UTTAR PRADESH JOURNAL OF ZOOLOGY

42(6): 1-11, 2021
ISSN: 0256-971X (P)

MODE OF TRANSMISSION AND INTENSITY OF AMOEBIASIS IN *Entamoeba histolytica* INFECTION: A CASE STUDY FROM GAYA DISTRICT OF BIHAR, INDIA BASED ON ANIMAL MODELS

EJAZ JAWAID[1*] AND C. D. P. SINHA[2]

[1]Department of Biotechnology, Gaya College, Gaya, Bihar 823001, India.
[2]Department of Zoology, Magadh University, Bodh Gaya, Bihar 824234, India.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Authors EJ and CDPS conceived the idea of the project. Author EJ conducted experiments. Authors EJ and CDPS analyzed the results and wrote the manuscript. Both authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Sirigireddy Sivajothi, Sri Venkateswara Veterinary University, India.
Reviewers:
(1) Thiago Pajeú Nascimento, Rural Federal University of Pernambuco, Brazil.
(2) Enrique Gonzalez Rivas, UNAM, Mexico.

Received: 14 January 2021
Accepted: 19 March 2021
Published: 26 March 2021

Original Research Article

ABSTRACT

Gaya district, located in the state of Bihar, India, is a hub for Hindu and Buddhist pilgrims. Every year millions of pilgrims visit this city. However, lack of proper sanitation facility in the city creates an unhygienic environment leading to several gastrointestinal diseases. One such disease prevalent in this region is Amoebiasis causes by *Entamoeba histolytica*. Amoebiasis is a significant cause of morbidity and mortality in the city. However, mode of transmission and intensity of this disease in this region has not been studied in detail till date. In this study, we aimed to understand the extent of the spread of amoebiasis in Gaya district and characterized its pathogenicity. We have used methods such as microscopy, staining, sedimentation, culture, and animal models for the identification and characterization of trophozoites and cysts. Our results revealed the presence of *Entamoeba histolytica* infection in both symptomatic and asymptomatic population but the virulence and pathogenicity of strains from symptomatic patients were found to be more severe. Infections could be detected by established protocols of microscopy without any added detection advantage from culture methods. Albino rats and guinea pigs both showed similar virulence and pathogenicity of the strains.

Keywords: Amoebiasis; *Entamoeba histolytica*; gastrointestinal symptoms; pathogenicity.

1. INTRODUCTION

Amoebiasis is an infectious disease, chronic in nature, involving the wall of the intestine. Primarily it is caused by *Entamoeba histolytica, an anaerobic parasite* [1]. Amoebiasis has a wide global distribution, an estimated 10% of the population of the world is infected with *E. histolytica /E.dispar.*

**Corresponding author: Email: ejazjawaid69@gmail.com;*

About 40,000 to 1,10,000 individuals die of invasive amoebiasis annually [2]. Clinical features during infection can range from asymptomatic colonization to amoebic dysentery and invasive extraintestinal amoebiasis leading to abscesses in liver [1]. Although most of the cases of amoebiasis are subclinical; intestinal invasion leading to dysentery, extra intestinal indices like liver abscess, cardiac, pleuropulmonary, cerebral, genitourinary, renal etc. are also observed [1].

E. histolytica measures 15-30 μm in diameter having hyaline ectoplasm abruptly detached from endoplasm, which occupies about one third of the whole trophozoite [3]. Hematoxylin staining discloses a nuclear membrane, wherein inner surface is lined with regular chromatin [4]. The small karyosomes which are intensely stained are located in the center. The circular or oval cysts measure 10-20 μm in diameter. Mature cysts contain four nuclei while immature cysts contain only one to three nuclei [1]

According to Salit E. et al. [5], its intestinal and extraintestinal form is widely prevalent in tropical and subtropical countries and is mainly transmitted by viable cyst which comes from chronic cases or convalescent carriers. The fecal contamination is found to be the primary mode of transmission either through drinking water or food [1,6]. In 90% cases it is subclinical, the reason for the variations in clinical manifestation remains unknown [7].

In certain studies, carried out in India, incidence of amoebiasis has been reported in about 25% of cases [8,9,10]. Unfortunately in Bihar and especially in Gaya district, which covers large geographical areas including both rural and urban population, no serious attempts have been made so far to find out the relationship between socioeconomic conditions and life style with modes of transmission and pathogenicity of *E. histolytica*. In our earlier publication [11] we discussed about the spread of amoebiasis in Gaya district in Bihar. Here, we discuss the pathogenicity of the strains of *E. histolytica* found among wide population of Gaya district by examining 50 samples each from five different regions.

2. MATERIALS AND METHODS

2.1 Sample Selection

Entire Gaya district were divided into five regions i.e. northern, eastern, southern, western and central regions. All these regions have unsuitable dumping sites for feces from domestic, sanitation management is not appropriate, and house-hold waste is not collected regularly.

2.2 Sample Collection

250 samples of stool, 50 from each region were collected aseptically which were then labelled, on the basis of site of collection as s1 to s50 and other details. These samples were immediately moved to the laboratory and analyzed systematically with the help of microscope to confirm the presence of cysts/trophozoites. A total of 206 cases were included in this study, which were divided into the following two groups:

Group -A: This group included 80 stool samples from patients having severe gastrointestinal symptoms and were clinically suspected to be suffering from amoebiasis.

