Evaluation of intraepithelial lymphocytic counts in pediatric duodenal endoscopic biopsies by routine staining and CD3 Immunostaining

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Aim
This study aimed to compare the intraepithelial lymphocytic (IEL) counts by hematoxylin and eosin (H&E) and by CD3 immunohistochemistry (IHC) in pediatric duodenal biopsies during routine diagnostic practice and to evaluate the interobserver variability between both methods.
Materials and methods
A retrospective study was conducted on 64 pediatric duodenal biopsies received from January 2017 to December 2018 at the Pathology Laboratory of Ain Shams University Hospitals and classified into three groups: group 1 with established coeliac disease by previous biopsy, group 2 with newly received cases suspicious of coeliac disease with positive anti-tissue transglutaminase with present or absent villous atrophy, and group 3 with normal or unknown anti-tissue transglutaminase. IEL count was evaluated by routine H&E and by CD3 immunohistochemistry by the two authors and categorized into normal (<25 lymphocytes), mildly raised (25–40 lymphocytes), and markedly raised (>40 lymphocytes). Agreements between stains, as well as interobserver agreement, were calculated.
Results
The overall mean IEL count per 100 enterocytes for H&E was 21.86 [95% confidence interval=18.34–25.38], and for IHC by CD3, it was 26.19 (95% confidence interval=22.52–29.85). The difference was highly significant (P=0.001), with highly significant substantial agreement between H&E and CD3 IHC results (P=0.001; κ=0.694). The overall interobserver agreement for H&E was almost perfect among the cases of all groups, and among groups 1, 2, and 3 (κ=0.946, 0.825, 0.831, 1, respectively). The overall interobserver agreement for CD3 IHC was almost perfect among the cases of all groups, and among groups 1, 2, and 3 (κ=0.975, 1, 1, and 0.956, respectively).
Conclusion
CD3 can aid in the detection of IELs but only in the proper clinical and serological setting.

Keywords:
CD3 immunohistochemistry, coeliac disease, intraepithelial lymphocytes, pediatric duodenal biopsy

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Introduction

Intraepithelial lymphocytes (IELs) are present in between the epithelial cells of the small and large intestine, and the majority of which are T-cell type [1]. Elevated counts of IELs along superficial duodenal villi have been considered as a sensitive marker of the effect of gluten [2–4]; therefore, duodenal endoscopic biopsies are often required when there is a clinical suspicion of celiac disease [5], to detect the typical triad of elevated IELs, crypt hyperplasia, and villous atrophy [6]. However, in individuals with 'latent' gluten sensitivity, only IEL occurs with absent villous atrophy [7,8]. These individuals should be diagnosed and treated with gluten-free diet as they may encounter gastrointestinal symptoms, osteopenia, or osteoporosis, as well as high risk of autoimmune diseases and mortality.

Elevated counts of IELs are not specific for celiac disease, as this could be found in several other disease entities, like tropical sprue, food protein intolerance, peptic duodenitis, *Helicobacter pylori*-associated gastritis, and parasitic and viral infections [2–4,9]. This warrants appropriate assessment of IELs in the proper clinical and serological contexts. Evaluation of IELs is made by examination of routine hematoxylin and eosin (H&E) stained sections. In challenging cases, immunohistochemistry (IHC) for CD3 to demonstrate intraepithelial T lymphocytes may be considered [5].

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Non-neoplastic gastrointestinal lesions in pediatric age group encompass a diversity of several diseases with increasing rates, owing to the substantial advancement in pediatric endoscopy. Previous studies comparing IEL counts by H&E with their counts by CD3 IHC show conflicting results [3,6], with only scarce research conducted on pediatric age group cases. Hence, the aim of the current study was to compare the IEL counts by H&E and by CD3 IHC in pediatric duodenal biopsies during routine diagnostic practice and to evaluate the interobserver variability between both methods.

Materials and methods

Tissue and patient data

This was a retrospective study conducted on 64 cases of pediatric endoscopic duodenal biopsies. Cases were obtained from the archives of the Pathology Laboratory of Ain-Shams University Hospital. These were all pediatric endoscopic duodenal biopsies received and diagnosed during the period from January 2017 to December 2018. Cases were obtained via endoscopic biopsy. The histopathology reports were reviewed to determine pertinent data including age, sex, clinical presentations, endoscopic findings, and results of serological tests if present. Hematoxylin and eosin-stained slides were examined to re-evaluate and verify the histopathologic diagnosis. Only cases with enough tissue were selected in the analysis.

