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## ***In vitro* Sensitivity of Different Brands of Antiamoebic Drugs (Metronidazole Tablets) Against Clinical Isolates of *Entamoeba histolytica* in Bangladesh**

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**Abstract:** The aim of the study was to evaluate *in vitro* sensitivity of different metronidazole tablets from Bangladeshi pharmaceuticals against clinical isolates of *E. histolytica*. Metronidazole tablets of 12 different brands were randomized from some big and small pharmaceuticals according to their business. The parasite count was adjusted to  $3 \times 10^6$  parasites  $\text{mL}^{-1}$  in a medium. *In vitro* drug sensitivity assay of the samples was carried out by using microtiter plates after treatment with different concentrations of metronidazoles. The viable parasites were counted by haemocytometer. No statistical significance was observed in terms of viable parasites with the metronidazole tablets from three big pharmaceuticals at the concentration of 2.3, 3.5 and 4.6  $\mu\text{M}$  when compared with the standard metronidazole. We conclude that brands from some big pharmaceuticals showed *in vitro* sensitivity against *E. histolytica*.

**Key words:** Amoebiasis, metronidazole, *Entamoeba histolytica*, drug resistance

### **INTRODUCTION**

*Entamoeba histolytica*, associated with high morbidity and mortality continues to be a major public health problem throughout the world. Asymptomatic individuals account for almost 90% of the infections (WHO, 1997). Poverty, ignorance, overcrowding, poor sanitation and malnutrition favour the transmission and increased disease burden (Walsh, 1988). Prevalence varies from country to country and within a country (Tanaka and Kaneda, 1991; Haque *et al.*, 2000; Blessmann *et al.*, 2002; Kang *et al.*, 1998).

Diarrheal diseases are the major causes of morbidity and mortality in children in developing countries. For example, in Bangladesh 1 in 30 children dies of diarrhea or dysentery by his or her fifth birthday (Petri *et al.*, 2000). Bacillary dysentery is most commonly caused by microorganisms belonging to the genus *Shigella*, whereas amoebic dysentery is an infection caused by the protozoan parasite *Entamoeba histolytica* (Kotloff *et al.*, 1999).

It is now generally accepted that what was earlier known as *E. histolytica* actually comprises two genetically distinct but morphologically indistinguishable species, *E. histolytica* and *E. dispar*, previously known as pathogenic and non-pathogenic *E. histolytica*,

respectively. The World Health Organization has reaffirmed the definition of amoebiasis as infection with *E. histolytica* sensu stricto with or without clinical manifestations (WHO, 1997). Only *E. histolytica* can cause intestinal and extraintestinal disease. Amoebiasis is a common problem in the developing world. Based on the Mexican National Serosurvey at least 8.4% of the population of Mexico had evidence of prior invasive amoebiasis with an estimated one million cases of amoebiasis and 1,000 deaths annually (Caballero-Salcedo *et al.*, 1994).

The amoebic infection is primarily treated by instituting antiamoebic therapy. The major drug of choice for treating invasive amoebiasis is metronidazole which is derived from 5-nitroimidazole. This agent kills the trophozoites by alterations in the protoplasmic organelles of the amoeba and by fatal destabilization of the DNA helix (Tocher and Edwards, 1994; Öztas *et al.*, 2003; Bansal *et al.*, 2004).

Amoebiasis in symptomatic patients is chiefly treated by antiamoebic drugs (Martínez-Palomo and Martínez Baez, 1983). The limited number of drugs available to treat amoebiasis needs a new approach to treat infected individuals (Pillai *et al.*, 1999). In asymptomatic individuals, inappropriate usage of drugs or overdosing could lead to drug resistance although drug resistance in

*E. histolytica* is not common yet. The quality of medicines available in some less-developed countries is inadequate in terms of content of active ingredient (Taylor *et al.*, 2001). The substandard and/or spurious drugs is the result of addition of incorrect amount of active ingredients; date expired sub-potent active ingredients and excipients; poor stability of active ingredients in the finished product and so on. Poor stability can also result from the excessive decomposition of active ingredient at elevated temperature and humidity in tropical countries like Bangladesh. The treatment with this type of substandard and/or spurious drug could also lead to drug resistance and could endanger patient's life.

Previous study has shown that preschool children in urban slum of Bangladesh are at great risk of acquiring *E. histolytica* infection, with almost half infected by five years of age (Haque *et al.*, 1999). There are about 100 pharmaceutical companies manufacture metronidazoles in Bangladesh. To our knowledge, no study is undertaken in Bangladesh till date to evaluate the *in vitro* drug sensitivity of metronidazole. Since amoebiasis is a major public health problem in Bangladesh, we realize that there is a need to check the *in vitro* drug sensitivity of metronidazole. Therefore, this pilot study was carried out to investigate the *in vitro* drug sensitivity of different brands of metronidazole manufactured by different pharmaceutical companies in Bangladesh against clinical isolates of *E. histolytica*.

