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Original Article

Determination of Diagnostic Accuracy of Anti Endomyseal Antibodies Detection by Indirect Immunofluorescence Using Human Umbilical Cord as a Substrate

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ABSTRACT

Objective: To evaluate the diagnostic accuracy of anti-endomyseal antibody (EMA) detection using indirect immunofluorescence (IIF) on human umbilical cord (HUC) substrate, compared with biopsy-confirmed celiac disease (CD), and to assess its agreement with the conventional primate esophagus (PE) substrate.

Study Design: Comparative cross-sectional study.

Place and Duration of Study: Department of Immunology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan conducted from Jun 2024 to Mar 2025.

Methodology: In this study 86 cases who were diagnosed as Celiac disease on duodenal biopsy, and 90 age matched controls were included. The control group included 40 paderaites patients, 40 healthy blood donor population and 10 patients with Hypertension. IgA deficiency was ruled out in all the patients. IIF was performed on both the cases and controls sample on both commercial slides with Primate esophagus and on in house using Human umbilical cord as a substrate respectively.

Result: The Sensitivity and Specificity of HUC for the detection of anti endomyseal antibodies came out to be 96.5% and 97.8% respectively. The Positive predictive value, Negative predictive value and diagnostic accuracy were 97.6%, 96.7% and 97.1% respectively. Agreement between HUC-EMA and PE-EMA was excellent, with perfect inter-observer concordance (κ = 1.00)

Conclusion: In house HUC is an excellent substrate for detection of Anti endomyseal antibodies.It can serve as a reliable alternate to the commercial slides using primate oesophagus in terms of sensitivity and specificity. Moreover, it is inexpensive, and readily available.

Keywords: Anti-Endomyseal Antibodies; Autoimmune Enteropathy; Celiac Disease; Diagnostic Accuracy; Human Umbilical Cord; Indirect Immunofluorescence; Primate Esophagus; Serologic Tests.

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INTRODUCTION

Celiac disease (CD) is a chronic immune-mediated enteropathy characterized by specific serological and histological abnormalities triggered by exposure to a dietary group of alcohol-soluble proteins gluten.[1] The untreated disease may lead to malabsorption, iron-deficiency anemia, growth failure, osteoporosis, infertility, and increased long-term morbidity. CD is increasingly recognized as one of the most common autoimmune gastrointestinal disorders worldwide.[2]

The global burden of celiac disease has increased substantially over recent decades.[2] Current epidemiological estimates suggest a worldwide sero prevalence of approximately 1.4%, while biopsy-confirmed prevalence is around 0.7%, indicating that a significant proportion of cases remain undiagnosed.[3–5] In comparison, higher prevalence rates have been reported in Europe and Oceania (approximately 0.8%).[4–5] In Pakistan, more than two million individuals may be affected, highlighting the need for accessible diagnostic approaches.[6] Serological testing is central to screening and includes anti-endomyseal antibodies (EMA), anti-tissue transglutaminase antibodies (tTG), and deamidated gliadin peptide antibodies (DGP). Among these, IgA-based EMA and tTG assays demonstrate the highest diagnostic accuracy, with reported sensitivity and specificity of anti-tTG IgA reaching 97% and 91%, while EMA IgA specificity approaches 99%.[7-9]

Indirect immunofluorescence (IIF) using primate esophagus is traditionally regarded as the gold standard substrate for EMA detection8. However, the use of animal-derived substrates is limited by ethical concerns, high cost, restricted availability, and regulatory constraints, particularly in low- and

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middle-income countries.[9] Human umbilical cord (HUC) has emerged as a promising alternative, with internationally reported sensitivity up to 98.5% and specificity approaching 100%, comparable to primate esophagus10. Despite encouraging evidence, local confirmatory evaluations remain scarce. Therefore, the present study is conducted to assess the diagnostic accuracy and feasibility of HUC-based EMA testing in a tertiary care setup by comparing its performance with biopsy-confirmed celiac disease and conventional primate esophagus-based IIF assay.

