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Original Research Article

Comparison of efficacy of various stains and immunohistochemistry in diagnosis of amoebic colitis

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Abstract
Background: *Amoebic colitis is a common parasitic infection especially in developing countries. It's a devastating disease and should be diagnosed accurately. This study was designed to analyse compare efficacy & reliability of Haematoxylin & Eosin (H&E), Periodic acid schiff's (PAS), Masson trichrome (MT)and immunohistochemical (IHC) marker in diagnosis of amoebic colitis, and to ascertain advantages and disadvantages of each stain used in amoebic colitis.*
Material & Methods: *We studied 30 cases reported as amoebic colitis on H&E and 10 cases of non-specific colitis. The pick-up rate of trophozoites of Entamoeba histolytica in H&E, PAS, MT and IHC was compared.*
Observation & Results: *Out of 30 cases reported as amoebic colitis on H&E, trophozoites were identified in 26 cases of fresh H&E stained slides, in 23 cases with PAS, in 15 cases with MT and 20 out of 30 cases were positive for trophozoites on IHC. Number of trophozoites identified was 5 times on IHC and 2 times on PAS in comparison to H&E. Whereas role of MT was found to be less significant in diagnosing amoebic colitis.*
Conclusion: *IHC was found to be superior in offering accurate diagnosis of amoebic colitis as it is highly efficacious and gives intense brown colour which makes recognition of trophozoites easier. Our study results suggest that by using IHC marker for amoebic colitis, even complicated cases can be diagnosed easily where they are difficult to be identified using other stains. Another advantage of using IHC is that it curtails false positive diagnosis of amoebiasis on H&E.*
Keywords: *Amoebic colitis, Immunohistochemistry, Masson's trichrome, Periodic acid schiff's.*

Introduction

Entamoeba histolytica (E. histolytica) is an enteric anaerobic parasite that causes about 500 million infections with a death rate of over 100,000 worldwide annually. [1]

There are at least eight species of amoebas (*E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni, E. polecki, Iodamoeba butschlii, and Endolimax nana*) which live in the human intestinal lumen.[2,3,4,5,6] These are generally accepted as commensal organisms except *E. histolytica*.[7, 8]

There are studies in literature which have shown the importance of IHC marker for *E. histolytica*

over Haematoxylin & Eosin (H&E) and Periodic acid schiff's (PAS) stain in diagnosing amoebic liver abscess ex vivo but literature search didn't reveal any study in which IHC marker has been used on paraffin sections of amoebic colitis on human tissue.

Material and Methods

Present study was conducted in the Department of Pathology, Histopathology section, University College of Medical Sciences & Guru Teg Bahadur Hospital, Delhi. Histopathology numbers of already diagnosed 30 cases of amoebic colitis and 10 cases of non-specific colitis on H&E between January 2011 and December 2014 were retrieved. Paraffin blocks of representative sections of these cases were retrieved. Four sections of each block were taken and stained with H&E, PAS, Masson trichrome (MT) and Immunohistochemistry (IHC) for *E. histolytica* (Thermo Fisher- *E. histolytica* polyclonal antibody, D42A).Study was conducted after obtaining ethical clearance from the Institutional Ethics Committee.

Procedure for each stain is as follows

H&E: Staining of the processed tissue sections was done according to Bancroft and Gamble. [9] Sections were brought to distilled water, nuclei stained with the alum haematoxylin and rinsed in running tap water. Thereafter, tissuedifferentiated with 0.3% acid alcohol and rinsed in running tap water.Sections stained with eosin for two minutes, dehydrated, cleared and mounted with distyrene, plasticizer and xylene (DPX, sigma-44581).

PAS: Staining was done according to standard protocol.[9] Sections deparaffinized and hydrated to water then oxidized in 0.5% periodic acid solution for 5 minutes and rinsed in distilled water. Schiff's reagent was placed for 15 minutes and then washed in lukewarm tap water for 5 minutes. Counterstaining was done by using alum haematoxylin for 1 minute and washed in tap water for 5 minutes, dehydrated, cleared and mounted with DPX.