Ethics statement

All patients who participated in this study signed a written, informed consent before performing the endoscopic biopsy. The study was approved by the Research Ethical Committee at Faculty of Medicine, Ain Shams University.

Immunohistochemical staining

Four-micrometer sections of formalin-fixed and paraffin-embedded samples of duodenal endoscopic biopsies were prepared. Immunohistochemical staining was performed using primary antibody: mouse monoclonal anti-CD3 (Clone: PS1, catalog number: NCL-CD3-PS1, Novocastra Antibodies, 1 : 200 dilution; Leica Biosystems Division of Leica Microsystems Inc., Buffalo Grove, Illinois, USA). Avidin–biotin–immunoperoxidase complex technique was used according to Hsu *et al.* [10] by applying the supersensitive detection kit (Biogenex, Fremont, California, USA). The prepared tissue sections were fixed on poly-l-lysine-coated slides overnight at 37°C. They were deparaffinized and rehydrated through graded alcohol series. Then the sections were heated in a microwave oven in 10 mmol/l citrate buffer (pH: 6.0) for 20 min. After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (DakoCytomation, Glostrup, Denmark) for 20 min, the sections were incubated overnight at 4°C with primary antibodies. Biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were then added. Finally, 3,3'-diaminobenzidine as the substrate or chromogen was used to form an insoluble brown product. Finally, the sections were counterstained with hematoxylin and mounted. Sections of tonsils were used as a positive control for CD3. Negative control sections were incubated with normal mouse serum instead of the primary antibody.

Interpretation of hematoxylin and eosin and immunohistochemical staining

Slides were examined by two pathologists (the authors) without prior knowledge of the clinicopathological data. At first, an IEL-rich area was chosen and marked on the H&E slide, and then its corresponding area was marked on its paired CD3 slide. The slides were examined in a random sequence (i.e. paired slides were not examined after each other), and IELs were counted. Membranous and cytoplasmic staining of CD3 in IELs was regarded as positive staining.

The cutoff values for IEL counts, by H&E and CD3 IHC (immunohistochemical) evaluation, were divided into three groups: normal (<25 per 100 enterocytes), mildly raised (25–40 per 100 enterocytes), and markedly raised (>40 per 100 enterocytes) [5].

Statistical analysis

Quantitative data were tested for normality with Shapiro–Wilk test and described as mean and SD. Categorical variables are expressed as frequencies and percentages. Paired *t*-test was used to compare between H&E and IHC. A significance level of P less than 0.05 was used in all tests. κ statistic was used to test agreement between H&E and IHC. κ values less than 0 indicated as no agreement, 0–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1 as almost perfect agreement. All statistical procedures were carried out using SPSS version 19 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

The 64 cases comprised 28 girls and 36 boys, with age range between 8 months up to 16 years. There were

three groups of cases; group 1 included 10 cases with established diagnosis of coeliac disease by a previous endoscopic biopsy, group 2 included 13 cases that were newly received as suspicious of coeliac accompanied with raised anti-tissue transglutaminase antibodies (αTTG) with or without the histological finding of villous atrophy, and group 3 included the remaining 41 cases that had either normal or unknown αTTG level.

The overall mean IEL count per 100 enterocytes for H&E was 21.86 [95% confidence interval (CI)= 18.34–25.38], and for IHC by CD3, it was 26.19 (95%CI=22.52–29.85). The difference was highly significant (P=0.001) (Table 1), with highly significant substantial agreement between H&E and CD3 IHC results (P=0.001; κ=0.694) (Table 2).

Table 1 Comparison between overall mean intraepithelial lymphocytic counts by hematoxylin and eosin and immunohistochemistry among all cases and within each group

	Mean	95%CI	SD	*P*	Significance
All groups					
Mean H&E	21.86	18.34–25.38	14.08	0.001	HS
Mean IHC	26.19	22.52–29.85	14.68		
Group 1					
Mean H&E	24.10	16.48–31.72	10.65	0.001	HS
Mean IHC	26.60	18.44–34.76	11.40		
Group 2					
Mean H&E	35.15	29.81–40.5	8.84	0.001	HS
Mean IHC	38.69	33.44–43.95	8.69		
Group 3					
Mean H&E	17.10	12.8–21.35	13.47	0.001	HS
Mean IHC	22.12	17.44–26.8	14.82		

H&E, hematoxylin and eosin; HS, highly significant; IHC, immunohistochemistry.