Group -B: This included 126 stool samples from patients not clinically diagnosed as amoebiasis and from healthy persons.

2.3 Analysis of Samples

A thin slide preparation of sample with saline and Dobell's iodine was observed under a light microscope. The trophozoites were studied on fresh dysenteric samples to evade their encystation. Various field lens particularly 40x objective were used before labelling as no cyst or trophozoite was found. Grading of encystation were reported according to [12] as scanty (1-3), few (4-10), moderate (11-20), many (21-40) very much (>40).

2.4 Microscopy

Samples from field were collected in Phenol Alcohol Formaldehyde fixative (P.A.F.) [13]. The specimens were examined within half an hour of reaching the laboratory and were cultivated in both Dobell's "Ehs" and N.I.H. medium. It was then incubated for 48 - 72 h at 37^0C. The cultured colonies were examined for vegetative form and trophozoite forms by formal-ether concentration technique [14] and the cysts of *E. histolytica* were examined by staining with Faust's rapid iron haematoxylin technique [15]. The vegetative form of *E. histolytica* was differentiated from that of *E. coli* and other *Entamoeba* strains based on morphology and nuclear details by wet mount staining technique [16].

2.5 Animal Experiments

The strains of *E. histolytica* were inoculated in albino mice and guinea pig experimental animal models in order to find any variation amongst the strains in their ability to produce lesions. Four weeks old albino mice

of both genders weighing 30-40 gm was used in the study including 12 as controls (Fig. 1A). 56 guineapigs of both genders weighing 250-300 gm was used in the experiment including 6 as controls (Fig. 1B). Total 21 strains of *E. histolytica* used for animal experiments (Albino mice -15 strains & guinea pigs - 6 strains) were from cases of acute amoebic dysentery (6 strains), amoebic colitis (3 strains), chronic amoebiasis (5 strains), patients clinically diagnosed with pain in abdomen (2 strains) and from patients without any history of gastrointestinal symptoms of amoebiasis (5 strains).

The Entamoeba strains were inoculated in the caecum of abdominal cavity under aseptic conditions. The abdomen was dissected and opened 2-3 cm long and the caecum was brought out. Culture fluid containing 300,000 to 350,000 trophozoites was inserted into the caecum. The caecum was then put back to the abdominal cavity and the abdomen was closed latched (Fig. 2).

Control animals were injected with the same amount of culture fluid without any trophozoites. 10 Albino mice and 2 guinea-pigs died either during anaesthesia or during the inoculation process and thus have been excluded from the study.

All the mice were sacrificed after 7-9 days and guineapigs after 10-11 days of inoculation under anaesthesia. The abdomen was opened along the left flank so that the entire abdominal wall could be reflected to the right side for a complete examination of all abdominal viscera.

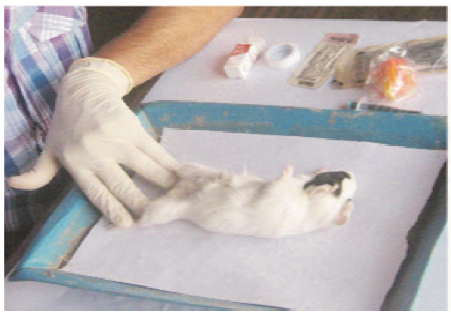

Fig. 1A. Albino mice model

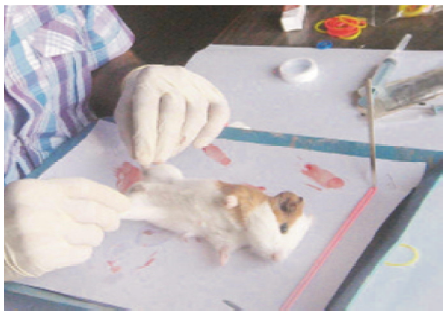

Fig. 1B. Guinea pig model

Fig. 1. *In-vitro* animal models anesthetized for *E. histolytica* mode of transmission and pathogenicity studies

SUTURING

INOCULATION

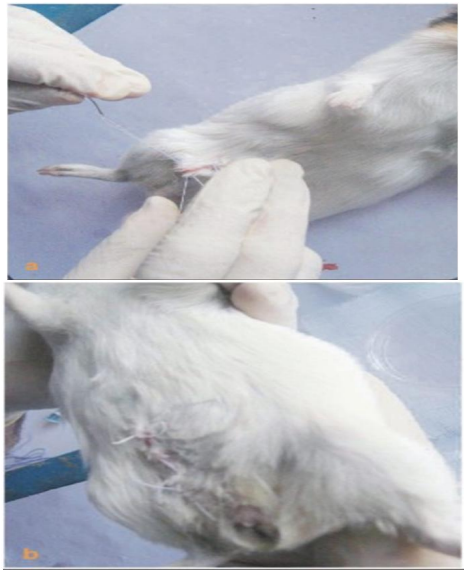

Fig. 2A. (Albino Mice)