Masson trichrome

Slides were stained according to conventional protocol described by Bancroft and Gamble.[9] Sections brought to distilled water, stained in Celestin blue for 5 minutes and rinsed in distilled water. Stained nuclei with alum haematoxylin for 5 minutes and washed in running tap water for 5 minutes then rinsed in distilled water. Thereafter sections were stained with Biebrich scarlet-acid fuchsin stain for 10 minutes and rinsed in distilled water. Sections treated with freshly prepared dodeca-molybdophosphoric acid for 10 minutes. Then stained with light green for 10 minutes. Rinsed excess stain from slide with alcohol and dehydrated, cleared and mounted with DPX.

Labelled Streptavidin biotin (LSAB) method of *E. Histolytica* Immunostaining

Staining was performed on processed tissue sections with some modifications of the standard protocol as described by Bancroft and Gamble. [9] Immunostaining for *E. Histolytica* was done to confirm presence of *E. histolytica* trophozoites. Three-four micron thick sections were taken on lysinated slides, deparaffinized with xylene and rehydrated with graded alcohol and washed with phosphate buffer saline (PBS) at pH 7.2. Slides were transferred to staining dish containing antigen retrieval solution in the microwave retrieval chamber for 15-20 minutes and washed with PBS at pH 7.2. Then endogenous peroxide blocking was done by hydrogen peroxide (4%) for 5-10 minutes. Slides were incubated with the primary antibody against *E. histolytica* for 120 minutes and washed with PBS at pH 7.2 for 10 minutes. Thereafter, slides were incubated with preformed avidin-biotinylated peroxide antibody complex for 20 minutes.

These were then, incubated with diaminobenzidine (DAB) solution for 10 minutes and rinsed with PBS at pH 7.2 and transferred to running tap water. Counterstaining was done by haematoxylin followed by rehydration and clearing. Mounting was done by using DPX.

Trophozoites of *E. Histolytica* were counted in 10 high power fields (HPF) in the same area of the

section in each stain. Efficacy of stains in terms of median number of trophozoites recognized per 10HPF were counted and compared.

Observation& Results

On H&E, trophozoites were recognized as round to oval structures ranging in size from 20-90µ (of size of small lymphocyte) having pyknotic nuclei, abundant vacuolated eosinophilic cytoplasm & variable number of ingested intact and/or fragmented red blood cells in the cytoplasm. Organisms were present in the necrotic background with presence of inflammatory cells including neutrophils, lymphocytes, macrophages & few plasma cells. Trophozoites were found, not only at the ulcer edges but also in the entire thickness of intestinal wall. (Fig 1A) Flask shaped ulcers were found in six out of 30 cases and three cases showed lymph-vascular involvement.

On PAS, trophozoites were identified as round to ovoid bodies with magenta colour cytoplasm. (Fig 1B)

On MT, trophozoites appeared as round to oval structures with green colour cytoplasm, black dot like nuclei and ingested RBCs within cytoplasm in green necrotic background. (Fig 1C)

Immunostaining for *E. Histolytica* was done and trophozoites appeared as intense brown coloured round to oval to fragmented structures in a pale blue background. (Fig 1D)

In some cases amoebic trophozoites were difficult to identify. Large macrophages, fragmented muscles and small capillaries many a times simulated amoebic trophozoites on H&E sections. (Fig 2A & B)

H&E, PAS, MT and IHC revealed trophozoites in 26,23,18 and 20 out of 30 cases diagnosed as amoebic colitis on routine H&E stain. Six cases reported as amoebic colitis on revised H&E stain, turned out negative for *E. histolytica* on IHC. None of the non-specific colitis cases (10) was positive for IHC. Trophozoite count was highest per 10 HPF on IHC as it was 5 times of H&E and 3 times of PAS stain. MT showed quite less significant results in comparison to other stains used and IHCfor *E. histolytica*.

