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CASE STUDY

Colitis caused by *Entamoeba histolytica* identified by real-time-PCR and fluorescence *in situ* hybridization from formalin-fixed, paraffin-embedded tissue

ANDREAS MÜLLER[1], HAGEN FRICKMANN[2,3*], EGBERT TANNICH[4], SVEN POPPERT[4†] and RALF MATTHIAS HAGEN[5†]

[1] Department of Tropical Medicine, Missioklinik, 97074 Würzburg, Germany

[2] Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, 20359 Hamburg, Germany

[3] Department of Medical Microbiology, Virology and Hygiene, University Medicine Rostock, 18057 Rostock, Germany

[4] Bernhard Nocht Institute for Tropical Medicine, 20359 Hamburg, Germany

[5] Department of Microbiology and Hospital Hygiene, Bundeswehr Central Hospital Koblenz, 56070 Koblenz, Germany

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ABSTRACT

Intestinal amoebiasis in a 35-year-old German patient with a 3 weeks travel history in Indonesia was initially misidentified as non-steroidal anti-inflammatory-drug associated colitis in colonoscopy and histopathological analysis. Furthermore, initial stool examination by microscopy and *Entamoeba* faecal antigen ELISA did not reveal any protozoan infection. When cessation of non-steroidal anti-inflammatory drug (NSAID) use and mesalazine treatment did not lead to clinical improvement, the patient presented to a specialist for tropical diseases. An intensive reinvestigation including a workup of formalin-fixed, paraffin-embedded colonic biopsies by molecular analysis with real-time PCR and fluorescence *in situ* hybridization (FISH) proofed the diagnosis of *Entamoeba histolytica* colitis. Molecular methods including real-time PCR and FISH for the diagnosis of amoebiasis from histopathological samples are rarely used for the diagnosis of *E. histolytica* infections. Bloody diarrhoea vanished after the onset of metronidazole treatment. In conclusion, the here-presented case demonstrates how modern molecular diagnostics may help to diagnose *E. histolytica*-associated colitis, even from difficult specimens like paraffin-embedded, formalin-fixed tissue.

KEYWORDS

Entamoeba histolytica, colitis, PCR, fluorescence *in situ* hybridization, formalin, paraffin

INTRODUCTION

Entamoeba histolytica is a pathogenic protozoan parasite which transmission on the faecal-oral route is associated with restricted hygiene conditions. Clinical manifestations comprise both ulcerative colitis and hepatic abscess formation, the latter being preferentially but not exclusively associated with male sex. Amoebic colitis may present with abdominal pain and severe, bloody "raspberry jelly-like" diarrhoea, amoebic liver abscess with a feeling of being severely ill, fever, leucocytosis and pain in the upper abdomen [1]. In case of amoebic colitis, amoebic cysts are shed in the patients' stool, however, cyst morphology does not allow

†Sven Poppert and Ralf Matthias Hagen equally contributed to this work.

*Corresponding author. Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany.
E-mail: Frickmann@bnitm.de

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a reliable discrimination of pathogenic *E. histolytica* from non-pathogenic, colonizing species like *Entamoeba dispar*. Microscopy is only conclusive if trophozoites with phagocytosed erythrocytes are seen in very fresh diarrhoea samples or if tissue invasion is histologically proven in tissue biopsies. If only cysts are seen, discrimination of *E. histolytica* from non-pathogenic species can be performed either by PCR or antigen testing [2]. While diagnostic accuracy of modern real-time PCR for the detection of *E. histolytica* in stool samples allows its application as a first-line diagnostic approach [3], antigen testing should only supplement prior microscopic assessments in order to achieve a sufficient pre-test probability in spite of known specificity problems of antigen tests for *E. histolytica* which limit their use for screening purposes [2]. In some cases of amoebic abscess of the liver, the parasites are no longer detectable in stool. In such instances of invasive infections, serology can help to set up the correct diagnosis [2].

