

## Primary pathogenicity analysis of a Chinese *Entamoeba histolytica* isolate

Muxia Luo<sup>1</sup>, Meng Feng<sup>1,2</sup>, Xiangyang Min<sup>1</sup>, Xueping Li<sup>1</sup>, Junlong Cai<sup>1</sup>, Hiroshi Tachibana<sup>2</sup>, Xunjia Cheng<sup>1,2,\*</sup>

<sup>1</sup>Department of Medical Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai, China;

<sup>2</sup>Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa, Japan.

### Summary

This study is the first to isolate an *Entamoeba histolytica* strain from Chinese amoebic patients and to conduct a detailed examination of its virulence. A fecal sample that contains cysts of *E. histolytica* was obtained from Guangxi province. The sample was cultured axenically and then cloned by limiting dilution, and named as XLAC. *In vitro* and *in vivo* tests were conducted to evaluate the virulence of the *Entamoeba* isolate. The *E. histolytica* strain XLAC was successfully cloned and cultured axenically. DNA regions that contain hexokinase, glucose-6-phosphate isomerase, phosphoglucomutase, and heavy subunit of lectin genes were amplified by PCR. The PCR products were then sequenced. Virulence analysis suggested that the XLAC strain was similar to the HM1:IMSS strain at the genetic level. *In vitro* and *in vivo* tests also implicated these strains to be similar. These findings may be attributed to the low expression levels of pathogenic genes obtained through real-time PCR. The XLAC strain restored its virulence after it was injected into hamster liver. This study may be a good model for studying virulence changes in *E. histolytica*.

**Keywords:** *Entamoeba histolytica*, lectin, apoptosis, virulence

### 1. Introduction

The enteric protozoan parasite *Entamoeba histolytica* causes an estimated 50 million cases of amoebic colitis and extraintestinal abscess, which result in 100,000 deaths annually (1). *Entamoeba dispar* is morphologically indistinguishable from *E. histolytica*, but it is nonpathogenic (2,3). *E. histolytica* infections have different clinical outcomes. Most infections remain asymptomatic, whereas some infected patients develop diarrhea and dysentery. Only a few infections develop extra-intestinal complications, such as liver abscess.

Several *E. histolytica* infections in China are reported every year. The average infection rate of amoebiasis in China was 0.949% in the 1990s. A 2006

survey of HIV-positive patients in China showed a serum-positive rate of 7.9% for *E. histolytica* (4). A recent study in seven provinces in China has shown a serum-positive rate of 0.53% to 9.04% for *E. histolytica* (5). However, insufficient pathogenic information on Chinese *E. histolytica* strains is currently available. The *E. histolytica* strain utilized in the current paper was obtained from Guangxi Province. *In vitro* and *in vivo* tests were conducted to evaluate the virulence of the *Entamoeba* isolate.

### 2. Materials and Methods

#### 2.1. Sample collection

Stool and blood samples were obtained from 120 villagers in August, 2011 in Xilin County, Guangxi Province, China.

#### 2.2. Indirect fluorescence antibody assay (IFA)

The IFA test was performed as previously described using formalin-fixed trophozoites smeared on glass

Luo MX and Feng M contributed equally to this work.

\*Address correspondence to:

Dr. Xunjia Cheng, Department of Medical Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai 200032, China.

E-mail: xjcheng@shmu.edu.cn

slides. Fluorescein isothiocyanate-conjugated goat immunoglobulin G (IgG) to whole human IgG (MP Biomedicals-Cappel, Solon, OH, USA) was used as the second antibody.

### 2.3. Culture conditions

A fecal sample that contains *Entamoeba* cysts was suspended in water for 24 h to remove *Blastocystis* spp. The sample was then cultured in modified Tanabe-Chiba medium (6) at 37°C. The trophozoites were treated with a cocktail of antibiotics and then cultured monoxenically with live *Crithidia fasciculata* in TYI-S-33 medium supplemented with 15% adult bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA) at 37°C. The trophozoites of the strain were cultured axenically in TYI-S-33 medium and then cloned through limited dilution.