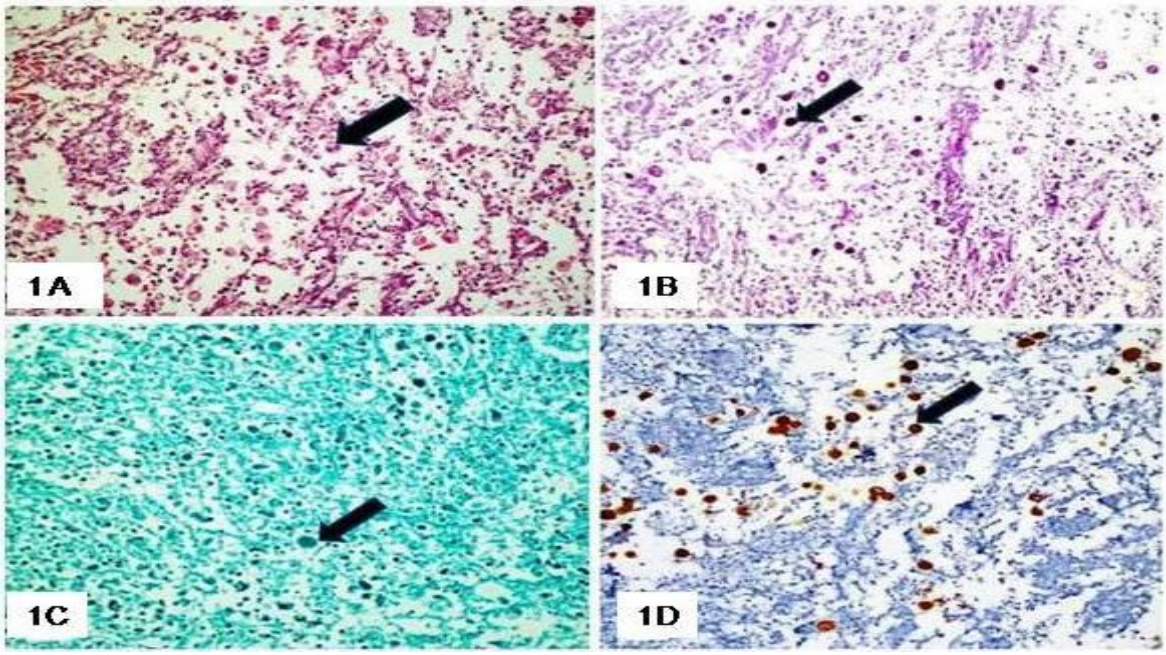


Fig 1 Section of colon A; shows pink coloured round to oval amoebic trophozoites lying in a necrotic background (H&EX200, original magnification) B; shows trophozoites stained magenta in colour (PASX200, original magnification) C; shows round to oval trophozoites having green coloured cytoplasm in necrotic background (MTX200, original magnification) D; shows intense brown colour stained trophozoites of *E. histolytica*, present in well contrast background. (*E. histolytica* antibody IHCX200, original magnification).

