital, Calicut 673008, Kerala E-mail: drvarghesethomas@gmail.com