In vitro sensitivity of *Carica Papaya* Seed extract against clinical isolates of *Entamoeba histolytica*

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A Research paper submitted to the Department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy

A Collaborative study between Department of Pharmacy, East West University and International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

CERTIFICATE

This is to certify that the thesis "Invitro sensitivity of Carica Papaya Seed extract against clinical isolates of *Entamoeba histolytica*" submitted to the Department of Pharmacy, East West University, Mohakhali, Dhaka in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Shonia Salma Shezuty (ID: 2006-1-70-005) under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed of this connection is duly acknowledged.

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ACKNOWLEDGEMENTS

First, I would like to give my thanks to my supervisors Mrs. Farhana Rizwan, Senior Lecturer and Dr. Sufia Islam, Associate Professor Department of Pharmacy, East West University for their continuous support in the research program. They were always there to listen and to give advice and responsible for involving me in this research project in the first place.

I am grateful to Dr. Rashidul Haque, Scientist and head of the department of Parasitology laboratory and Md. Abdullah Siddique for their support in my study at International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

I am gratefully acknowledged to Professor Dr. Muniruddin Ahmed, Pro vice chancellor of East West University and Professor Dr. Chowdhury Faiz Hossain, Chairperson Department of Pharmacy, East West University for their inspiration in my study.

I am especially thankful to all the participants without whose enthusiastic co-operation this study would not have been completed.

Last, but not least, I want to thank my parents, for giving me life in the first place, for educating me with aspects from sciences and for unconditional support and encouragement.

ABSTRACTS

Background: *Entamoeba histolytica* is the etiological agent of amoebic dysentery and amoebic liver abscess. Amoebiasis is one of the most common health problems in the developing countries. Almost 10% people of the world suffer from amoebiasis.Poverty, ignoranc, overcrowding, poor sanitation and malnutrition favor transmission and increased disease burden.

Objectives: To find out the *in vitro* sensitivity of Carica papaya seed extract against clinical isolates of *Entamoeba histolytica.*

Materials and methods: *Entamoeba histolytica* isolates collected from the stool and cultured in different culture medium. Carica papaya seed extract prepared in different concentrations of 0.12 mg/ml, 0.24 mg/ml, 0.49 mg/ml, 0.98 mg/ml, 1.97 mg/ml, 3.95 mg/ml and 7.98 mg/ml using the Di-methyl sulphoxide (DMSO) solvent and incubated at 24 hours and 48 hours respectively. After incubation the isolates of viable *Entamoeba histolytica* were calculated.

Result: Viable count of the *Entamoeba histolytica* in each concentration of Carica papaya seed extract was compared to the initial concentration and with the control. After 24 hours of incubation concentration of 7.89 mg/ml shows no presence of *Entamoeba histolytica.* After 48 hours of incubation concentrations of 0.98 mg/ml, 1.97 mg/ml, 3.95 mg/ml, 7.89 mg/ml shows no presence of the clinical isolates of *Entamoeba histolytica.*

Conclusion: The results show that the *in vitro* sensitivity of Carica papaya seed extract against the Clinical isolates of *Entamoeba histolytica.*

INTRODUCTION

1.1 Carica papaya

Papaya (*Carica papaya* L.) is a popular and economically important fruit tree of tropical and subtropical countries. The fruit is consumed world-wide as fresh fruit and as a vegetable or used as processed products. The Spaniards gave it the name 'papaya' and took the plant to The Philippines, from where it expanded to Malaya and finally India in 1598 (Jaime, 2007).

When first encountered by Europeans, papaya was nick named 'tree melon'. Although the term papaya is most commonly used around the world, the fruit is also known as 'kapaya', 'lapaya', 'tapayas' in the Philippines, 'dangan dangan'. In Africa, Australia, and Jamaica, the fruit is commonly termed 'paw-paw', while other names such as 'papaw' are also heard. The French refer to the fruit as 'papaya' (Jaime, 2007).

The papaya tree (*Carica papaya*) is cultivated in tropical countries and the fruit is appreciated by its flavor as well as by its easy digestion and nutritive value (Monti, 2004).