Colitis caused by *E. histolytica* may present with non-specific symptoms as well as with non-specific or even misleading endoscopic or histopathological findings [4–6]. Furthermore, the disease is rare in Western industrialized countries, while it is still occasionally imported from tropical sites of endemicity [7]. Therefore, it is likely to be overlooked or, even more dangerous, confused with immunological diseases like the chronic inflammatory diseases (CID) Crohn's disease or ulcerative colitis [6, 8–20] or even Behçet's disease [21] and carcinoma [22] requiring completely different therapeutic approaches. Enteric tuberculosis, pseudomembranous colitis and even proctitis-associated sexually transmitted infections have been described as potential infectious differential diagnoses as well [23–25]. Further, *E. histolytica*-induced colitis can severely aggravate pre-existing chronic inflammatory disease of the gut [26–36].

Modern molecular diagnostic methods can help to prevent medical malpractice due to a missed diagnosis of *E. histolytica*-associated colitis, if they are early performed to confirm or exclude the suspicion of amoebiasis. To the authors' best knowledge, we describe the first case of an *E. histolytica* associated enterocolitis, which was diagnosed from formalin-fixed, paraffin-embedded tissue by the combined use of real-time PCR and fluorescence *in situ* hybridization (FISH), although the general feasibility of this diagnostic approach has been described already 10 years ago [37].

CASE REPORT

A 35-year-old German male patient initially presented to a specialist for internal medicine with complaints of diarrhoea mixed with blood and mucous for 3 weeks. The diarrhoea had already started during the last week of a 3-week trip through Indonesia and became blood-stained after his return to Germany. Fever, severe abdominal pain or nausea were denied. No pre-existing chronic medical diatheses were known; however, the patient had used the non-steroidal anti-inflammatory drug (NSAID) ibuprofen frequently for the past one year due to recurrent pain after luxation of the right shoulder joint. At the time of initial presentation, a stool examination by microscopy and faecal antigen ELISA for *Entamoeba* did not reveal any parasites, a full blood count and serum C-reactive protein (CRP) were unremarkable.

Colonoscopy demonstrated multiple ulcerations, predominantly in the coecum and ascending colon on a red and hyper-vulnerable mucosa. Several diverticula were observed in the sigmoid colon as well as a few mucosal ulcerations in the rectum. Ulcerative colitis and recto-proctitis in association with NSAID abuse were suspected based on the macroscopic findings with a differential diagnosis of infection-associated colitis. Initial histopathological analysis of several biopsies taken from the coecum, ascending colon and rectum showed a colonic mucosa with irregular hyperplastic crypts, focal hyper-regeneration and proliferation of goblet cells. In summary, ulcerations in the florid stage with associated non-specific inflammation were diagnosed particularly in the coecum and ascending colon. So, the histopathological findings were considered as compatible with clinically suspected NSAID-associated colitis. The patient was asked to stop NSAID use and received oral mesalazine treatment.

However, strict abstinence from NSAIDs associated with oral application of mesalazine did not lead to clinical improvement. Therefore, the patient presented to a specialist for tropical diseases 2 months later. He still complained of an imperative urge to defecate, associated with diarrhoea mixed with blood and mucous. Physical examination was unremarkable except of a slight tenderness on palpation in the left lower abdomen. Re-evaluation consisted of stool analysis for pathogenic bacteria including *Clostridioides difficile* toxin and parasites, level of faecal PMN (polymorpho-nuclear) elastase, serum antibodies for *E. histolytica* and IgE level. Due to his considerable complaints, the patient received an empirical antibiotic treatment consisting of metronidazole 500 mg t.i.d. (ter in die = three times a day) and ciprofloxacin 500 mg b.i.d. (bis in die = twice a day) before results of specific diagnostic tests were available.