## MATERIALS AND METHODS

**Collection of sample:** About 160 products of metronidazole (tablets and suspension) are manufactured by different pharmaceutical companies in Bangladesh. All the pharmaceutical companies are divided into two groups as big and small pharmaceutical according to their business in Bangladesh. Name of the pharmaceuticals from each group was arranged alphabetically. Four big and 8 small companies producing metronidazole tablets were selected randomly for this study. Thus 12 brands of metronidazole tablets were collected from retail medicine shop from different areas of Bangladesh.

Metronidazole tablets of 12 different brands were coded as MT01, MT02, MT03, MT04, MT05, MT06, MT07, MT08, MT09, MT10, MT11 and MT12. The products of big pharmaceutical companies are coded as MT01, MT02, MT03 and MT04. From MT05 to MT12 are the products of small pharmaceutical companies. The samples were properly checked for their physical appearance, name of the manufacturer, batch number, manufacturing date, expiry date, manufacturing license number, D.A.R. number and maximum retail price at the

time of purchase. This investigation was performed in the Parasitology Laboratory, Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) during March, 2006 to December, 2007.

**Preparation of antimicrobial agents (standard and sample):** The standard metronidazole drug used in the study was collected as pure salt from Sanofi-Aventis Ltd. Dhaka, Bangladesh. Standard metronidazole (8 mg) was weighed and dissolved in 1 mL of distilled water. The stock solution was stored in a refrigerator. All the samples of metronidazoles (MT01 to MT12) were prepared in a similar manner.

**Clinical isolates:** Clinical isolates of *E. histolytica* were harvested from 24 h old cultures and suspended in a LYI-S-2 medium. Axenic medium (LYI-S-2) consists of liver digest, yeast extract, iron, serum (Table 1).

The parasite count was adjusted to  $3 \times 10^6$  parasites  $\text{mL}^{-1}$  in medium by haemocytometer (Mukhopadhyay and Chaudhuri, 1996; Bansal *et al.*, 2004). Isolation is usually achieved by growing the species in an environment that was previously sterilized and was thereby rid of contaminating organisms.

***In vitro* drug sensitivity assay:** Drug sensitivity assay of the samples was carried out by using microtiter plates. In row A 200  $\mu\text{L}$  of the standard and the samples were given. In all other rows (B-H) the 100  $\mu\text{L}$  medium was added and dilutions of the drugs were performed down the plate mixed properly. One hundred microlitre of the medium from the last row (H) was discarded to maintain the equality of the concentration of the drugs. The final concentrations of the drugs were 0.07, 0.14, 0.29, 0.58, 1.1, 2.3 and 4.6  $\mu\text{M}$ . Further 100  $\mu\text{L}$  of parasite suspension ( $3 \times 10^6$  parasites  $\text{mL}^{-1}$ ) was added to all the rows (A-H). Each test included control (without drug) and blank wells (medium only). Then plastic strip was used to cover the plate. Plates were incubated at  $37^\circ\text{C}$  and examined after 1,

Table 1: Quantitative composition of LYI-S-2

Ingredients	Amount
Potassium phosphate dibasic	1.0 g
Potassium phosphate monobasic	0.6 g
Sodium chloride	2.0 g
Yeast extract	25.0 g
Liver extract	5.0 g
Glucose	10.0 g
Cysteine	1.0 g
Ascorbic acid	0.2 g
Ferric ammonium citrate	2.0 mL
1 M sodium hydroxide	7.5 mL
Water to (distilled/deionized)	880.0 mL
Bovine serum	15.0%
Vitamin mix	2.0%

2 and 4 h under a microscope to check for the presence of amoebae. After 4 h the plate was taken from the incubator. Then the viable parasites were counted by haemocytometer under microscope in each of the rows. For another sets of experiments the final concentrations of the drugs were 1.1, 2.3, 3.5 and 4.6  $\mu\text{M}$ .

**Statistical analysis:** The data were analyzed using SPSS for windows version 12.0 (SPSS, Lead Technologies, Inc., USA). Descriptive statistics were done by one-way ANOVA and Post Hoc Tests, a probability level of 0.05 was considered statistically significant.

### RESULTS AND DISCUSSION

The study was conducted with different brands of metronidazole tablets manufactured by different pharmaceutical companies. Twelve brands are checked for their *in vitro* drug sensitivity against clinical isolates of *E. histolytica*.