Figure 1: *Carica Papaya* L.

1.1.2 Nomenclature

The classification of papaya has undergone many changes over the years. The genus Carica was previously classified under various plant families, including Passifloraceae, Cucurbitaceae, Bixaceae, and Papayaceae. However it is presently placed under Caricaceae, a plant family incorporating 35 latex-containing species in four genera, Carica, Cylicomorpha, Jarilla and Jacaratia (Jaime, 2007).

Carica consists of 22 species and is the only member of the Caricaceae that is cultivated as a fruit tree while the other three genera are grown primarily as ornamentals (Burkill 1966). Cylicomorpha is the only member of the Caricaceae that is indigenous to Africa, and consists of two species. Jacaratia, found in tropical America, consists of six species. Jarilla, from central Mexico consists of only one species (Jaime, 2007).

Recently, another taxonomic revision was proposed and supported by molecular evidence that genetic distances were found between papaya and other related species. Some species that were formerly assigned to Carica were classified in the genus Vasconcella. Accordingly, the classification of Caricaceae has been revised to comprise Cylicomorpha, Carica, Jacaratia, Jarilla, Horovitzia and Vasconcella, with Carica papaya the only species within the genus Carica (Jaime, 2007).

Kingdom: Plantae – Plants
Subkingdom: Tracheobionta – Vascular plants
Superdivision: Spermatophyta – Seed plants
Division: Magnoliophyta – Flowering plants
Class: Magnoliopsida – Dicotyledons
Family: Caricaceae
Genus: Carica
Species: Papaya
Common Names: Papaya, chich put, fan kua, kavunagaci, lechoso, lohong si phle, mapaza, mu kua, papailler, papaw, papaye, papayer, pawpaw tree, pepol, tinti, wan shou kuo, betik petik, gandul, katela gantung, kates, kepaya, kuntaia
Part Used: Leaves, fruit, seed, latex (Britton, 1926).

1.1.3 Description

Commonly and erroneously referred to as a "tree", the plant is properly a large herb growing at the rate of 6 to 10 ft (1.8-3 m) the first year and reaching 20 or even 30 ft (6-9 m) in height, with a hollow green or deep-purple stem becoming 12 to 16 in (30-40 cm) or more thick at the base and roughened by leaf scars. The leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal petioles 1 to 3.5 ft (30-105 cm) long, hollow, succulent, green or more or less dark purple. The blade, deeply divided into 5 to 9 main segments, each irregularly subdivided, varies from 1 to 2 ft (30-60 cm) in width and has prominent yellowish ribs and veins. The life of a leaf is 4 to 6 months. Both the stem and leaves contain copious white milky latex (Morton, 1987).

Papaya is a fast-growing tropical herb. The stem is single, straight and hollow and contains prominent leaf scars. Papaya exhibits strong apical dominance rarely branching unless the apical meristem is removed, or damaged. Palmately-lobed leaves, usually large, are arranged spirally and clustered at the crown, although some differences in the structure and arrangement of leaves have been reported with Malaysian cultivars. Generally, papaya cultivars are differentiated by the number of leaf main veins, the number of lobes at the leaf margins, leaf shape, stomata type, and wax structures on the leaf surface, as well as the color of the leaf petiole (Jaime, 2007).

Generally, the fruit is melon-like, oval to nearly round, some what pyriform, or elongated club-shaped, 6 to 20 in (15-50 cm) long and 4 to 8 in (10-20 cm) thick; weighing up to 20 lbs (9 kg). Semi-wild (naturalized) plants bear miniature fruits 1 to 6 in (2.5-15 cm) long. The skin is waxy and thin but fairly tough. When the fruit is green and hard it is rich in white latex. As it ripens, it becomes light or deep-yellow externally and the thick wall of succulent flesh becomes aromatic, yellow, orange or various shades of salmon or red. It is then juicy, sweetish and somewhat like a cantaloupe in flavor; in some types quite musky. Attached lightly to the wall by soft, white, fibrous tissue, are usually numerous small, black, ovoid, corrugated, peppery seeds about 3/16 in (5 mm) long, each coated with a transparent, gelatinous aril (Jaime, 2007; Morton, 1987).