Routine laboratory parameters including full as well as differential blood count, erythrocyte sedimentation rate (ESR), serum total protein and electrophoresis were within the normal range. Serum transaminase levels were only slightly elevated (ASAT 30 U/l with a normal range (NR) of 0–30 U/l, ALAT 65 U/l with NR of 0–50 U/l). The IgE-level was slightly elevated (49 kU/l with NR < 20 kU/l). No pathogenic bacteria were detected applying stool culture for *Salmonella*, *Shigella*, *Yersinia* or *Campylobacter*. An assay for *C. difficile* toxin remained negative. A microscopical stool analysis for cysts and ova was performed on the day of the patient's first presentation to the tropical medicine outpatient department. Scanty *Entamoeba coli* cysts were detected but no definite pathogenic organisms. A real-time multiplex PCR for *Giardia duodenalis*, *E. histolytica* and *Cryptosporidium parvum* from the same faecal sample remained negative. The level of the faecal PMN elastase was markedly elevated (1,382 ng g^{-1} faeces, NR < 60 ng g^{-1})

indicating an inflammatory process in the colon or distal parts of the ileum. Serological tests for antibodies against *E. histolytica* were performed using an in-house immunofluorescence test (IFT), indirect haemagglutination (IHA) and an enzyme immuno assay (EIA). In detail, IFT was 1:128 (positive >1:64), IHA was 1:256 (positive >1:64), and 100 antibody units were measured in EIA (positive >14).

The pathologist was contacted and asked to reassess the biopsy samples obtained two months ago for the presence of amoebae. As the assessment may be quite difficult using routine stains, the formalin-fixed and paraffin-embedded biopsies were sent to the Bernhard-Nocht Institute for Tropical Medicine in Hamburg (Germany) for further analysis.

Paraffin was removed and DNA was extracted from thick tissue slices as previously described [37, 38]. Afterwards, real-time multiplex PCR for *E. histolytica* and *E. dispar* was performed [2, 37, 39]. Positive reactions for *E. histolytica* were observed in two tissue samples at cycle thresholds Ct 27 and Ct 32, respectively. The in-house PCR protocol was run on a RotorGene 6000 real-time cycler (Qiagen, Hilden, Germany) based on primers and probes published by Verweij and colleagues [39] as well as by Kebede and colleagues [40], targeting a 308-base-pair fragment occurring in 200-copies per parasite on an episomal plasmid [41]. A 98.4% sequence homology between *E. histolytica* and *E. dispar* [42] allowed a discrimination based on species-specific probes. In detail, the applied primers were Ehd-239F 5′-ATTGTCGTGGCATCCTAACTCA-3′, Ehd-88R 5′-GCG GACGGCTCATTATAACA-3′, the applied probes as well as quencher-reporter-compositions were histolytica-96T 5′-JOE-TCATTGAATGAATTGGCCATTT-BHQ1-3′ for *E. histolytica* and dispar-96T 5′-Cy5-TTACTTACATAA ATTGGCCACTTTG-BHQ2-3′ for *E. dispar*. The PCR reactions were run in 25 µL volumes comprising a reaction mix as follows: 12.5 µl HotStarTaq master-mix (Qiagen, Hilden, Germany), 2.5 µl DNA template, a final concentration of 5 mM $MgCl_2$ in the reaction tube, a final concentration per primer in the PCR reaction of 125 nM, and a final concentration per probe in the PCR reaction of 175 nM. The final volume was added to 25 µL using PCR-grade water. The cycle conditions comprised an initial denaturation step at 95 °C for 15 min. For the first 9 cycles of the 45 cycles of the PCR run, a touchdown program decreased the temperature from 64 °C to 60 °C in 0.5 °C steps for the annealing step. Each cycle consisted of denaturation for 15 s at 95 °C, annealing applying the temperature profile as described above for 30 s and elongation at 72 °C for 30 s. The minimum detectable genomic equivalent as titrated with positive control plasmids is in the 10^3 copy range as described elsewhere [43]. Quality control comprised positive controls based on DNA of the *E. histolytica* strain HM-1:IMSS (ATCC 30459) and the *E. dispar* strain HX-2:CDC (ATCC 30931) as well as a PCR-grade water-based negative control of the extraction and amplification process. To control the effectiveness of extraction and amplification, an internal control PCR based on a 89-base-pair fragment of the phocid herpesvirus 1 *gB* gene as described elsewhere [44] within a plasmid was included in the PCR reaction. The plasmid was titrated to achieve cycle threshold values of 25, the applied oligonucleotides comprised the forward primer PhHV-267s 5′-GGGCGAATCACAGATTGAATC-3′, the reverse primer PhHV-337as 5′-GCGGTTCCAAACGTAC CAA-3′ and the probe PhHV-305tq 5′-Cy55-TTTTTATGT GTCCGCCACCATCTGGATC-BBQ-3′. Based on the authors' experience from previous evaluation studies [3, 43], there is no clear cut-off cycle threshold value for specific versus non-specific amplification. In case of low target DNA concentrations and partial inhibition within the sample, late signals with high cycle threshold values can nevertheless indicate specific amplification in case of typical exponential amplification curves, while negative samples should be free of *Entamoeba*-specific amplification signals. Under standard conditions in non-inhibited samples, however, specific amplification is expected in the cycle threshold value range ≤35.