METHODOLOGY

This study was conducted as a comparative cross-sectional diagnostic accuracy evaluation at Department of Immunology, Armed Forces Institute of Pathology, Rawalpindi from August 2024 to March 2025 after approval from ethics committee of Armed Forces Institute of Pathology ERB Certificate [FC-FC21-9/READ-IRB/24/3359] issued on16 August 2024.

The sample size was calculated according to sensitivity and specificity calculator with Sensitivity of HUC as 98.5%.[10] Specificity as 100% Prevalence of disease = 7%6and by taking confidence interval of 95%, the appropriate sample size came out to be 80. In this study 86 cases who were diagnosed with Celiac disease on duodenal biopsy were included, and 90 age matched controls were included.

The primary objective was to assess the analytical performance and feasibility of anti-endomyseal antibody (EMA) detection by indirect immunofluorescence (IIF) using human umbilical cord (HUC) as a substrate, with duodenal biopsy as the reference standard for celiac disease diagnosis. In addition, the study evaluated analytical agreement between HUC-based EMA testing and the conventional primate esophagus (PE) substrate. The study was designed as a confirmatory method-comparison investigation and did not aim to introduce a novel diagnostic assay.

Inclusion Criteria: The study population comprised patients with biopsy-confirmed celiac disease and a control group without a prior diagnosis of celiac disease. Cases were defined as individuals with compatible clinical features and duodenal biopsy findings demonstrating Marsh–Oberhuber grade ≥2 changes, interpreted by experienced histopathologists while patients were consuming a gluten-containing diet. Controls were recruited from patients presenting with non-gastrointestinal conditions such as upper respiratory tract infection. Controls did not undergo systematic screening using duodenal biopsy, tissue transglutaminase serology, or HLA typing; therefore, the possibility of undiagnosed or subclinical celiac disease among controls was acknowledged.

Exclusion Criteria: Patients receiving any immunosuppressive treatment were excluded. Hemolyzed and Lipemic samples as assessed by visual inspection were also excluded from the study. Moreover, Patients with borderline histological findings (Marsh grades 0–1), equivocal biopsies, or known IgA deficiency were excluded to ensure diagnostic certainty.

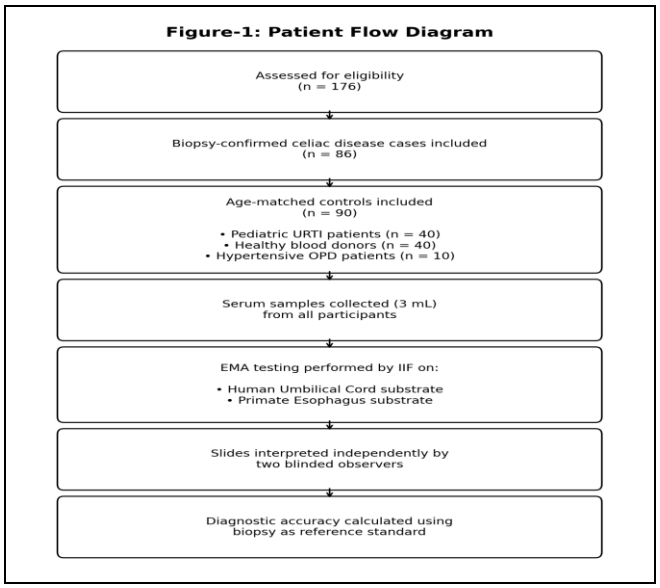
Human umbilical cord tissue was obtained prospectively from the Department of Gynecology, CMH Rawalpindi, after administrative approval from the Head of Department. Samples were collected from anonymized postpartum females after obtaining written informed consent. Only tissue routinely discarded after delivery was used. The principal investigator personally transported the tissue in sterile normal saline to the Immunology Department, AFIP, for immediate processing. Privacy, confidentiality, and biosafety precautions were ensured throughout, and the study was approved by the AFIP Ethical Review Board.