Papaya grows best in a well drained, well aerated and rich organic matter soil, pH 5.5-6.7 (Morton, 1987). Water logging of soils often results in the death of trees within 3-4 days (Storey, 1985).

The plants are frost-sensitive and can only be grown between latitudes 32′ N and S, with optimal growth at 22-26°C and an evenly distributed rainfall of 100-150 cm. Some, however, are able to survive the high humidity of equatorial zones. The best fruit develops under full sunlight in the final 4-5 days to full ripeness on the tree (Jaime, 2007).

1.1.4 Distribution

Though the exact area of origin is unknown, the papaya is believed native to tropical America, perhaps in southern Mexico and neighboring Central America. It is recorded that seeds were taken to Panama and then the Dominican Republic before 1525 and cultivation spread to warm elevations throughout South and Central America, southern Mexico, the West Indies and Bahamas and to Bermuda in 1616. Spaniards carried seeds to the Philippines about 1550 and the papaya traveled from there to Malacca and India (Morton, 1987).

Papaya, *Carica papaya* L., is one of the major fruit crops cultivated in tropical and sub-tropical zones. Worldwide over 6.8 million tones (Mt) of fruit were produced in 2004 on about 389,990 Hector. Of this volume, 47% was produced in Central and South America (mainly in Brazil), 30% in Asia, and 20% in Africa. The papaya industry in Brazil is one of the largest world-wide that continues to show rapid growth, on a 151% increase in total area cultivated over the past decade (16,012 ha in 1990 to 40,202 ha in 2000) and a 164% increase in the quantity produced during the same period (642,581 to 1,693,779 fruits from 1990 to 2000). In 11 years, the volume exported increased 560% from 4,071 t to 22,804 t in 2001 and 38,760 t in 2005. Although papaya is mainly grown (>90%) and consumed in developing countries, it is fast becoming an important fruit internationally (Jaime, 2007).

The mountain papaya is native to Andean regions from Venezuela to Chile at altitudes between 1,800-3,000 m. The 'babaco' or 'chamburo' is commonly cultivated in mountain valleys of Ecuador; plants are slender, up to 3 m high, and pentagonal fruits reach 30 cm in length. Compared to the well known tropical papaya, C. papaya, fruits of the mountain papayas tend to be smaller in size and less succulent (Jaime, 2007). In some areas, bisexual types are in greatest demand. In South Africa, round or oval papayas are preferred (Morton, 1987).

1.1.5 Cultivation

Papayas are usually grown from seeds. Unlike the seed of many tropical species, papaya seed is neither recalcitrant nor dormant and are classified as intermediate for desiccation tolerance (Ellis et al., 1991). Germination occurs within 2-4 weeks after sowing. While seeds may be sowed directly in the orchard, some orchards are started with established seedlings (6-8 weeks after germination). Whether direct seeding or transplanting is practiced, a number of seeds or transplants are sown per planting site since the sex of a given plant cannot be determined for up to 6 months after germination (Gonsalves, 1994), although molecular methods for detection are now available (Gangopadhyay, 2007). At this time, plants are thinned to achieve the desired sex ratio and to reduce competition between plants, which would later affect fruit production. For dioecious varieties, a ratio of one male to 8-10 female plants is recommended to maximise yield whereas one bisexual plant is left in each planting position (Jaime, 2007).

Papaya trees are fast-growing and prolific and can often result in widely-separated internodes; the first fruit is expected in 10-14 months from germination and in general the fruit takes about 5 months to develop. Soil application of paclobutrazol, a growth retardant, at 1000 mg/L resulted in reduced overall height and reduced height at which first flowers bud; it did not affect the start of production or yield. Fruit production may occur following either self-pollination or cross-pollination and is affected by pollinator efficiency or abundance. Honeybees, thrips, hawk moths have been reported as pollinators of papaya. Although the floral morphology in papaya plants suggests insect pollination, various authors have indicated that wind pollination may also be important (Jaime, 2007).