In addition, fluorescence *in situ* hybridization (FISH) analysis in tissue was performed as described previously [37]. For the assessment, 4–6 µm thick paraffin sections were cut. Deparaffinization was conducted as follows: The slides were washed in 100% xylene twice for ten minutes each, followed by 100% ethanol and 75% ethanol for ten minutes each, and additional ten minutes in 100% methanol. Hybridization was performed using standard conditions at 30% formamide and 46°C that are also suitable for many previously described DNA probes [45–47]. In detail, two *E. histolytica* specific, Cy3-labeled probes EH_18S 193 (5′-Cy3-TTCATTGAAT GAATTGGCC-3′) and EH_18S 840 (5′-Cy3-TCTAGAAA CAATGCTTCTCTAT-3′) were applied in combination. Probe design had been performed using the ARB-software [48, 49] and *in-silico* specificity testing using the "probecheck" software [50]. The probes had been tested with culture isolates of *E. histolytica* and *E. dispar*, where they had shown specificity for *E. histolytica* after adjusting optimal binding conditions at 30% formamide in hybridization buffer (data not shown). A broad *in-vitro* specificity testing against a broad spectrum of other pathogens had not been performed, because *E. histolytica* can easily be identified by micromorphology and the nucleic morphology in DAPI counter-stain in case of a positive FISH reaction. Tissue-counter-staining was performed with the FAM-labelled probe EUK502 (5′-FAM-ACCAGACTTGCCCTCC-3′) with specificity for eukaryotic cells [51]. The detailed staining process after the fixation step in methanol of the above-mentioned deparaffination procedure was as follows. Hybridization with final probe concentrations of 10 pmol μL^{-1} in the hybridization buffer was conducted at 46°C for one hour in a moist chamber in an incubator in the dark. The hybridization buffer comprised 30% formamide, 1 M sodium chloride and 20 mM Tris-HCl. Subsequently, the slides were shortly rinsed with preheated washing buffer with as little exposition to light as possible and incubated for an additional 15 min at 46°C in washing buffer (2 M NaCl, 0.1 M EDTA, 0.4 M TRIS-HCl, 0.2% SDS (sodium dodecyl sulfate)) without exposition to light. The rinsing step has to be performed rapidly to avoid cooling of the pre-heated washing buffer.

After this washing step, the slides were covered with the mounting medium "Vectashield with DAPI" (Vector Laboratories, Burlingame, CA, USA) based on the non-intercalating DNA stain 4′,6-diamidino-2-phenylindole (DAPI). Subsequently, fluorescence was analysed using an upright Leica DM5000B fluorescence microscope (Leica, Wetzlar, Germany) equipped with a Leica DFC 360 FX camera. Images were acquired and processed using the Openlab 5.1 software (Improvision, Coventry, United Kingdom) (Fig. 1).

In addition, presence of *E. histolytica* was confirmed by traditional PAS (Periodic Acid Schiff) staining in histological slides (Fig. 2). *Entamoeba* spp. were associated with an increased number of eosinophilic granulocytes and B-lymphocytes as well as with deposited fibrin fibres. Phagocytosis of erythrocytes by some of the parasites was demonstrated as well, thus confirming the diagnosis *E. histolytica*. Further, amoebae with phagocytosed erythrocytes within the trophozoites could be demonstrated (Fig. 3).