### 2.4. PCR analysis and sequence

Genomic DNA was extracted from the axenic cultures using a QIAamp DNeasy kit (Qiagen, Valencia, CA, USA) (7). The genomic DNA was subjected to PCR for the amplification of *hexokinase (HXK)*, *glucose-6-phosphate isomerase (GPI)*, *phosphoglucomutase (PGM)*, and *heavy subunit of lectin genes (LecHgl)*. The primers and PCR conditions for *E. histolytica* were based on previously described procedures (8,9). PCR was performed briefly in a 50 µL reaction mixture using TaKaRa Ex-taq<sup>®</sup> DNA Polymerase (Takara, Dalian, China).

The PCR products were subjected to direct sequencing after purification using a QIAquick PCR purification kit (Qiagen) using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA). The reactions were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence data were analyzed using ClustalX Ver. 1.83 (Conway Institute UCD Dublin, Dublin, Ireland).

### 2.5. Erythrophagocytosis assay

Type O human erythrocytes from healthy donors were washed and suspended in TYI-S-33 medium. Erythrocytes ( $2 \times 10^7$ ) were incubated with  $2 \times 10^5$  trophozoites in 0.4 mL TYI-S-33 medium at 37°C for 10 min. After being lysed with free and adherent erythrocytes through the addition of distilled water, the trophozoites were fixed and stained with a 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) solution containing hydrogen peroxide (Sinopharm Group Co. Ltd., Shanghai, China). The number of ingested erythrocytes was determined by examining 300 trophozoites. The experiments were repeated three times. Statistical analysis was performed using Student's *t*-test.

### 2.6. Erythrocyte adherence assay

The trophozoites ( $2 \times 10^5$ ) were incubated with  $2 \times 10^7$  type O human erythrocytes for 5 min at 4°C. The erythrocyte-trophozoite suspension was then fixed in 2.5% glutaraldehyde (Sigma-Aldrich). Afterwards, the erythrocytes were washed with PBS and stained with a 3,3-diaminobenzidine (Sigma-Aldrich) solution containing 0.2% H<sub>2</sub>O<sub>2</sub> (Sinopharm Group Co. Ltd.). The number of amoeba with at least three erythrocytes was scored by examining 300 trophozoites. The experiments were repeated three times. Statistical analysis was performed using Student's *t*-test.

### 2.7. Apoptosis in Jurkat cells

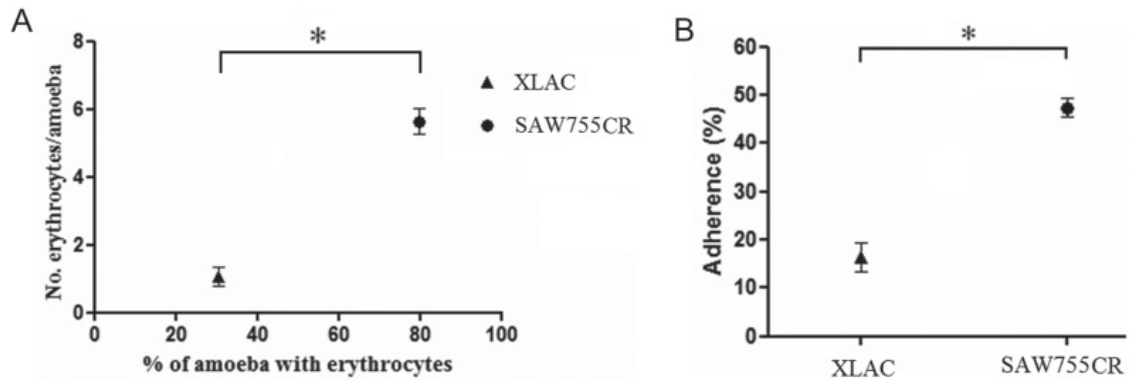
*Entamoeba* trophozoites were washed with and suspended in RPMI 1640 medium (Gibco, Life Technologies). The trophozoites ( $2 \times 10^4$ ) were incubated with  $10^5$  Jurkat cells (The Cell Bank of Chinese Academy of Science, Shanghai, China) for 20 min at 37°C. The cells were then washed twice and stained with FITC-conjugated annexin V (Sigma-Aldrich). The numbers of apoptosis and total cells were determined under a microscope. The experiments were repeated three times. Statistical analysis was performed using Student's *t*-test.