1.1.6 Harvesting and Storage

Papayas are hand harvested at the color break stage or when they have started to ripen as judged by the appearance of skin yellowing. Fruits are collected in smooth surfaced plastic crates or in clean collection bags and thereafter transferred into large lug collection bins. Fruits are sorted at the field according to color stages and defects. They are subsequently washed in packing sheds and, in some, countries subjected to vapor heat treatment or double

dip hot water treatment to kill insects and their larvae (42°C x 30 min followed by 20 min or more at 49°C) (Nishijima, 1995; Jaime, 2007).

The vapor heat treatment raises the temperature of the fruit center to about 47.5°C over a period of 6-8 hours. After this treatment, fruits are cooled to 30°C in water. Hot water treatment or hot water treatment with fungicides is usually adopted to control decay. Exposure of papaya fruit to high temperatures results in the disruption of softening. The pattern of ripening related events such as the change in skin color, climacteric respiration, ethylene production, 1-aminocyclo-propane-1-carboxylic acid (AAC) content, net ethylene forming enzyme (EFE) activity and internal carotenoid synthesis are also altered by the high temperature treatments. The response of papaya to heat treatments depends on maturity, growing season and temperature changes. Chemical treatments can cause fruit damage and reduce the external fruit quality (Jaime, 2007).

Storage temperature depends on the type of papaya cultivar. The storage temperature usually ranges between 10-13.5°C. Papaya harvested at color break stage can be stored in cold storage at 7°C for 14 days and will ripen normally when transferred to room temperature. Storage below 10°C is known to cause chilling injury. Symptoms of chilling injury occur in mature green fruits or in 60% yellow fruits as skin scald, hard lumps in the pulp around the vascular bundles, water soaking of flesh and high susceptibility to decay (Jaime, 2007).

1.1.7 Chemistry

C. papaya contains many biologically active compounds. Two important compounds are chymopapain and papain which are widely known as being useful for digestive disorders and disturbances of the gastrointestinal tract. Papaya-derived papain, caricain, chymopapain, and glycine endopeptidase can survive acidic pH conditions and pepsin degradation. However, at low pH, a conformational transition that instantaneously converts their native forms into molten globules that are quite unstable and rapidly degraded by pepsin. Thus, they may need to be protected against both acid denaturation and proteolysis for them to be effective in the gut after oral administration for the control of gastrointestinal nematodes (Jaime, 2007).

Apart from papain and chymopapain, *C. papaya* contains many biologically active compounds. *C. papaya* lipase, or CPL, a hydrolase, is tightly bonded to the water-insoluble

fraction of crude papain and is thus considered as a "naturally immobilized" biocatalyst. Applications of CPL: (i) fats and oils modification, derived from the sn-3 selectivity of CPL as well as from its preference for short-chain fatty acids; (ii) esterification and inter-esterification reactions in organic media, accepting a wide range of acids and alcohols as substrates; and (iii) more recently, the asymmetric resolution of different non-steroidal anti-inflammatory drugs (NSAIDs), 2-(chlorophenoxy)propionic acids, and non-natural amino acids (Jaime, 2007).

The papaya Kunitz-type trypsin inhibitor, a 24-kDa glycoprotein, when purified, stoichiometrically inhibits bovine trypsin in a 1:1 molar ratio (Azarkan, 2006). A novel α-amylase inhibitor from *C. papaya* seeds was recently shown to be effective against cowpea weevil (Callosobru chus maculatus) (Farias, 2007).

1.1.8 Diseases

As with many tropical crops, papaya is host to various species of pests and pathogens. In 1990, Singh reported that of the 39 arthropods that infest papaya, 4 insect and mite species are major pests of papaya. More important than mite and insect pests are pathogens that reduce plant vigor and affect fruit quality. In most regions papaya, which is classified as a perennial, is grown as an annual given the reduction of productive years to 1-2 years because of parasitic infestations (Jaime, 2007).