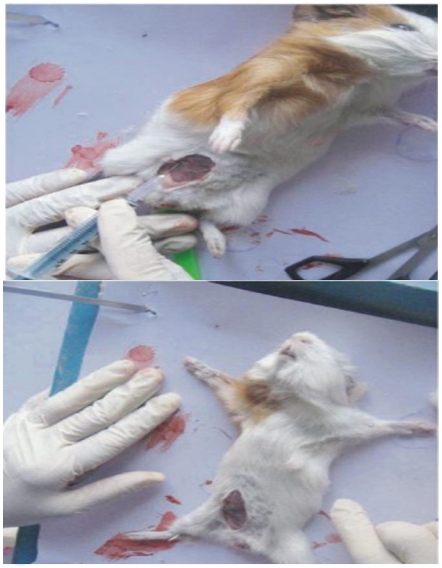

Fig. 2B. (Guinea Pig)

Fig. 2. Inoculation of trophozoites in caecum of albino mice (Fig. 2A) and guinea pig (Fig. 2B) and suturing of abdominal cavity

The caecum was examined first for any thickening, contraction, change in the shape and any other abnormality. It was then opened with scissors to visualize the contents and to examine the interior wall for congestion and ulceration (Fig. 3A). The cecal materials were examined microscopically for the occurrence of amoebae, pus cells or red blood cells and the cecal material was cultured in Dobell's "Ehs" medium (Fig. 3B). The amoebae found were identified on morphological grounds (Fig. 4). The occurrence or absence of amoebae was documented along with other variations seen in the cecal wall and contents (Fig. 5). The "cecal scoring" was done according to Jones [17] and Neal [18]. The condition of wall was scored along with the contents and a mean was taken. The maximum cecal score possible was 4. The strains of *E. histolytica* tested were ranked according to the cecal core.

3. RESULTS AND DISCUSSION

3.1 Direct Microscopy, Formol ether Concentration Technique and Culture Methods

A total of 206 stool specimens were studied for the occurrence of ova, cysts and trophozoites. In the present study 48% stool specimens examined were positive for ova, cysts and vegetative forms of parasites (Table 1). A similar study by Prakash et al. [19] showed that 38.1% of the stool samples were positive for parasite by direct microscopy, formol-ether concentration and culture methods. In our study, 25% stool specimens (Table 2) showed the presence of *E. histolytica*, which is similar to Vinayak et al. [20] who reported 20% stools to be positive for *E. histolytica*, from patients having abdominal complaints. A higher proportion of stool samples from cases of severe gastrointestinal complaints showed the presence of *E. histolytica* than from cases with mild complaints or those having no symptoms of amoebiasis. In cases with severe gastro-intestinal symptoms, *E. histolytica* was detected in 31.2% cases. On the other hand, only 21.2% stool samples from cases with mild or no symptoms or not clinically diagnosed as amoebiasis were positive for E. histolytica (Table 3). These findings are in line with previous reports. Shrivastav [21], Misra et al. [22], Prakash and Tandon [10], and Vinayak [23] reported 35.6%, 30.0%, 18.4% and 20.0% stool samples, respectively, to be positive for *E. histolytica* from cases with severe gastrointestinal trouble.

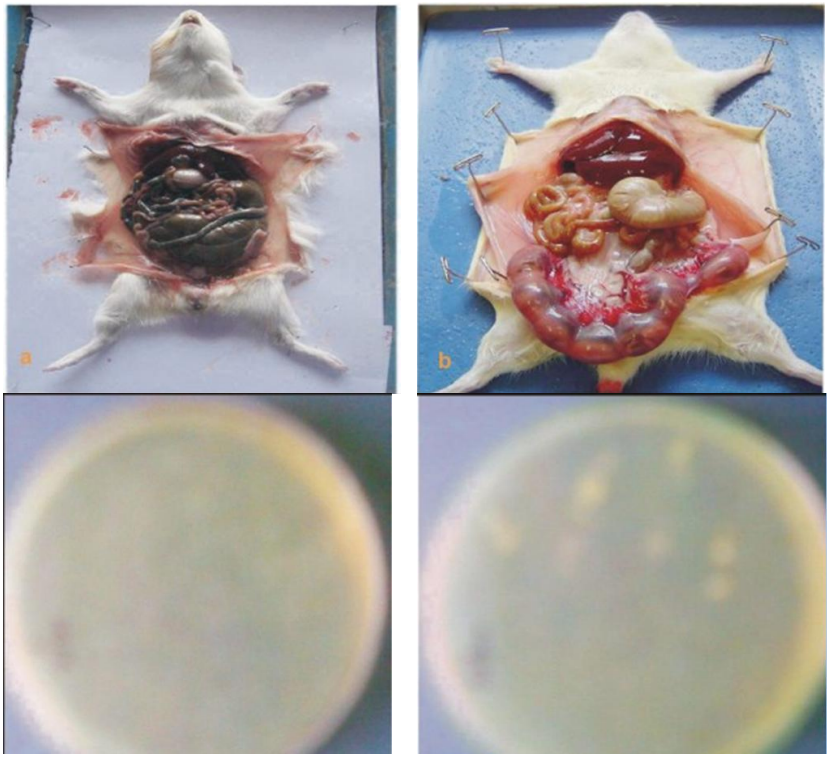

Fig. 3A. Albino Mice **Fig. 3B. Guinea Pig**

Fig. 3. Examination of abdominal viscera for morphological and physiological changes post inoculation of trophozoites (Fig. 3A) and culturing of infected cecal materials (Fig. 3B)