### 2.8. Expression of the heavy subunit of lectin genes

Total RNAs of *E. histolytica* trophozoites were isolated using an RNeasy mini kit (Qiagen) and used for cDNA synthesis using a GeneAmp RNA PCR kit (Applied Biosystems). A reaction mixture that contains SYBR Premix Ex Taq (Takara), specific primers, and the cDNAs was used for quantitative real-time PCR analysis. The primer pairs used were previously described (10). Forty cycles of amplification were performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The fluorescence intensity in each cycle was also recorded using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The relative quantification of the data obtained from the ABI PRISM 7500 Sequence Detection System software version 2.0.1 (Applied Biosystems) was performed by the comparative CT method using actin genes as internal standards. The experiments, including the culture of trophozoites and the isolation of RNA, were repeated three times.

### 2.9. Hepatic challenge with *E. histolytica*

Sixteen 6-week-old male hamsters (Shanghai Songlian Experimental Animal Farm, Shanghai, China) were used. The hamsters were challenged with intrahepatic inoculation of  $10^6$  *E. histolytica* trophozoites into the left lobe of the liver. The hamsters were sacrificed 7 d after the challenge. The percentage of the abscessed



**Figure 1. Erythrophagocytosis and erythrocyte adherence of *Entamoeba histolytica*.** (A), Erythrophagocytosis of *E. histolytica* XLAC and SAW755CR strains. (B), Erythrocyte adherence of *E. histolytica* XLAC and SAW755CR strains. \* $p < 0.001$ .

liver was calculated as the weight of the abscess divided by the recorded weight of the liver before abscess removal. The *E. histolytica* trophozoites were again subjected to the erythrophagocytosis assay after being injected into hamster liver.

### 3. Results and Discussion

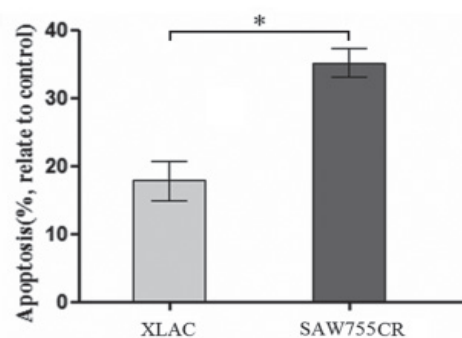
The present study was processed during 2012. Among the 120 stool samples, 11 were discovered to be positive for *Entamoeba* species by microscopy. One *E. histolytica* infection, named XLAC, was finally defined via the IFA assay. The XLAC strain was successfully cloned and cultured axenically.

The DNA regions of the XLAC strain that contain *LecHgl* and isozyme genes were amplified by PCR. The PCR products were sequenced directly. The nucleotide sequences of the *LecHgl*, *HXK*, and *PGM* genes from XLAC were identical to those of the HM1:IMSS strain. The nucleotide sequences of the *GPI* gene from XLAC were identical to those of the SFL-3 and BF-841 strains. These sequences had one nucleotide substitution. However, no differences in amino acid sequences were observed between the XLAC and HM1:IMSS strains.

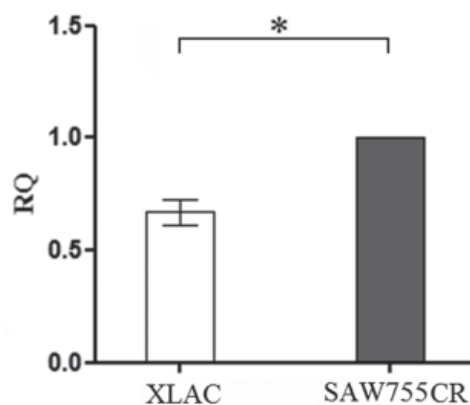
The XLAC strain was evaluated for erythrophagocytosis. The SAW755CR strain was used as the control. The rates of erythrocyte-ingesting trophozoites of the XLAC and SAW755CR strains were 30.7% and 79.3%, with 1.1 and 5.7 ingested erythrocytes per trophozoite, respectively (Figure 1A). A significant difference in erythrophagocytosis was observed between the XLAC and SAW755CR strains ( $p < 0.001$ ).

The adherence of the XLAC and SAW755CR strains to human erythrocytes is shown in Figure 1B. The adherence rates of the trophozoites of the XLAC and SAW755CR strains to human erythrocytes were 16.3% and 47.4%, respectively. A significant difference in adherence was found between the XLAC and SAW755CR strains ( $p < 0.001$ ).