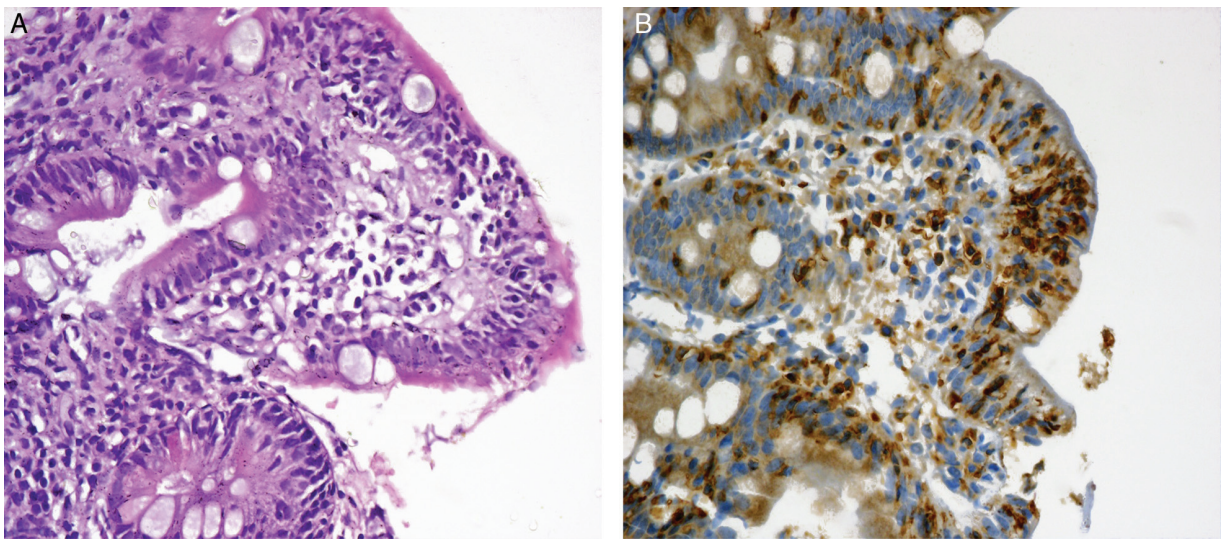
When the overall mean IEL count per 100 enterocytes was calculated as per groups of cases, there was a highly significant perfect agreement between H&E and CD3 IHC results in group 1 of established coeliac disease cases (P=0.001; κ=1) (Fig. 1a and b). There was a highly significant substantial agreement between H&E and CD3 IHC in group 2 cases of newly received cases suspicious of coeliac with or without the histological finding of villous atrophy but with high αTTG (P=0.001; κ=0.683). There was a highly significant moderate agreement between H&E and CD3 IHC results in group 3 cases where αTTG was normal or unknown (P=0.001; κ=0.482) (Table 2).

Table 2 The agreement between hematoxylin and eosin and CD3 immunohistochemistry in intraepithelial lymphocytic counts among all cases and within each group

IHC	H&E [*n* (%)]			κ	*P*
	0	1	2		
All groups					
0	29 (78.4)	0	0	0.694	0.001 (HS)
1	8 (21.6)	14 (77.8)	0		
2	0	4 (22.2)	9 (100.0)		
Group 1					
0	4 (100.0)	0	0	1.000	0.001 (HS)
1	0	5 (100.0)	0		
2	0	0	1 (100.0)		
Group 2					
1	7 (77.8)	0		0.683	0.001 (HS)
2	2 (22.2)	4 (100.0)			
Group 3					
0	25 (75.8)	0	0	0.482	0.001 (HS)
1	8 (24.2)	2 (50.0)	0		
2	0	2 (50.0)	4 (100.0)		

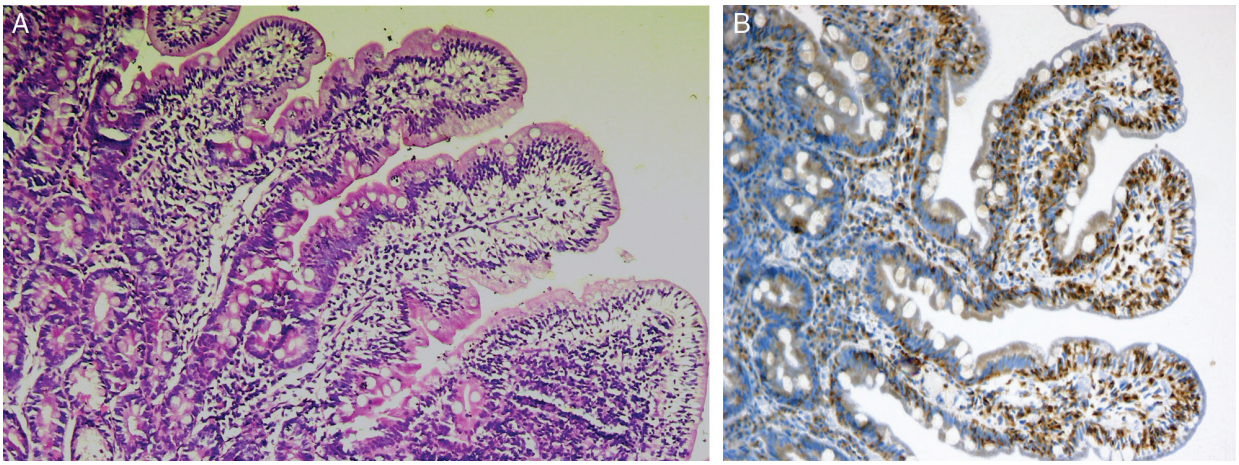
0=normal, 1=mildly raised, 2=markedly raised. H&E, hematoxylin and eosin; HS, highly significant; IHC, immunohistochemistry.