Table 2 shows the mean viable parasites count from clinical isolates after treatment with different brands of metronidazole tablets (MT01 to MT04). No statistical significance has been observed in terms of viable parasites with the brands MT01, MT02 and MT03 when compared with the standard metronidazole at the concentration of 2.3, 3.5 and 4.6  $\mu\text{M}$ . MT02 at the concentration 1.1  $\mu\text{M}$  and MT04 at the concentrations of 1.1, 2.3 and 3.5  $\mu\text{M}$  are significantly different when compared with the standard metronidazole in terms of viable parasite count.

Table 3 and 4 shows the mean viable parasites count from clinical isolates after treatment with different brands of metronidazole tablets (MT05 to MT12). All the values in terms of viable parasites are significantly different when compared between samples and standard metronidazole at different concentrations.

Figure 1 show percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets (MT01, MT02, MT03 and MT04). The mean  $\text{IC}_{50}$  of standard metronidazole tablet is 0.07  $\mu\text{M}$ . The mean  $\text{IC}_{50}$  of MT01, MT03 and MT4 against *E. histolytica* are less than 0.14  $\mu\text{M}$ . However,  $\text{IC}_{50}$  of MT02 is more than 0.14  $\mu\text{M}$ .

Figure 2 shows percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets (MT05, MT06, MT07 and MT08). The mean  $\text{IC}_{50}$  of MT05, MT06, MT07 and MT08 against *E. histolytica* is 0.29  $\mu\text{M}$  and above but less than 0.30  $\mu\text{M}$ .

Figure 3 shows percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets

Table 2: The mean viable parasites count from clinical isolates after treatment with different brands of metronidazole tablets (MT01 to MT04)

Conc. ( $\mu\text{M}$ )	STD	MT01	MT02	MT03	MT04
4.6	22±0.6	25±1	26±0.6	26±1	27±1.0
3.5	31±0.6	37±1	38±2.0	37±1	39±1.0*
2.3	44±0.6	49±1	50±0.6	50±1	53±2.0**
1.1	86±3.0	93±3	95±1.0***	93±1	100±0.6****

Results expressed as mean±SD, \*p = 0.047, \*\*p = 0.003, \*\*\*p = 0.007, \*\*\*\*p = 0.000 vs. standard metronidazole

Table 3: The mean viable parasites count from clinical isolates after treatment with different brands of metronidazole tablets (MT05 to MT08)

Conc. ( $\mu\text{M}$ )	STD	MT05	MT06	MT07	MT08
4.6	22±0.6	69±1	57±3	78±1	90±2
3.5	31±0.6	90±3	73±1	105±2	97±2
2.3	44±0.6	98±1	94±4	110±3	109±4
1.1	86±3.0	134±3	121±1	146±3	137±3

Results expressed as mean±SD, p = all values are significantly different vs. standard metronidazole

Table 4: The mean viable parasites count from clinical isolates after treatment with different brands of metronidazole tablets (MT09 to MT12)

Conc. ( $\mu\text{M}$ )	STD	MT09	MT10	MT11	MT12
4.6	22±0.6	102±1	111±0.6	50±1	48±3
3.5	31±0.6	133±4	144±2.0	64±4	64±1
2.3	44±0.6	155±1	150±2.0	74±1	71±3
1.1	86±3.0	189±1	181±4.0	108±1	114±1

Results expressed as mean±SD, p = all values are significantly different vs. standard metronidazole

(MT09, MT10, MT11 and MT12). The mean  $\text{IC}_{50}$  of MT09 and MT10 are about 0.58  $\mu\text{M}$ . The mean  $\text{IC}_{50}$  of MT11 and MT12 is about 0.14.

This study with different brands of metronidazole in Bangladesh showed different sensitivity of the drugs against clinical isolates of *E. histolytica*. Results showed that the metronidazole tablets from three big pharmaceutical companies are as good as standard metronidazole at different concentrations (2.3, 3.5 and 4.6  $\mu\text{M}$ ).

Treatment failure among amoebiasis patients often raises the possibility of drug resistance (Ayala *et al.*, 1990). In the present study, the *E. histolytica* clinical isolates maintained by *in vitro* cultivation in axenic medium were subjected to drug susceptibility tests against metronidazole tablets.