Jurkat cells were incubated with *E. histolytica* trophozoites for 20 min at a 5:1 ratio. The relative



**Figure 2. Apoptosis in Jurkat cells.** Jurkat cells were incubated with *E. histolytica* trophozoites for 20 min at a 5:1 ratio. The relative apoptosis rates to the blank control of the XLAC and SAW755CR strains were 17.8% and 35.2%, respectively. \* $p < 0.001$ .



**Figure 3. Expression of the heavy subunit of lectin genes.** The quantitative expression of the *LecHgl* gene in the XLAC and SAW755CR strains was measured. Real-time reverse transcription PCR was performed using actin genes as internal standards. The mean value of the relative expression level of XLAC *LecHgl* to SAW755CR *LecHgl* was estimated to be 0.669. \* $p < 0.001$ .

apoptosis rates to the blank control of the XLAC and SAW755CR strains were 17.8% and 35.2%, respectively (Figure 2). A significant difference in relative apoptosis rate was observed between the XLAC and SAW755CR strains ( $p < 0.001$ ).

**Table 1. Hamsters challenged with an intrahepatic inoculation of *Entamoeba histolytica* trophozoites of the XLAC strain**

Group	Injectant	No. of hamsters with abscesses/Nos. challenged	Percentage of liver abscess in hamster liver (%; mean $\pm$ S.D.)
Control	TYI-S-33 medium	0/6	0
Test	XLAC trophozoites	10/10	21.7 $\pm$ 17.8

Real-time reverse transcription PCR was performed to measure quantitatively the expression of the *LecHgl* genes in the XLAC and SAW755CR strains. The results were analyzed by the comparative CT method using actin genes as internal standards. The experiments were repeated in triplicate. The mean value of the relative expression level of XLAC *LecHgl* to SAW755CR *LecHgl* was estimated to be 0.669 (Figure 3). A significant difference in the expression level of *LecHgl* was found between the XLAC and SAW755CR strains ( $p < 0.001$ ).

Ten hamsters developed amebic liver abscesses 7 d after they were challenged with an intrahepatic inoculation of XLAC strain trophozoites. The mean mass of the abscesses was 21.7% of the liver mass (Table 1). By contrast, no amebic liver abscess formation was observed in the PBS control group. The presence of erythrophagocytosis in the XLAC strain after passage hamster liver was also evaluated. The original XLAC strain was used as the control. The rate of erythrocyte ingestion after trophozoite passage hamster liver was 61.7%, which was higher than the 37.7% rate of the original XLAC strain. The number of ingested erythrocytes after XLAC trophozoite passage hamster liver was 2.3 per trophozoites, which was higher than the 1.15 per trophozoites of the original XLAC strain. The XLAC strain restored its virulence after being injected into hamster liver.

The ability of *E. histolytica* trophozoites to invade the colon and other tissues depends on several pathogenic factors. One of the most important factors is the galactose- and *N*-acetyl-D-galactosamine-inhibitable cell surface lectin of the ameba. The lectin mediates the adherence of trophozoites to human colonic mucins, colonic epithelial cells, neutrophils, and erythrocytes. The lectin is also important in the cytolytic event that follows adherence (11,12). Zymodeme analysis was employed to discriminate the virulent *E. histolytica* and the nonvirulent *E. dispar*. The analysis indicates that *HXK* is a key enzyme and that *GPI* and *PGM* are also useful. The amino acid sequences of the *LecHgl*, *HXK*, *GPI*, and *PGM* genes from XLAC were identical to those of the HM1:IMSS strain. This result suggests that the virulence of the XLAC strain is similar to that of the HM1:IMSS strain.

In Japanese amebiasis patients, the crude antigen of the Asian *E. histolytica* strain HK9 generates a higher serum-positive rate than that of the Mexican strain HM1:IMSS (unpublished data). This result suggests that the local *E. histolytica* strain is more suitable

for diagnosing amebiasis. No axenic *E. histolytica* strain has been isolated from China in the past years. This study is the first to isolate the *E. histolytica* strain XLAC from Chinese amebic patients. Detailed examination of its virulence may play an important role in further diagnostic studies of Chinese amebiasis.