EMA detection was performed using indirect immunofluorescence on both HUC and Primate esophagus substrates. Blood sample of 3ml was taken from the participants in a plain tube under sterile conditions. After centrifuging the blood sample, the separated serum was collected and IIF test for EMA was performed on Euro immune kit according to the protocol provided by the manufacturer. 1:10 diluted Patient sera sample was applied on the HUC smears and incubated in moist chamber for 20 minutes followed by washing thrice with PBS. The smears were then incubated with fluorescein-isothiocyanate anti-human IgA (FITC Ig A) at a 1:100 dilutions in PBS for 20 minutes. The sections were washed again thrice with PBS and mounted using glycerin and cover slips.

Slides were examined using fluorescence microscopy at standardized magnification. EMA positivity was defined by the presence of the characteristic reticular fluorescence pattern along smooth muscle fibers. The study methodology is summarized as a patient flow diagram (Figure-1).

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 27.0. The Shapiro–Wilk test showed that age was not normally

distributed; therefore, it was summarized as median (IQR) and compared between groups using the Mann–Whitney U test. Categorical variables, including gender, study groups, and indirect immunofluorescence (IIF) results, were presented as frequencies and percentages, and compared using the Chi-square test. Diagnostic performance was assessed by calculating sensitivity, specificity, positive and negative predictive values, likelihood ratios, and diagnostic accuracy using the Primate esophagus substrate as the reference standard. A *p*-value of <0.05 was considered statistically significant.



RESULTS

A total of 176 participants were included in this study, consisting of 86 biopsy-confirmed cases of celiac disease and 90 controls. Overall median age was 11.00 (30.00 – 7.00) years. Among celiac disease cases, the median age was 10.00 (25.75 – 6.00) years while control group had a median age of 19.50 (32.25 – 7.00) years, with a borderline significant difference between the groups *p*-value=0.050. Regarding gender distribution, males and females each comprised 88 (50.0%) of the total participants. Among celiac disease cases, 38(44.2%) were males and 48(55.8%) were females, whereas the control group included 50(55.6%) males and 40(44.4%) females. There was no statistically significant difference in gender distribution between the groups (*p*-value=0.132) shown in Table-I.

On qualitative assessment, the fluorescence pattern in positive samples appeared as a characteristic reticular staining of the endomyseal tissue localized around the umbilical vessels. This pattern was consistently observed in confirmed cases. (figure 2a and 2b). A small number of discrepancies were noted, including three cases that were biopsy-positive but negative on HUC, and two controls that showed faint staining. Despite these minor variations, the overall interpretability of the test remained straightforward.

Table-I-Demographic Characteristics of Study Participants (n=176)

Variable	Total participants (n = 176)	Celiac disease cases(n=86)	Controls (n=90)	*p*-value
Age in Years (median (IQR)	11.00 (30.00 – 7.00)	10.00 (25.75 – 6.00)	19.50 (32.25 – 7.00)	0.050
Gender				
Male	88 (50.0%)	38 (44.2%)	50 (55.6%)	0.132
Female	88 (50.0%)	48 (55.8%)	40 (44.4%)	

IQR=Interquartile Range

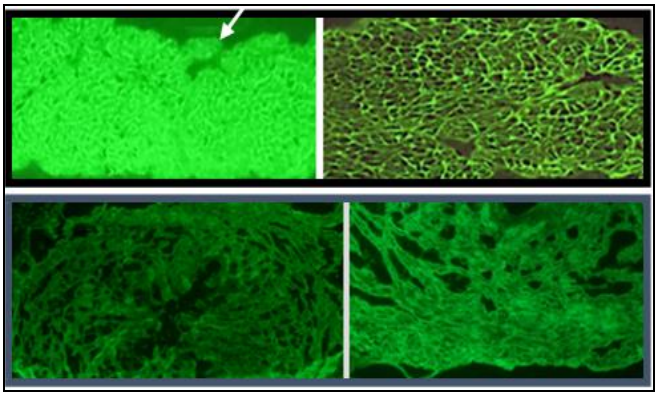


Figure-2: A and 2B: IgA anti endomyseal antibodies staining the connective tissue around venous walls of HUC (arrow) on IIF(20X)(40X