The  $\text{IC}_{50}$  value ( $\mu\text{M}$ ) against *E. histolytica* after treatment with standard metronidazole is about 0.07  $\mu\text{M}$ . The  $\text{IC}_{50}$  values of all the drugs except MT09 and MT10 are comparable with the standard metronidazole. However, all the products of small pharmaceutical companies are not as sensitive as the products of some big pharmaceuticals in terms of their *in vitro* sensitivity against the clinical

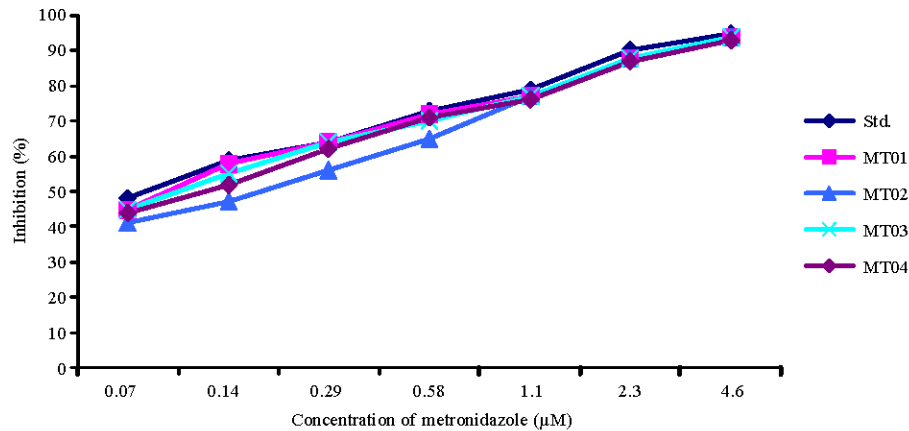


Fig. 1: Percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets (MT01 to MT04)

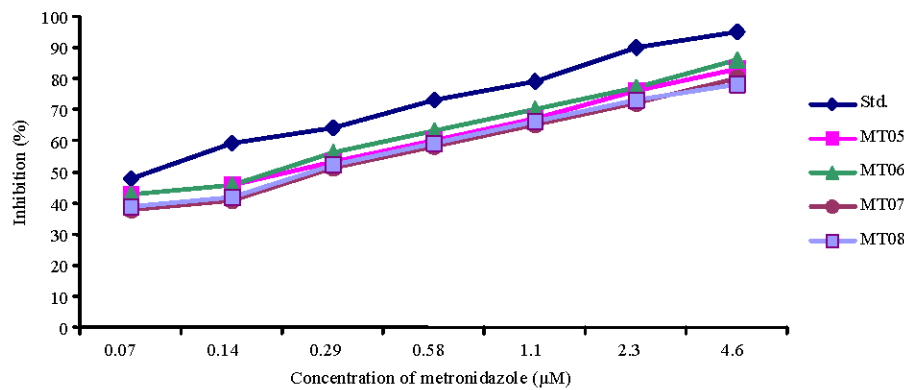


Fig. 2: Percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets (MT05 to MT08)

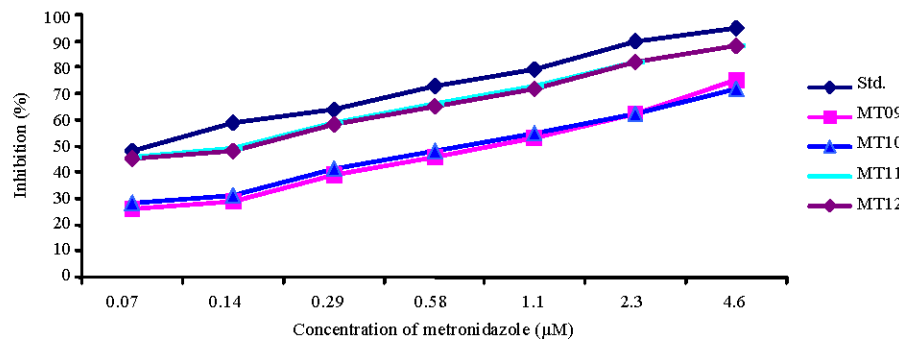


Fig. 3: Percentage inhibition of non-viable *E. histolytica* by different brands of metronidazole tablets (MT09 to MT12)

isolates. Out of four big pharmaceuticals only one is a multinational and rests of them are local pharmaceuticals.

We conclude that the three big pharmaceuticals including the two local and one multinational companies in Bangladesh are manufacturing the metronidazoles which are as sensitive (*in vitro*) as standard metronidazole against the clinical isolates of *E. histolytica*. Monitoring

the random drug sensitivity of different brands of metronidazole was helpful to get an impression about the possible emergence of resistance of metronidazole in future in Bangladeshi patients. Further studies are needed to test other brands of metronidazole available in Bangladesh. Increased awareness and continued surveillance for the possible emergence of resistance

among clinical isolates is necessary for the ultimate prevention and control of amoebiasis. The findings of this study are also very helpful to make awareness of both physicians and consumers to select quality products.

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