The major pests that attack papaya foliage, fruit and roots include fruit flies, the two-spotted spider mite, the papaya whitefly (Trialeuroides varibilis), and nematodes (Jaime, 2007).

Papaya is susceptible to more than a dozen fungal pathogens. Phytophthora (Phytophthora palmivra) root and fruit rot, anthracnose (Collectricum gloerosporioides), powdery mildew (Oidium caricae) and black spot (Asperisporium caricae) are, however, the more important fungal pathogens (Zhu, 2004; Jaime, 2007).

Phytophthora rot or blight is a common disease of papaya particularly in rainy periods and in heavy, poorlydrained soils. Phytophthora palmivora, the etiological agent, attacks the fruit, stem, and roots of papaya plants. The first manifestations of root rot are seen in the lower leaves (Jaime, 2007).

1.1.9 Food uses

Ripe papayas are most commonly eaten fresh, merely peeled, seeded, cut in wedges and served with a half or quarter of lime or lemon. Sometimes a few seeds are left attached for those who enjoy their peppery flavor but not many should be eaten. The flesh is often cubed or shaped into balls and served in fruit salad or fruit cup. Firm-ripe papaya may be seasoned and baked for consumption as a vegetable. Ripe flesh is commonly made into sauce for shortcake or ice cream sundaes, or is added to ice cream just before freezing; or is cooked in pie, pickled, or preserved as marmalade or jam. Papaya and pineapple cubes, covered with sugar sirup, may be quick-frozen for later serving as dessert. Half-ripe fruits are sliced and crystallized as a sweetmeat (Morton, 1987).

Table 1: Nutrient content of ripe papaya (Jaime, 2007)

Constituent	Appropriate value	Constituent	Appropriate value	Constituent	Appropriate value
Water	89 %	Calcium	24 mg	Sodium	3 mg
Calories	39 kcal	Iron	0.1 mg	Niacin	0.34 mg
Protein	0.61 g	Phosphorous	5 mg	Pantothenic acid	0.22 mg
Fat	0.14 g	Potassium	257 mg	Vitamin A	1094 IU
Carbohydrate	9.8 g	Magnesium	10 g	Vitamin E	0.73 mg

Source: USDA Nutrient Database for Standard Reference, Release 18 (2005).

Papaya juice and nectar may be prepared from peeled or unpeeled fruit and are sold fresh in bottles or canned. In Hawaii, papayas are reduced to puree with sucrose added to retard gelling and the puree is frozen for later use locally or in mainland USA in fruit juice blending or for making jam (Morton, 1987).

Unripe papaya is never eaten raw because of its latex content. [Raw green papaya is frequently used in Thai and Vietnamese cooking.] Even for use in salads, it must first be peeled, seeded, and boiled until tender, then chilled. Green papaya is frequently boiled and served as a vegetable. Cubed green papaya is cooked in mixed vegetable soup. Green papaya is commonly canned in sugar syrup in Puerto Rico for local consumption and for export (Morton, 1987).

Green papayas for canning in Queensland must be checked for nitrate levels. High nitrate content causes detinning of ordinary cans, and all papayas with over 30 ppm nitrate must be packed in cans lacquered on the inside. Australian growers are hopeful that the papaya can be

bred for low nitrate uptake. A lye process for batch peeling of green papayas has proven feasible in Puerto Rico. The fruits may be immersed in boiling 10% lye solution for 6 minutes, in a 15% solution for 4 minutes, or a 20% solution for 3 minutes. They are then rapidly cooled by a cold water bath and then sprayed with water to remove all softened tissue. Best proportions are 1 lb (.45 kg) of fruit for every gallon (3.8 liters) of solution (Morton, 1987).

Young leaves are cooked and eaten like spinach in the East Indies. Mature leaves are bitter and must be boiled with a change of water to eliminate much of the bitterness. Papaya leaves contain the bitter alkaloids; carpaine and pseudocarpaine, which act on the heart and respiration like digitalis, but are destroyed by heat. In addition, two previously undiscovered major D1-piperideine alkaloids, dehydrocarpaine I and II, more potent than carpaine, were reported from the University of Hawaii in 1979. Sprays of male flowers are sold in Asian and Indonesian markets and in New Guinea for boiling with several changes of water to remove bitterness and then eating as a vegetable. In Indonesia, the flowers are sometimes candied (Morton, 1987).