Table 1. Incidence of different types of parasites

	No of cases	Percentage
Number positive for ova, cysts, or trophozoites forms	99	48.05
E. histolytica	52	25.2
E. hartmanni	0	0
E. coli	60	29.10
G. lamblia	39	18.9
I. butschlii	22	10.6
Trichomonas species	18	8.7
E. nana	19	9.2
Chilomastixmesnill	4	1.9
Hookworm	11	5.3
Ascaris lumbricoides	7	3.3
Hymenolepis nana	6	2.9
Enterobius vermicularis	4	1.9
Trichuris trichura	1	0.48

All the samples were studied by direct microscopy, formol ether concentration technique and culture method for *E. histolytica*. Percentage distribution for each method is provided in Table 2. Direct microscopic examination could reveal only 19.4% samples as positive for *E. histolytica*, on the other hand, 24.7% were positive for *E. histolytica* by formol-ether concentration technique. Prakash et al. [24] found an additional detection of 10.6% percent cases for all parasitic infestation by formol-ether concentration technique. Ritche, Pan and Hunter [25] had shown that formalin ether centrifuged sedimentation technique could concentrate parasitic objects in stools and maintain the diagnostic integrity of such objects. Prakash et al. [24] pointed out that there was minimal distortion of cysts by formol-ether concentration technique.

Culture examination of stool for amoebae has gained importance from the diagnostic point of view, as the motility of trophozoites can be clearly seen and also the trophozoites can be stained by wet mount preparation to give the nuclear details. Faust [26] advocated the use of culture method for isolation of *E. histolytica* in obscure cases and in cases of protracted colitis. Several workers [27-29] have demonstrated that cultivation of stool for *E. histolytica* is better than direct microscopic examination of stool. Tandon et al. [10] found an additional 2.9 percent samples to be positive by culture over formol-ether concentration material.

In the present study, out of 52 cases of *E. histolytica*, 40 (19.2%) cases were detected by direct microscopy, 51 (24.7%) by formol-ether concentration technique and 50 (24.2%) cases by culture method. Two cases, which were positive by formol- ether technique, were missed by culture method while one case which was missed by formol-ether concentration was picked up by culture. Culture method in this study was found not to be better compared to formol-ether concentration technique though it yielded a better result than direct microscopy. Thus, direct microscopy along with formol ether concentration appears to be an efficient method of first line detection.

In group A, out of 25 cases of *E. histolytica*, 24 were isolated in Dobell's medium and 23 were isolated in N.I.H. medium. In group B, out of 27 cases of *E. histolytica* infestations, 26 were isolated in each of the two media. As far as culture medium is considered, both Dobell's medium and N.I.H. medium seem to be equally efficient. Maintenance of culture was done in N.I.H. medium as there was no serum in it. Hence, the

Table 2. Incidence of *E. histolytica* as revealed by direct microscopy, formol-ether concentration technique and culture methods

Group	Total number of cases	Total number of positive cases	Number of positive cases by direct microscopy	Number of positive cases by formol ether concentration technique	Number of positive cases by culture methods
A	80	25	21	25	24
B	126	27	19	26	26
Total	206	52	40	51	50
		25.2%	19.4%	24.7%	24.2%

Table 3. Clinical diagnosis of the cases with incidence of isolation of *E. histolytica*

Clinical diagnosis	Number of cases studied	Number of positive cases	Percentage
Group A			
Acute amoebic dysentery	15	3	20
Amoebic colitis	18	7	38.8
Chronic amoebiasis	47	12	25.5
Group B			
Pain in abdomen	45	11	24.4
Apparently healthy persons	52	7	13.4
Clinically diagnosed as peptic ulcer	15	4	26.6
Clinically labelled as Helminthic infestation	14	3	21.4

chances of bacterial contamination were less. Extended subcultures and incubation did not result in any extra-positive case.

Presence of *E. histolytica* was distinguished from other parasites by the presence of sharp finger like pseudopodia and clear distinction of ectoplasm and endoplasm (Fig. 4). About 25-30 % of the trophozoites could be stained in wet preparation by 1% brilliant green in citrate buffer (pH 4.4) in three to four minutes and nuclear differentiation was clear with small centrally placed karyosome.