Figure-3: A and 3B: IgA anti endomyseal antibodies staining Muscularis mucosae of primate esophagus on IIF 20X, 40X

Inter-observer reliability was excellent. All slides were reviewed independently by two observers, and both reported identical findings across all samples. This resulted in a Cohen's kappa value of 1.00, reflecting perfect agreement and indicating that the test results were highly reproducible with minimal subjective variation. Based on these findings, the diagnostic performance of HUC-based immunofluorescence was found to be very high. The sensitivity was 96.51% and specificity was 97.78%. Similarly, the positive predictive value was 97.65%, and the negative predictive value was 96.70%. The overall diagnostic accuracy reached 97.16% Shown in Table-II. The likelihood ratios further supported the strength of the test, with a positive likelihood ratio of

43.43 and a negative likelihood ratio of 0.04, indicating strong ability to both confirm and exclude disease. It is important to note, however, that predictive values should be interpreted cautiously, as they may be influenced by the study design and the proportion of cases and controls.

Table-II: Diagnostic Accuracy of Inhouse HUC slides for the detection of Anti endomyseal antibodies (n=176)

	Positive on HUC	Negative on HUC
Positive on Primate Esophagus	83	2
Negative on Primate Esophagus	3	88

Sensitivity= TP/(TP +FN)= 83/83+3×100= 96.5%
Specificity= TN / (TN +FP) =88/88+2×100= 97.8%
Positive Predictive Value = TP/ (TP+FP)=83/83+2×100 = 97.6%
Negative Predictive Value= TN/(TN+FN =88/88+3×100= 96.7%
Diagnostic Accuracy= (TP +TN)/All Patients=83+88/176×100=97.1%

TP=True positive, FN=False negative, FP=False positive, TN=True negative

DISCUSSION

The findings of this study demonstrate high sensitivity and specificity for EMA detection using HUC and show excellent analytical agreement with the conventional primate esophagus (PE) substrate. These results support the feasibility of HUC as an alternative substrate for EMA detection under controlled laboratory conditions.

The results of the study are comparable with the available international studies, that have demonstrated EmA detection using various tissue substrates. Two different studies that utilized primate esophagus as a substrate, have reported sensitivity ranging from 95% to 100% and specificity between 96% and 99%.[11,12] Our sensitivity of 96.5% is comparable to these studies. Similarly, our specificity of 97.8% agrees with the findings of studies conducted in European and American populations, which reported specificities of over 95%.[12,13]

Our results support the validity of HUC in a wider diagnostic setting than the study by Bahia et al, in that study both the monkey esophagus and the umbilical cord was used as substrates.[13] Bagnasco et al. also reported high accuracy rates comparable to our findings, highlighting the potential utility of human umbilical cord parts as a substrate for detecting IgA EmA in relatives of patients with coeliac disease.[14] Furthermore, our results corroborate those of Carroccio *et al*., who assessed EmA detection in pediatric patients and found a similar sensitivity range.[15]

It is important to emphasize that this investigation does not claim methodological novelty. Human umbilical cord has previously been validated as a suitable substrate for EMA detection in studies from Europe, South America, and other regions.[16,17] The contribution of the present work lies in its confirmatory and implementation-oriented nature, demonstrating that comparable diagnostic performance can be achieved in a local tertiary care laboratory using an in-house prepared human-derived substrate. Such confirmatory evaluations remain relevant, particularly in healthcare systems where access to commercially prepared or animal-derived substrates may be limited by cost, ethical considerations, or regulatory constraints.