Young stems are cooked and served in Africa. Older stems, after peeling, are grated, the bitter juice squeezed out, and the mash mixed with sugar and salt.

In India, papaya seeds are sometimes found as an adulterant of whole black pepper. Collaborating chemists in Italy and Somalia identified 18 amino acids in papaya seeds, principally, in descending order of abundance, glutamic acid, arginine, proline, and aspartic acid in the endosperm; and proline, tyrosine, lysine, aspartic acid, and glutamic acid in the sarcotesta. A yellow to brown, faintly scented oil was extracted from the sundried, powdered seeds of unripe papayas at the Central Food Technological Research Institute, Mysore, India. White seeds yielded 16.1% and black seeds 26.8% and it were suggested that the oil might have edible and industrial uses (Morton, 1987).

1.1.10 Medicinal Properties

Antibiotic: Extracts of ripe and unripe papaya fruits and of the seeds are active against gram-positive bacteria. Strong doses are effective against gram-negative bacteria. The substance has protein-like properties. The fresh crushed seeds yield the aglycone of glucotropaeolin benzyl isothiocyanate (BITC) which is bacteriostatic, bactericidal and fungicidal. A single effective does is 4-5 g seeds (Morton, 1987).

In a London hospital in 1977, a post-operative infection in a kidney-transplant patient was cured by strips of papaya which were laid on the wound and left for 48 hours, after all modern medications had failed (Morton, 1987).

Anti-Fungal: Papaya milk latex inhibits fungal growth, especially that of *Candida albicans* and thus would be useful in the treatment of skin eczema caused by this fungus (Giordani, 1991).

Anti-Fertility: Extracts of papaya seeds could be used as a contraceptive in rats, specifically two principal compounds, MCP I and ECP I (the code names of the major purified compounds of methanol and ethyl acetate subtractions of the benzene chromatographic fraction of the chloroform extract of the seeds of *C. papaya*, respectively; demonstrated that the methanol sub fraction or MSF of the seeds of C. papaya, a putative male contraceptive, could be safely used in rats as a male anti-fertility agent (Jaime, 2007).

Anti-Microbial: Extracts from fruits showed effective anti-microbial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas* sp. and Shigella sp (Jaime, 2007).

Ulcer-Protective: The effects of *Carica papaya* Linn on exogenous ulcer and histamine-induced acid secretion were studied. The latex of the un-ripened fruit of *C. papaya* was effective in protecting the exogenous ulcer. It significantly lessened the acid secretion induced by intravenous infusion of histamine in chronic gastric fistulated rats. Crystalline papain was also effective in protecting the exogenous ulcer and in decreasing the histamine-induced acid secretion in rats. The conclusion is that papain is the active principle in *C. papaya* that exerts the ulcer-protective effect (Chen, 1981).

Antioxidant: The fermented papaya preparation has the antioxidant properties. This property is related to both hydroxyls scavenging as well as iron chelating properties. This may be one of potential mechanism contributing to enhance wound healing (Mahmood, 2005).

Wound Healing: Aqueous extracts of *Carica papaya* leaves accelerates the progression of wound healing activity. Tropical treatment of mush pulp of *Carica papaya,* which contains papain and chymopapaine for pediatric infected burns, appeared to be effective for desloughing necrotic tissue, preventing infection and providing a granulating to wound (Mahmood, 2005).

Collagen, the major protein of the extracellular matrix, is the component that ultimately liberates free hydroxyl proline and its peptides. Therefore, measurement of hydroxyproline can be used as an index for collagen turn over. Increase in hydroxyproline content indicates increased collagen synthesis corresponding to enhanced wound healing (Gurung, 2009).

Anti-amoebic activity: The seeds and fruits demonstrated in vitro anti amoebic activity. (Okeniyi, 2007; Jaime, 2007).