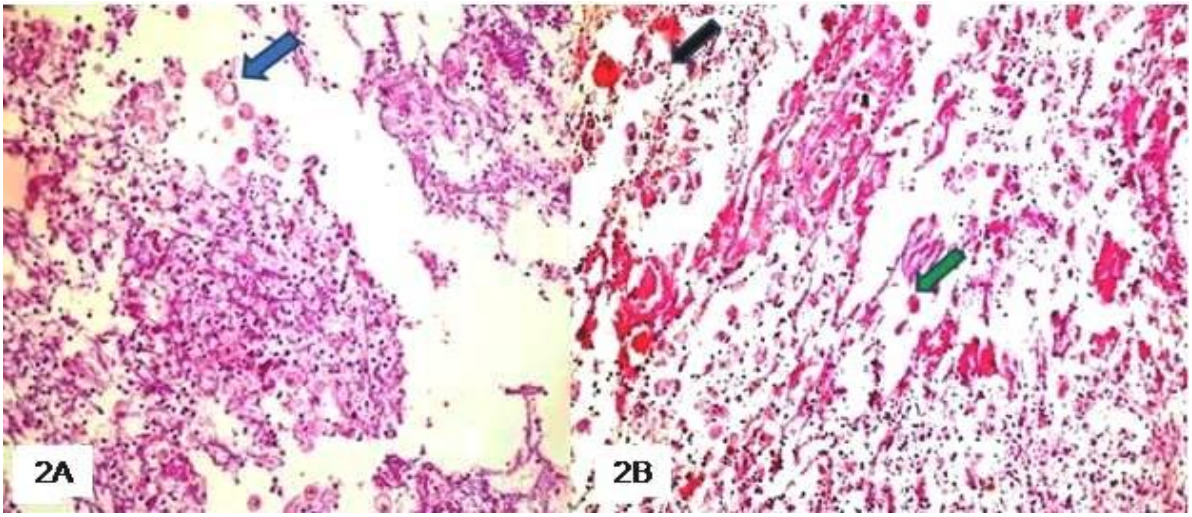
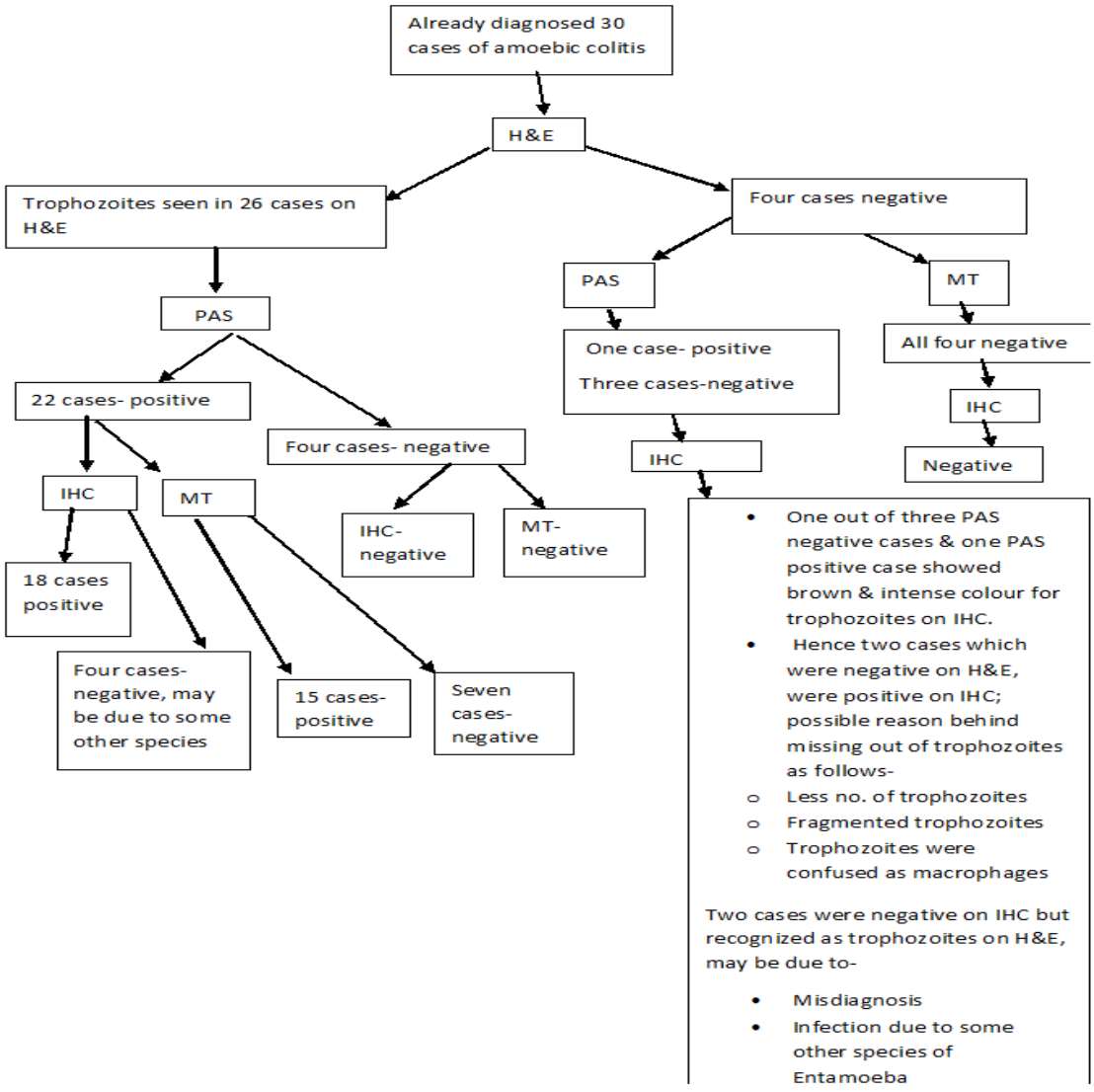


Fig 2 A; shows small capillaries filled with 2-3 RBCs mimicking trophozoites of *E. histolytica* (H&EX200, original magnification)B; round to oval shaped muscle fragments simulating as trophozoites. (H&EX200, original magnification)

Chart: Flow chart shows the comparison of different stains and IHC in identifying trophozoites of *E. histolytica* based on present study result.