A notable methodological strength of this study is the use of duodenal biopsy as the sole reference standard for celiac disease diagnosis. By avoiding the use of EMA testing itself as a reference, the study minimizes circular reasoning in diagnostic accuracy estimation. Additionally, the assessment of inter-observer agreement demonstrated perfect concordance between independent readers, highlighting the interpretive reliability of EMA pattern recognition when performed by trained personnel in a standardized laboratory setting.

Despite these strengths, several limitations must be carefully considered when interpreting the findings. Foremost among these is the comparative cross-sectional study design, which is known to introduce spectrum bias. By comparing well-defined, biopsy-confirmed celiac disease cases with apparently non-celiac controls, the study creates an artificial separation between diseased and non-diseased groups. This design excludes patients with borderline histological findings (Marsh grades 0–1), atypical clinical presentations, IgA deficiency, or low-titer sero-positivity subgroups that commonly present diagnostic challenges in routine.

The construction of the control group represents an additional limitation. Controls were not systematically evaluated using duodenal biopsy, tissue transglutaminase serology, or HLA typing. Consequently, the presence of undiagnosed or subclinical celiac disease among control participants cannot be excluded. Apparent false-positive EMA results observed in the control group may therefore represent true, previously unrecognized disease rather

than analytical error. This introduces a degree of verification bias and underscores the challenges inherent in defining a truly disease-free control population for celiac disease research.

Another important consideration relates to the preparation and standardization of the HUC substrate. While the substrate was prepared consistently using an in-house protocol, the study did not formally evaluate critical analytical parameters such as batch-to-batch variability, slide-to-slide reproducibility, antigen stability, or fluorescence decay over time. These factors are essential for ensuring assay robustness and reproducibility, particularly if the method is to be adopted beyond a single laboratory setting. The absence of such evaluations limits conclusions regarding the scalability and routine diagnostic implementation of HUC-based EMA testing.

The interpretation of predictive values also warrants careful consideration. Positive and negative predictive values were calculated but must be interpreted with caution, as the artificial prevalence of celiac disease inherent to a comparative cross-sectional design distorts these measures. For this reason, likelihood ratios were emphasized as more robust, prevalence-independent indicators of diagnostic performance. The observed likelihood ratios suggest that HUC-based EMA testing provides meaningful diagnostic discrimination in biopsy-confirmed disease, although these estimates should be validated in prospective clinical cohorts.

Although the study does not include formal cost-effectiveness analysis, the use of a human-derived substrate such as HUC may offer practical advantages in certain settings.[18] Ethical concerns related to animal tissue use, supply limitations, and regulatory restrictions on primate materials can pose barriers to routine EMA testing in some regions.[19,20] In such contexts, HUC may represent a viable alternative, provided that rigorous standardization and quality control procedures are established.

In summary, this study contributes confirmatory evidence supporting the analytical equivalence of human umbilical cord and primate esophagus substrates for EMA detection by indirect immunofluorescence. The findings should be interpreted as a preliminary step toward broader implementation rather than definitive validation. Future research should focus on prospective, STARD-compliant diagnostic accuracy studies in consecutive patients with suspected celiac disease, incorporate borderline and atypical phenotypes, and include comprehensive assessments of substrate reproducibility, stability, and cost-effectiveness. Only through such rigorous evaluation can the clinical utility and generalizability of HUC-based EMA testing be fully established.

CONCLUSION

Human umbilical cord is a sensitive and specific substrate for the detection of anti-endomysial antibodies by indirect immunofluorescence. Its diagnostic performance is comparable to primate esophagus, making it a practical and reliable alternative for routine laboratory use, particularly in resource-limited settings.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

HM & HNT: Data acquisition, data analysis, critical review, approval of the final version to be published.

MH & MB: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

MAH & IK: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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