Burn: The fresh leaves and extract of the plant *Carica papaya* are a traditional herbal treatment in developing countries for burns and skin infection. It gives the protective action (Mahmood, 2005).

Asthma: The smoke from dried papaya leaves relieves asthma attacks (Jaime, 2007).

Stomachic: The dried leaf infusion is taken for stomach troubles in Ghana and it is used as a purgative (Jaime, 2007).

Fibrinolytic: This properties help to remove slough from wounds. There may also be a proteolytic effect on bacteria, resulting from the production of a coagulum that immobilize microorganisms and protects the host against bacterial infection (Mahmood, 2005).

1.1.11 Folk Uses

In tropical folk medicine, the fresh latex is smeared on boils, warts and freckles and given as a vermifuge In India; it is applied on the uterus as an irritant to cause abortion. The unripe fruit is sometimes hazardously ingested to achieve abortion. Seeds, too, may bring on abortion. They are often taken as an emmenagogue and given as a vermifuge. The root is ground to a paste with salt, diluted with water and given as an enema to induce abortion. A root decoction is claimed to expel roundworms. Roots are also used to make salt (Morton, 1987).

Crushed leaves wrapped around tough meat will tenderize it overnight. The leaf also functions as a vermifuge and as a primitive soap substitute in laundering. Dried leaves have been smoked to relieve asthma or as a tobacco substitute. Packages of dried, pulverized leaves are sold by "health food" stores for making tea, despite the fact that the leaf decoction is administered as a purgative for horses in Ghana and in the Ivory Coast it is a treatment for genito-urinary ailments. The dried leaf infusion is taken for stomach troubles in Ghana and they say it is purgative and may cause abortion (Morton, 1987).

1.2 *Entamoeba histolytica*

Entamoeba histolytica is the etiological agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40–50 million symptomatic cases of amoebiasis occur annually and 70,000 to 100,000 deaths due to this infection. There are two distinct, but morphologically identical species of Entamoeba: *Entamoeba histolytica*, is pathogenic (Walsh, 1986).

Amoebiasis is one of the most common health problems in the world. It is estimated that annually about 480 million people develop clinical amoebiasis and at least 40,000 die (Ghosh, 1998).

Entamoeba histolytica-associated infection is a public health problem in many developing countries, where it is responsible for severe morbidity and mortality (Ghosh, 2000).

Nitroimidazoles are highly potent anti-protozoal agents with well-established indications. The drugs which are used in the amoebiasis are metronidazole, tinidazole, ornidazole, diloxanide furoate, emitine, chloroquine, secnidazole etc (Devendra Bansal, 2006).

Since the discovery of metronidazole, a number of this group, the treatment of amoebiasis has been revolutionized (Rossignol, 1984; Rosenblatt, 1987).

Worldwide there is the higher incidence of amebiasis in developing countries. Diarrheal diseases continue to be 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 her fifth birthday (Haque, 2003).

1.2.2 Definition

According to the World Health Organization (WHO) and Pan American Health Organization (PAHO), *Entamoeba histolytica* is the protozoan parasite causing amebiasis, regardless of associated symptomatology (Pritt, 2008).

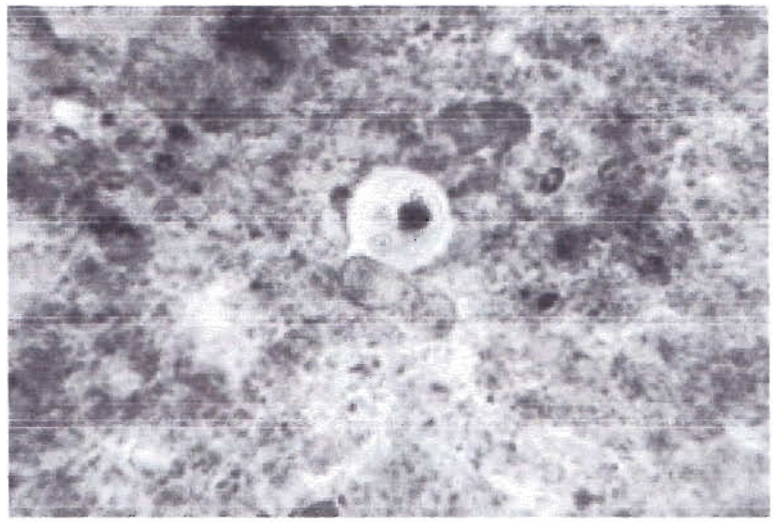

Figure 2: *Entamoeba histolytica* cyst.