Figure 1



Paired sections from a case in group 1 of markedly raised intraepithelial lymphocytic count. (a) Hematoxylin and eosin, ×400; (b) CD3 immunohistochemistry, ×400 (original figure).

Figure 2



Paired sections from a case in group 3 that was originally assigned as having normal intraepithelial lymphocytic count by hematoxylin and eosin but was assigned as having a markedly raised count by CD3 immunohistochemistry. (a) Hematoxylin and eosin, ×200; (b) CD3 immunohistochemistry, ×200 (original figure).

Table 3 The agreement between the two readers (interobserver agreement) regarding hematoxylin and eosin among all cases and within each group

H&E reader 2	H&E reader 1 [*n* (%)]			κ	*P*
	0	1	2		
All groups					
0	36 (97.3)	0	0	0.946	0.001 (HS)
1	1 (2.7)	17 (94.4)	0		
2	0	1 (5.6)	9 (100.0)		
Group 1					
0	3 (75.0)	0	0	0.825	0.001 (HS)
1	1 (25.0)	5 (100.0)	0		
2	0	0	1 (100.0)		
Group 2					
1	8 (88.9)	0		0.831	0.001 (HS)
2	1 (11.1)	4 (100.0)			
Group 3					
0	33 (100.0)	0	0	1.000	0.001 (HS)
1	0	4 (100.0)	0		
2	0	0	4 (100.0)		

H&E, hematoxylin and eosin; HS, highly significant; IHC, immunohistochemistry.

The overall IEL counts per 100 enterocytes by H&E and CD3 IHC within each of the 3 groups of the study are summarized in Table 1, where using CD3 IHC did not raise any of the previously diagnosed cases of coeliac disease of group 1 to higher counts, but CD3 did lead to different assessments in two (15.4%) of 13 cases of group 2, raising the cases from mildly raised IEL counts to markedly raised counts. The highest difference in overall mean IEL counts between H&E and CD3 IHC was among group 3 cases, which showed raised IEL counts of 8 (24.2%) of 33 cases from the normal range by H&E to the mildly raised (six cases) and to the markedly raised (two cases) categories (Fig. 2a and b).

The overall interobserver agreement for H&E was almost perfect among cases of all groups, and among groups 1, 2, and 3 (κ=0.946, 0.825, 0.831, and 1, respectively) (Table 3). The overall interobserver agreement for CD3 IHC was almost perfect among cases of all groups, and among groups 1, 2, and 3 (κ=0.975, 1, and 0.956, respectively) (Table 4).

Discussion

Raised counts of IELs have been encountered in many pathological conditions like drug reactions, infections, small bowel bacterial overgrowth, tropical sprue, and a variety of autoimmune disorders, but it is considered as

Table 4 The agreement between the two readers (interobserver agreement) regarding immunohistochemistry among all cases

IHC reader 2	IHC reader 1 [*n* (%)]			κ	*P*
	0	1	2		
All groups					
0	28 (96.6)	0	0	0.975	0.001 (HS)
1	1 (3.4)	22 (100.0)	0		
2	0	0	13 (100.0)		
Group 1					
0	4 (100.0)	0	0	1.000	0.001 (HS)
1	0	5 (100.0)	0		
2	0	0	1 (100.0)		
Group 2					
1	7 (100.0)	0		1.000	0.001 (HS)
2	0	6 (100.0)			
Group 3					
0	24 (96.0)	0	0	0.956	0.001 (HS)
1	1 (4.0)	10 (100.0)	0		
2	0	0	6 (100.0)		

H&E, hematoxylin and eosin; HS, highly significant; IHC, immunohistochemistry.

an important histopathological factor in the diagnosis of coeliac disease [5].