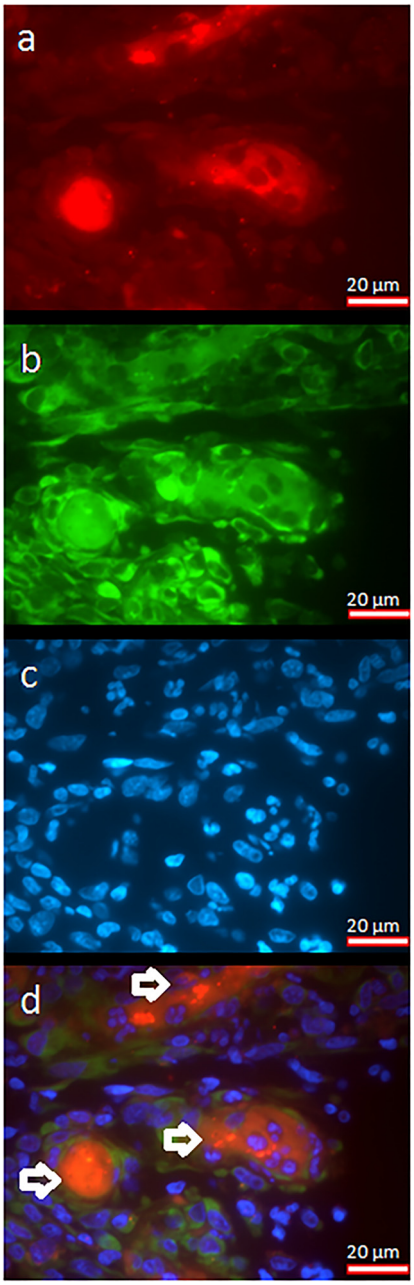

Fig. 1. Demonstration of *Entamoeba histolytica* in tissue by FISH (630× magnification). a) red Cy3 stain = *E. histolytica* specific probes. b) green FAM stain = probe with specificity for eukaryotic cells. c) blue DAPI stain = non-intercalating nucleic stain, d) orange = merged visualization of the amoebae (marked with arrows)

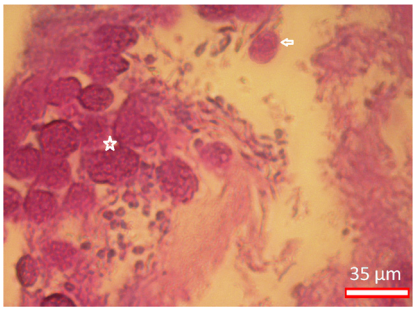

Fig. 2. E. histolytica in enteric tissue in PAS staining. The photograph was taken at 630× magnification. The asterixis indicates a cluster of amoebae, the arrow a single amoeba

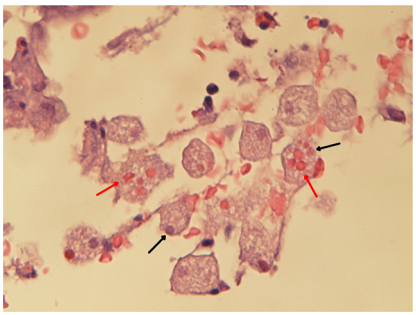

Fig. 3. E. histolytica with phagocytosed erythrocytes. Red arrows: phagocytosed erythrocytes. Black arrows: nuclei of the trophocytes

The bloody diarrhoea stopped at the first day after initiation of a therapy with metronidazole. After confirmation of *E. histolytica*-induced colitis, the metronidazole dose was increased to 500 mg q.i.d. (quarter in die = four times a day) for 10 days and ciprofloxacin was stopped. After metronidazole treatment, paromomycin 500 mg t.i.d. for 10 days was added as luminal amoebicide. The symptoms of the patient resolved completely.