Prakash [16] performed wet staining of cultures by several dyes and got the best result with the above mentioned procedure. Prakash et al. [19] made a comparative study of permanent staining techniques and found Faust's Iron hematoxylin techniques to be a rapid and good method. Out of a total of 19 slides stained by them for *E.histolytica*, majority showed

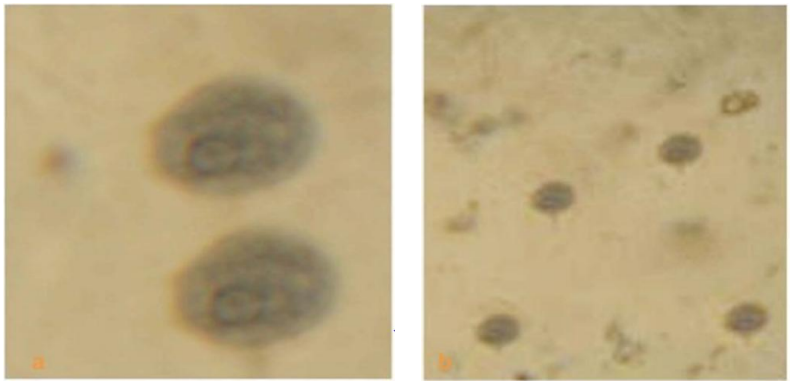

Fig. 4A.

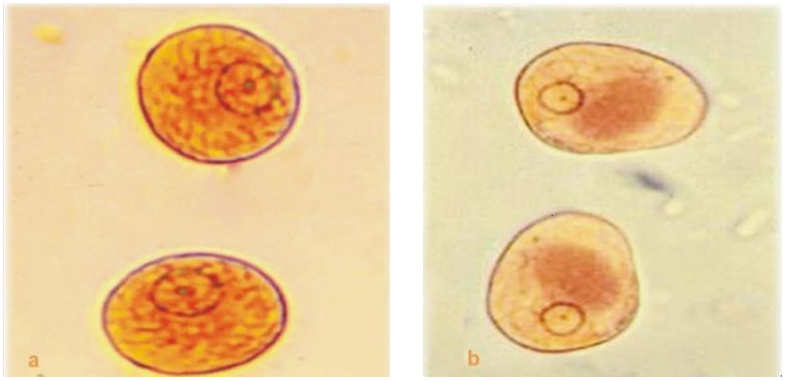

Fig. 4B.

Fig. 4. Microscopic examination of caecal contents cultured post 48 hours of incubation showed presence of amoebae as observed in albino mice (Fig. 4A) and guinea pig (Fig. 4B)

good and excellent nuclear details. In the present study, 32 slides were stained for *E. histolytica* by Faust's Iron hematoxylin. In 4 slides nuclear details were poor, in 10 slides nuclear details were fair, in 10 slides nuclear details were good, and in 8 slides nuclear details were excellent (Table 4), confirming that permanent staining could be a reliable first line diagnostic method to confirm *E. histolytica's* presence.

3.2 Results of Animal Experiment

21 strains of *E. histolytica* isolated from cases of acute amoebic dysentery, amoebic colitis, chronic amoebiasis, pain in abdomen and asymptomatic cyst passers, successfully infected 62.5-100 % of albino mice and guinea pigs Stewart and Jones [30] published their discoveries on infectivity and virulence of diverse strains of *E. histolytica* and reported them to be variable. These authors found that one strain contributed virulent infection and diseased over 70% of rats whereas, the infectivity of three other strains was associated to their virulence for the tissues. Neal [18] displayed that virulence of diverse strains were sovereign of infectivity. The current work confirmed the findings of Neal [18] in general, except that the strains from asymptomatic cyst passers produced lesser degree of infectivity.

In the present study 128 albino rats were inoculated with 15 strains of *E. histolytica* isolated from diverse cases. The results revealed the existence of strains of *E. histolytica*, which significantly vary in their virulence to these animals. The lesions, if present, were always reported in the caecum, sporadically colon was also invaded. The degree of participation of the caecum by different strains was variable. The caecal scoring in albino mice and guinea-pigs was determined based on morphological changes in caecal wall (Fig. 5) and contents of the caecum and mean of the two values was taken as the "caecal score" (Table 5). The virulence was adjudged according to "caecal score" [17,18,31]. As suggested by other workers, strains having a "caecal score" above 2 were considered as virulent and invasive, whereas those with "caecal score" below 2 were considered as non-invasive and non-virulent [31]. The *E. histolytica* strains isolated from acute amoebic dysentery cases produced almost similar caecal score in albino rats and guineapigs. 4 strains were tested in albino mice, with each strain injected in 8-10 animals. The albino mice and the guinea-pigs infected with strains isolated from acute amoebic dysentery had caeca which were grossly ulcerated and showed marked contraction. There was thickening and variations in shape and size of caeca along with a plentiful discharge of mucus so that there was slight or no solid debris in the lumen. In every infected animal, the ulceration was formed. In cross section of these ulcers, the mucosa was totally eroded; the amoebae, leukocytes and necrotic tissue were seen in the erosion. Lesions of these types have already been described by Neal [18,32], Singh et al. [33], and Gleeson and Healy [34]. The amoebae

Table 4. Permanent staining done by iron-haematoxyline method showed nuclear details for *E. histolytica*

	No. of samples stained	-	+	++	+++
E. histolytica	32	4	10	10	8

-, +, ++, +++ stands for poor, fair, good, excellent details respectively for reliable diagnosis