*E. histolytica* loses its virulence after a long *in vitro* culture. However, *E. histolytica* can restore its virulence by injecting it into hamster liver (13,14). In the present study,  $10^6$  *E. histolytica* trophozoites injected into hamster liver can induce liver abscess, whereas  $5 \times 10^5$  *E. histolytica* trophozoites cannot (data not shown). The rate of erythrocyte ingestion after the injection of XLAC trophozoites was higher than that of the original XLAC strain. The XLAC strain restored its virulence after it was injected into hamster liver. This study can be a good model for studying the virulence changes in *E. histolytica*.

#### Acknowledgements

This work was supported by the National Science Foundation of China (Grant No. 81171594) (to XC) and by KAKENHI (Grant No. 23117009) (to HT).

#### References

- Walsh JA. Problems in recognition and diagnosis of amebiasis: Estimation of the global magnitude of morbidity and mortality. *Rev Infect Dis.* 1986; 8:228-238.
- Diamond LS, Clark CG. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *J Eukaryot Microbiol.* 1993; 40:340-344.
- Zaki M, Meelu P, Sun W, Clark CG. Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. *J Clin Microbiol.* 2002; 40:1271-1276.
- Chen Y, Zhang Y, Yang B, Qi T, Lu H, Cheng X, Tachibana H. Seroprevalence of *Entamoeba histolytica* infection in HIV-infected patients in China. *Am J Trop Med Hyg.* 2007; 77:825-828.
- Yang B, Chen Y, Wu L, Xu L, Tachibana H, Cheng X. Seroprevalence of *Entamoeba histolytica* infection in China. *Am J Trop Med Hyg.* 2012; 87:97-103.
- Yoshimura M, Ebukuro M. A modified Tanabe-Chiba medium for detection of *Entamoeba histolytica*. *Jikken Dobutsu.* 1988; 37:321-324.
- Feng M, Cai J, Yang B, Fu Y, Min X, Tachibana H, Cheng X. Unique short tandem repeat nucleotide sequences in *Entamoeba histolytica* isolates from China. *Parasitol Res.* 2012; 111:1137-1142.
- Beck DL, Tanyuksel M, Mackey AJ, Haque R, Trapaidze N, Pearson WR, Loftus B, Petri WA. *Entamoeba*

- histolytica*: Sequence conservation of the Gal/GalNAc lectin from clinical isolates. *Exp Parasitol.* 2002; 101:157-163.
9. Tachibana H, Yanagi T, Pandey K, Cheng XJ, Kobayashi S, Sherchand JB, Kanbara H. An *Entamoeba* sp. Strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. *Mol Biochem Parasitol.* 2007; 153:107-114.
  10. Beck DL, Boettner DR, Dragulev B, Haque R, Trapaidze N, Pearson WR, Loftus B, Petri WA. Identification and gene expression analysis of a large family of transmembrane kinases related to the Gal/GalNAc lectin in *Entamoeba histolytica*. *Eukaryot Cell.* 2005; 4:722-732.
  11. Petri WA, Haque R, Mann BJ. The Bittersweet interface of parasite and host: Lectin-Carbohydrate interactions during Human Invasion by the parasite *Entamoeba histolytica*. *Annu Rev Microbiol.* 2002; 56:39-64.
  12. Stanley SL Jr. Amoebiasis. *Lancet.* 2003; 22:1025-1034.
  13. González-Garza MT, Castro-Garza J, Cruz-Vega DE, Vargas-Villarreal J, Carranza-Rosales P, Mata-Cárdenas BD, Siller-Campos L, Said-Fernández S. *Entamoeba histolytica*: Diminution of erythrophagocytosis, phospholipase A2, and hemolytic activities is related to virulence impairment in long-term axenic cultures. *Exp Parasitol.* 2000; 96:116-119.
  14. Olivos A, Ramos E, Nequiz M, Barba C, Tello E, Castañón G, González A, Martínez RD, Montfort I, Pérez-Tamayo R. *Entamoeba histolytica*: Mechanism of decrease virulence of axenic cultures maintained for prolonged periods. *Exp Parasitol.* 2005; 110:309-312.

(Received March 20, 2013; Revised April 7, 2013; Accepted April 12, 2013)