Methods used to assess IELs vary. Some authors recommend taking counts throughout the whole length of the villous per 100 enterocytes, and according to current guidelines, counts more than 25 per 100 enterocytes are abnormal [11,12]. Other studies counted IELs in villous tips [13,14]. Moreover, some authors favor the application of immunohistochemical stains for T lymphocytes even among histologically normal duodenal biopsies in spite of lack of supporting evidence [8,15,16].

In the current study, the overall mean IEL count per 100 enterocytes by H&E was 21.86 (95% CI=18.34–25.38), and by CD3 IHC, it was 26.19 (95%CI=22.52–29.85), which is lower than the counts in Cooper *et al.* [5], which were 35.2 (95% CI=30.0–40.8) by H&E and 49.7 (95% CI=44.1–55.3) by CD3 IHC. This may be attributed to the fact that the intestinal density of IELs increases with age [3]. Hence, we would have lower range of IEL counts because our study is conducted on pediatric age group.

The present study showed a highly significant difference between the overall counts of IELs by H&E and CD3 IHC as well as highly significant agreement between them among all of the cases and within each of the three groups included in the study, with higher counts detected by CD3 IHC, which might favor combining both techniques together for better detection of IEL counts. This goes well with what Balasubramanian *et al.* [9] and Nasseri-Moghaddam *et al.* [17] had suggested that the utilization of CD3 IHC would aid the detection of IELs much easier as some IELs have irregular nuclear outline that might mimic polymorphonuclear cells, and also some IELs may resemble epithelial cells.

Our results showed that compared with H&E staining alone, using CD3 IHC did not raise any of the previously diagnosed cases of coeliac disease of group 1 to higher counts, but CD3 did lead to different assessments in 2 (15.4%) of 13 cases of group 2, raising the cases from mildly raised IEL counts to markedly raised counts. This was in agreement with Cooper *et al.* [5], where none of the previously diagnosed cases of coeliac disease were raised to higher counts by CD3 IHC, whereas one of seven cases newly received as suspicious for coeliac with positive serology were raised to higher IELs counts by CD3 IHC.

The highest difference in overall mean IEL counts between H&E and CD3 IHC in the current study was among group 3 cases, which showed raised IEL counts of 8 (24.2%) of 33 cases from the normal range by H&E to the mildly raised (six cases) and to the markedly raised (two cases) categories. These results go well with the results of Cooper *et al.* [5] whose cases that had normal or unknown αTTG showed raised IEL counts by CD3 such that 4 (22.2%) of 18 cases with normal counts were raised to mildly raised (three cases) or markedly raised (one case) categories. However, this warrants caution to avoid overdiagnosis upon the utilization of CD3 IHC in evaluating IEL counts in absence of clinical suspicion and serological evidence of coeliac disease. Moreover,

the current cutoff value for H&E that is settled for being greater than 25 IEL per 100 enterocytes should not be applied for CD3 IHC which detects higher counts of IELs, and therefore, this necessitates setting a lower cutoff value for CD3 IHC to avoid overdiagnosis, as stated by Cooper *et al.* [5] and Balasubramanian *et al.* [9].

The current study showed that the highest agreement between H&E and CD3 IHC was among groups 1 and 2, where κ=1 for each group, which might suggest a potential role for CD3 IHC in the follow-up of cases with a previous established diagnosis of coeliac disease, as well as in cases clinically suspicious of coeliac with positive serology with or without the histological finding of villous atrophy (early lesions). This was partly in agreement with Mubarak *et al.* [6] who stated that to identify all early lesions of coeliac disease (with raised IEL count), which are not explained by other conditions, and at the same time not overdiagnose any of them, CD3 staining should be performed when there is a discrepancy between serology and histology.

The present study also analyzed the interobserver agreement between the two pathologists (the authors) and found a highly significant almost perfect agreement in both the H&E and CD3 IHC staining, but the κ value was slightly higher for CD3 IHC than that of H&E. This was unlike Cooper *et al.* [5] who only had moderate interobserver agreement. This discrepancy could be attributed to the fact that in the study conducted by Cooper *et al.* [5], six observers were included, unlike our study, which was conducted only by two observers. Other causes for the discrepancy may include different immunohistochemical techniques, sample size, and age group of cases between both studies.

Conclusion

In conclusion, CD3 IHC aids in better evaluation of IELs as suggested by its almost perfect agreement with H&E in the current study. However, its use should only be in the appropriate clinical and serological setting and after setting new cutoff values validated by additional studies.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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