Discussion

Among parasitic diseases, amoebic infection is the third most common cause of death after malaria and schistosomiasis.[10] Amoebiasis occurs worldwide; but its prevalence is disproportionately high in developing countries because of poor socioeconomic status.

In some areas the overall prevalence of amoebic infection is as high as 50 percent.[11] Amoebiasis is prevalent throughout India and according to Mukharjii *et al*, prevalence rate is 3.6% in India.[12] Jain *et al* and Singla *et al* have found that intestinal perforation by amoebic colitis remains a common and an important cause for emergency colectomy.[13,14]

Many serological techniques have been used for detection of amoebic infection so far. [15, 16,17, 18,19] But they are not much informative as antibodies remain in blood for longer period even after recovery.Hence, routinely H&E and PAS stain are used to confirm presence of amoebic trophozoites.MT stain has also been used as a special stain in identification of amoebas in some studies.[20]

In present study, we have done MT and IHC for *E. histolytica* in all the cases, apart from H&E and PAS. Where H&E stained slides revealed trophozoites in 26 cases. Four cases didn't show any trophozoite on revised H&E. This denotes wrong diagnosis of these cases which could be due to confusion between macrophages and trophozoites. PAS was positive in 23 cases and negative in seven cases. Trophozoites of *E. histolytica* stained magenta in colour on PAS. PAS stains the glycogen containing structures present within the cytoplasm of the cells therefore sometimes macrophages also took the stain. Hence in view of the similarities in morphology and size, it often becomes difficult to differentiate trophozoites from macrophages in PAS stain and create dubious situation in making diagnosis.[11] Seven cases negative on PAS could be explained due to less number of trophozoites, or fragmented trophozoites or cases wrongly diagnosed on H&E stain.

On MT stain trophozoites were identified in only 15 cases. It was quite difficult to differentiate between trophozoites and macrophages until former had ingested RBCs. Also there was less colour contrast between trophozoites and macrophages. These findings suggest that MT is not much helpful over and above the H&E. In a study done by Gilmar *et al*, PAS stain was found to be more sensitive than H&E, Mallory trichrome and Phosphotungstic acid-haematoxylin stain in picking up amoebic trophozoites.[15] In the present study also, PAS stain turned out to be superior to H&E and MT as the number of trophozoites per high power field was high on PAS stain in comparison to these two stains.

Literature search revealed that although IHC has been used for recognization of amoebic trophozoites but most of them are experimental studies.[11] No study has been found in literature in which IHC has been used for diagnosing amoebic colitis in human tissue.Ning *et al* in their study on induced amoebic liver abscess found that IHC is best in making diagnosis of amoebic liver abscess over routinely used stains.

In our study IHC was positive in 20 out of 30 cases of amoebic colitis while 10 cases were negative. It could be due to ulcer caused by some other strains of amoeba besides *E. histolytica* as IHC marker was specific for *E. histolytica* or cases were misdiagnosed on H&E. Identification of trophozoite was easy on IHC because of its intense brown colour against well contrast background. Therefore number of trophozoites per 10HPF was also high on IHC, approximately 5 times of H&E. (see the chart)

Conclusion

H&E remains a good routine stain for diagnosing amoebic colitis in view of characteristic flask shaped ulcer and background enzymatic necrosis. Amongst the special stains PAS is a good supplementary stain whereas MT is much less sensitive for diagnosing amoebiasis. IHC proved to be a sensitive marker in recognization of trophozoites in cases where their number was less.

IHC was found to be superior especially in those cases where trophozoites were confused with macrophages or in cases where fragmented trophozoites were seen. Thus IHC for *E. histolytica* can be taken as gold standard for diagnosing amoebic colitis. Strains like *E. Dispar* and E. *Moshkovskii* have not been tested on tissue sections by IHC so far. Negative staining for IHC in 10 cases in our study calls for the immunostaining for these strains**.**

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