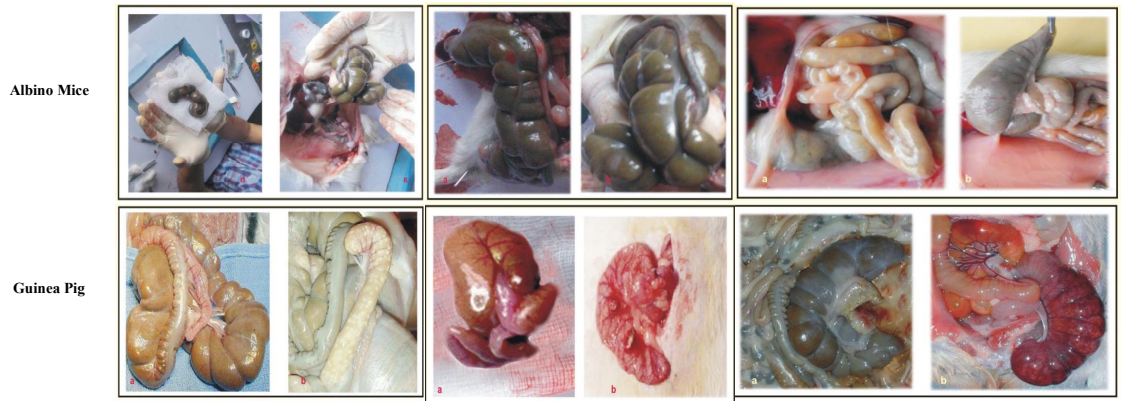

Fig. 5. Morphological changes recorded in caecal wall based on caecal scoring, Fig. 5A is in Albino Mice, Fig. 5B is in Guinea Pig

observed had ingested bacteria and trichomonads when isolated from intact caeca, while amoeba from caeca showing ulceration, had ingested host cell fragments and R.B.C. Strains from cases of chronic amoebiasis, pain in abdomen and amoebic colitis were also found to be invasive and virulent to albino rats. In all the cases caeca of rats showed marked local thickening and extensive contraction but ulceration was seen only in few cases.

3 strains from asymptomatic cyst passers were tested in mice. The strains were found to be non-invasive and infected a somewhat lower percentage of albino rats and guinea pigs than other strains when examined after 7-9 days and 10- 11days, respectively. The albino mice and guineapigs inoculated with these strains displayed no ulceration on macroscopic and microscopic inspection, although huge numbers of amoebae were reported in the lumen and slightly mucoid material were reported in the contents. These strains were capable of causing marked local thickening and contraction of the caeca in few individual mice. The control animals injected with culture fluid without amoebae showed no changes either macroscopically or microscopically.

Strains isolated from asymptomatic cases were unable to produce ulceration in any of the rats. There was only a slight thickening and contraction of the caeca of rats and the contents were slightly mucoid, however, trophozoites of *E. histolytica* were always isolated from the gut lumen.

Jones [17] was the first worker to produce infection in 4 weeks old rats weighing between 30-40 gr. Ulceration of caeca was produced within 6-7 days of infection. Neal [18] also followed the same method of inoculation and was able to produce lesions including gross ulceration in rats within 7-9 days of inoculation. Neal [18] and Neal and Vincent [35,36] also found that there was variation in the virulence of the isolated strains of *E. histolytica*. The strains collected from acute amoebic dysentery were invasive and virulent, whereas, strains from 'carriers' were non-invasive and avirulent. Neal and Bird [31] isolated 2 types of strains of *E. histolytica* from tropical areas. They reported that all the 3 strains of *E. histolytica* isolated from cases of acute amoebic dysentery were invasive and virulent to rats and the remaining 5 strains from cases not suffering from symptoms attributable to the parasite were non-invasive and avirulent.

In the present study virulence of a few strains of *E. histolytica* was also tested in guinea-pigs. The results found were almost similar to those in rats. Fifty guinea-pigs were inoculated with 6 strains of *E. histolytica* isolated from cases of acute amoebic dysentery, chronic amoebiasis and asymptomatic cyst passers. The strains of *E. histolytica* recovered from cases of acute amoebic dysentery produced almost same "caecal score" as in the albino rats. Contrary to the findings of some workers [37,38], the ulcers, were not found to be covered by pseudo-membrane. The strains of *E. histolytica* from cases of chronic amoebiasis were also virulent and invasive, but all the guinea-pigs did not show ulceration. There was, however, thickening and contraction of the wall. The contents of the lumen were mucoid in nature in all the cases. Large number of trophozoites could be seen from the contents of the lumen.