DISCUSSION

E. histolytica, an infectious agent that causes bloody diarrhoea and liver abscess, is world-widely distributed in the tropics [1] but most frequently imported from the Americas and South-Central Asia [7]. Unfortunately, the symptoms are often non-specific [52–61] and can mislead to false diagnoses like chronic inflammatory disease or, as described above, NSAID-associated colitis. Radiographic patterns can considerably vary as well [62, 63]. Misdiagnosis is likely, because *E. histolytica* becomes increasingly rare in stool samples of returnees from the tropics [64], amoebiasis

courses are regularly chronic, and latency is common [1]. The exceptionally high antibody titres against *E. histolytica* found in our case may be explained as a consequence of the 3-month history of illness. Misidentification of *E. histolytica* associated colitis with conventional methods [4, 5] as CID has already been described [6], increasing the risk of severe courses due to anti-inflammatory therapy.

The here-presented case demonstrates how modern molecular diagnostics may help to diagnose *E. histolytica* associated colitis, even from difficult specimens like paraffin-embedded, formalin-fixed tissue. Numerous real-time PCR procedures that specifically identify *E. histolytica* have been described [2, 39, 65–71]. However, application of real-time PCR for the detection of *E. histolytica* from formalin-fixed, paraffin-embedded tissue is feasible [37] but rarely applied. This special sample material is a challenge for PCR procedures not only because of the difficult DNA extraction due to paraffinization, but also because of the formalin-induced cross-linking between the DNA strands and proteins [38]. However, after successful DNA preparation, real-time PCR targeting short sequences can be successfully applied from paraffin-embedded tissue yet after decades as previously shown for leishmaniae [72] and amoebae [37].

It remains unclear why real-time PCR remained negative from the assessed stool sample. Sensitivity of real-time PCR for the detection of *Entamoeba* spp. from stool samples is generally higher than sensitivity of traditional microscopy that further fails to discriminate *E. histolytica* from the closely related but non-pathogenic *E. dispar* [1, 73–76]. Very low parasite density at the time of testing, as also suggested by concomitantly negative microscopy, might have caused the negative results, even though there was no sign of sample inhibition in real-time PCR. It is a well-known phenomenon among microscopists that the excretion of cysts may vary widely from day to day, although systematic assessments of this phenomenon in untreated patients are lacking in international literature for obvious ethical reasons.

Diagnostic *E. histolytica* FISH for paraffin-embedded, formalin-fixed such tissue samples is feasible as demonstrated in a retrospective assessment [37] but rarely applied for diagnostic purposes, though immunofluorescence for the detection of the parasite had been established already decades in the past [77]. PAS staining proofed to be suitable to identify *Entamoeba* spp. within the tissue samples as well in a consecutively performed staining, but requires experience to reliably discriminate parasites from tissue artefacts or to exclude the differential diagnosis of CID [78]. FISH provides an easily detectable contrast to the surrounding tissue that allows the correct identification even to less experienced investigators in no more than two-and-a-half hours including deparaffination. According to the authors' experience, FISH should not be applied to samples that are older than a decade, e.g., for retrospective analyses, as we had failed to get convincingly positive fluorescence signals from 20-year-old specimens showing many *E. histolytica* cysts with phagocyted erythrocytes in concomitant PAS staining of neighbouring slides in a previous assessment [37]. This phenomenon confirms high vulnerability of RNA during the preparation and fixation steps including formamide fixation and paraffin-embedding requiring RNAse free materials which are often unavailable in the routine histopathology laboratory.

After identification of the causative agents, prognosis of *E. histolytica*-associated colitis under therapy is good [79] as this case report demonstrates, even if severe and immunocompromising underlying disease pre-exists [80]. Immunosuppression in combination with overlooked amoebiasis, in contrast, may lead to unfavourable outcomes [81]. Therefore, it is important to consider *E. histolytica* as causative agent in cases of long-lasting, bloody diarrhoea, in particular if suspicion is raised by an indicative history of travelling through tropical countries. Thereby, molecular diagnosis may lead to rapid and reliable diagnosis of *E. histolytica* either alone or in mixed infections [82] and is feasible even from difficult material like paraffin-embedded, formalin-fixed tissue samples.

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