Table 5. Caecal scoring [13,14]

	Morphological changes	**Caecal scoring**
Caecal wall	Normal	0
	Slight thickening	1
	Marked local thickening and contraction with slight hyperaemia. Ulcers may/may not be observed.	2
	Extensive thickening and contraction, Gross hyperaemia. Ulcers may or may not be seen.	3
	Caecum shapeless, extensive thickening, gross contraction, ulceration and severe hyperaemia	4
Caecal contents	Normal	0
	No trophozoite by microscopic examination. Caecal contents slightly less solid than normal.	1
	Microscopic examinations show 1-10 trophozites under low power field. Slightly mucoid caecal content	2
	Microscopy reveals 20-40 trophozoites. Few pus cells may be seen. Mucoid content, some solid matter still present	3
	Microscopy reveals -80 trophozoites. Plenty of pus cells and red blood cells. No solid matter seen, only white and yellow mucus content present	4

Table 6. Infectivity rate and caecal score for different strains of *E. histolytica*

	Average infectivity rate in guineapigs	Average infectivity rate in albino mice	Average caecal score in guineapigs	Average caecal score in albino mice
Acute amoebic dysentery	93.7%	97.9%	3.02	3.63
Amoebic colitis	92.1%	-	2.70	-
Chronic amoebiasis	81.4%	86.1%	2.60	2.63
Pain in abdomen	85.4%	-	2.38	-
Asymptomatic cyst passers	68.7%	79.5%	1.59	1.78

The strains of *E. histolytica* from asymptomatic cyst passer infected a low percentage of guinea-pigs and were avirulent and non-invasive. Nevertheless, in all the cases trophozoites could be recovered from the lumen. Thus, in the present study, it was observed that at least two different strains of *E. histolytica* are prevalent in the population of Gaya, which differ in their virulence. A number of workers have shown the high susceptibility of guineapigs to *E. histolytica* within 10-11 days of injection of trophozoites in the caecum [37-39]. Neal [40] commented that guinea-pigs react in the same manner as rats to infection with virulent and avirulent strains. Krupp and Faust [38] showed the existence of virulent and avirulent strains of *E. histolytica* on the basis of their experimental work in guineapigs. This work also reestablished the above fact confirming that both albino rats and guinea pigs could be used to establish and study the pathogenicity of *E. histolytica*.

4. CONCLUSION

This study concludes that *E. histolytica* is endemic with varying degree of pathogenicity in the Gaya district population causing variable gastrointestinal symptoms. The strains in most of the cases are easily identifiable by direct microscopy and staining protocols accompanied by formol-ether concentration method. In our study, culture methods to identify *E. histolytica* were not found to be better than microscopy or concentration methods. Strains from symptomatic and asymptomatic carriers differed in pathogencity, although they didn't differ much in infectivity thus delinking infectivity and pathogenicity in case of *E. histolytica.* This study also established that there are at least two different strains of *E. histolytica* in the population of Gaya district varying in their pathogenicity. In spite of much progress in the field, mystery still remains about the different strains of *E. histolytica* and their pathogenicity [41]. In future, PCR based methods aided with microscopic examination might prove to be more informative.

ETHICAL APPROVAL

Authors declare that "Principles of laboratory animal care" were followed during this study. All experiments have been examined and approved by the ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kantor M, Abrantes A, Estevez A, Schiller A, Torrent J, Gascon J, Hernandez R, Ochner C. *Entamoeba histolytica*: Updates in clinical manifestation, pathogenesis, and vaccine development. Can J Gastroenterol Hepatol. 2018;4601420.
2. Saidin S, Othman N, Noordin R. Update on laboratory diagnosis of amoebiasis. Eur J Clin Microbiol Infect Dis. 2019;38(1):15-38.
3. Mi-ichi F, Ishikawa T, Tam VK, Deloer S, Hamano S, Hamada T, et al. Characterization of *Entamoeba histolytica* adenosine 50 - phosphosulfate (APS) kinase; validation as a target and provision of leads for the development of new drugs against amoebiasis. PLoS Negl Trop Dis. 2019;13(8).
4. Skinner BM, Johnson EEP. Nuclear morphologies: Their diversity and functional relevance. Chromosoma. 2017;126: 195–212.
5. Salit IE, Khairna K, Gough K, Pillai DR. A possible cluster of sexually transmitted *Entamoeba histolytica*: Genetic analysis of a highly virulent strain. Clinical Infectious Diseases. 2009;49346–353.
6. Ravdin JI. Pathogenesis of amoebiasis: An overview. Amoebiasis: Human Infection by *Entamoeba histolytica*. Ed by Ravdin JI. Wiley. New York. 1988;166-176,3.

7. Neal RA. Experimental studies on *Entamoeba* with reference to speciation. Advances in Parasitology. Ed by Ben Dawes. Academic Press, New York. 1966;4:1.
8. Stephen ED, Dunbar L. Notes on the dysenteries of the Madras district. Indian J Med Res. 1930;18:149.
9. Chuttani PN, Grewal MS, Ashraf SM. The cyst passer. Indian Practitioner. 1968;21:714-718.
10. Tandon BN, Tandon HD, Prakash O. A study of chronic colonic diarrhoea and dysentery. I. Clinical, Endoscopic and Coprological Study. Indian J Med Res. 1966; 54:623-628.
11. Jawaid E, Sinha CDP. Amoebiasis in rural and urban population of Gaya district in Bihar. Proc Zool Soc India. 2016;15:75-78.
12. Cheesbrough M. District laboratory practice for tropical countries. Cambridge University Press, USA. 1998;190-203.
13. Burrows RB. A new fixative and technics for the diagnosis of intestinal parasites. Am J Clin Path. 1967;48:342-346.
14. Ridley DS, Hawgood BC. The value of formol-ether concentration of faecal cysts and ova. J Clin Pathol. 1956;9:74–76.
15. Faust EC. In craig and faust's clinical parasitology. Ed by Lea and Febiger, Philadelphia, USA.1937;623.
16. Prakash O. Trial of some dyes for wet staining of amoebae in cultures. Indian J Med Res. 1967;55:536-537.
17. Jones WR. The experimental infection of rats with *Entamoeba histolytica*; with a method for evaluating the anti-amoebic properties of new compounds. Ann Trop Med Parasitol. 1946; 40:130-140.
18. Neal RA. Some observations on the variation of virulence and response to chemotherapy of strains of *Entamoeba histolytica* in rats. Trans R Soc Trop Med Hyg. 1951;44: 439-52.
19. Prakash O, Bhujwala RA, Vinayak VK. The value of extended incubation and subcultures in the isolation of *Entamoeba histolytica* from stool specimens. Indian J Med Res. 1967;55: 538-42.
20. Vinayak VK, Tandon BN, Prakash O. A comparative evaluation of formol-ether, zinc sulphate and magnesium sulphate concentration techniques for diagnosis of helminthic ova and protozoal cysts. Indian J Med Res. 1967;55:134-138.
21. Shrivastav JB. Incidence and epidemiology of intestinal parasites with special reference to intestinal amoebiasis and giardiasis. Ann Biochem Exp Med. 1960;20:529-44.
22. Misra SS, Misra RN, Bajpai CM, Jhingran SG. Intestinal amoebiasis. Some observations on the laboratory techniques in the diagnosis. J Indian Med Assoc. 1966;16: 554-558.
23. Vinayak VK. Thesis submitted for M.D., Microbiology, AIIMS; 1967.
24. Prakash O, Tandon BN, Bhalla I, Ray AK, Vinayak VK. Indirect hemagglutination and ameba-immobilization tests and their evaluation in intestinal and extraintestinal amebiasis. Am J Trop Med Hyg. 1969;18:670 – 675.
25. Ritchie LS, Pan C, Hunter GW. A comparison of the zinc sulfate and the MGL (formalin-ether) technics. J Parasit. 1952;38:16.
26. Faust EC. Modern criteria for the laboratory diagnosis of amoebiasis. Am J Trop Med Hyg. 1952;1:140-145.
27. Craig CF, St. John JH. The value of cultural methods in surveys for parasitic amebae of man. Amer Jour Trop Med. 1927;S1-7:39-48.
28. Tonney FO, Hoeft GL, Spector BK, The threat of amoebiasis in the food handler. The J Am Med Assoc. 1933;101:1638-1639.
29. Tsuchiya H. Further studies on the cultivation of *Entamoeba histolytica* and a complement fixation test for amoebiasis. J Lab Clin Med. 1934;19:495-504.
30. Stewart GT, Jones WR. The pathology of an experimental amoebic infection in the rat. Ann Trop Med Parasitol. 1948;42:33-45.
31. Bird RG, Neal RA. The virulence of strains of *Entamoeba histolytica* from Bahrain. Persian Gulf Trans R Soc Trop Med Hyg. 1962;56:87-90.
32. Neal RA, Robinson GL, Lewis WP, Kessel JF Comparison of clinical observations on patients infected with *Entamoeba histolytica*, with serological titres of their sera and virulence of their amoebae to rats. Trans R Soc Trop Med Hyg. 1968;62:69-75.
33. Singh BN, Das SR, Saxena U. Virulence of strains of *Entamoeba histolytica* from India, with an account of a method for obtaining cent percent infection in rat. Ann Biochem Exp Med. 1963;23:237-242.
34. Healy GR, Gleason NN. Studies on the pathogenicity of various strains of *Entamoeba histolytica* after prolonged cultivation, with observations on strain differences in the rats employed. Am J Trop Med Hyg. 1966;15:294-299.
35. Neal RA, Vincent P. Strain variation in *Entamoeba histolytica*. II. The effect of serial liver passage on the virulence. Parasitology. 1956;46:173-182.

36. Vincent P, Neal RA. Duration of invasiveness of *Entamoeba histolytica* maintained *in-vitro*. Parasitology. 1960;50:449-452.
37. Carrera GM, Faust EC Susceptibility of the guinea pig to *Entamoeba histolytica* of human origin. Am J Trop Med Hyg. 1949;29: 647-667.
38. Krupp IM, Faust EC. Parasitologic surveys in Cali, dep. del Valle, Colombia. IV. Experimental studies in guinea pigs of the pathogenic capacity of *Entamoeba histolytica* from infected persons in Ward Siloé Cali. J. Parasit. 1959;45:449.
39. Chaudhuri RN, Saha TK, Roy N. Amebiasis—an Experimental Study. Am J Trop Med Hyg. 1960;506-511.
40. Neal RA. Virulence in *Entamoeba histolytica*. Trans Roy Soc Trop Med Hyg. 1957;51:313-319.
41. Carrero JC, Reyes-Lopez M, Serrano-Luna J, Shibayama M, Unzueta J, León-Sicairos N, et al. Intestinal amoebiasis: 160 years of its first detection and still remains as a health problem in developing countries. Int J Med Microbiol. 2020;310(1): 151358.