1.2.3 Scientific classification of *Entamoeba histolytica*

Common name: Ameba

Kingdom: Protista

Phylum: Protozoa

Class: Lobosea

Order: Amoebida

Family: Entamoebidae

Genus: *Entamoeba*

Species: *histolytica*

1.2.4 Historical Background of *Entamoeba histolytica*

The trophozoite form of *Entamoeba histolytica* was described by Losch in 1875 from organisms found in a patient with chronic dysentery. The clinical evidence of the isolation of this organism with dysentery was described by Councilman and LaFleur in 1891.

Quincke and Roos described the cyst form in 1893, and Schaudinn named the organism *Entamoeba histolytica*in 1903. In 1925, Brumpt showed that quadrinucleate cysts of *Entamoeba histolytica* were a complex of two species. He differentiated a pathogenic, invasive form from a nonpathogenic, noninvasive one that he called Entamoeba dispar. Clark and Diamond reexamined Brumpt's claim in light of recent biochemical, immunological, and genetic studies. They redescribed the invasive parasite, retaining the name *Entamoeba histolytica* and set it apart from the non invasive parasite, E.dispar (Marshal, 1997).

1.2.5 Characteristics of *Entamoeba histolytica*

Genus and Species	*Entamoeba histolytica*
Etiologic Agent of	Amoebiasis; Amoebic dysentery; Extraintestinal Amoebiasis, usually Amoebic Liver Abscess = "anchovy sauce"); Amoeba Cutis; Amoebic Lung Abscess ("liver-colored sputum")
Infective stage	Cyst
Definitive Host	Human
Portal of Entry	Mouth
Mode of Transmission	Ingestion of mature cyst through contaminated food or water
Habitat	Colon and Cecum
Pathogenic Stage	Trophozoite
Locomotive apparatus	Pseudopodia ("False Foot")
Motility	Active, Progressive and Directional
Nucleus	'Ring and dot' appearance: peripheral chromatin and central karyosome
Mode of Reproduction	Binary Fission
Pathogenesis	Lytic necrosis (it looks like "flask-shaped" holes in Gastrointestinal tract sections (GIT)

Type of Encystment	Protective and Reproductive
Lab Diagnosis	Most common is Direct Fecal Smear (DFS) and staining (but does not allow identification to species level); Enzymeimmunoassay (EIA); Indirect Hemagglutination (IHA); Antigen detection – monoclonal antibody; Polymerase Chain Reaction PCR for species identification. Culture: From faecal samples - Robinson's medium, Jones' medium
Trophozoite Stage	
Pathognomonic/Diagnostic Feature	Ingested RBC; distinctive nucleus
Cyst Stage	
Chromatoidal Body	'Cigar' shaped bodies (made up of crystalline ribosomes)
Number of Nuclei	1 in early stages, 4 when mature
Pathognomonic/Diagnostic Feature	'Ring and dot' nucleus and chromatoid bodies

I. *Entamoeba histolytica* is an anaerobic parasitic protozoan, part of the genus *Entamoeba*. It infects predominantly humans and other primates. When cysts are ingested in fecally contaminated food or water. After ingestion and passage through the stomach, the organism excysts and emerges in the large intestine as an active trophozoite. Trophozoites multiply by simple division and encyst as they move further down the large bowel. Cysts are then expelled with the feces and may remain viable in a moist environment for weeks to months (Pritt, 2008).

II. *Entamoeba histolytica*, as its name suggests (*histo–lytic* = tissue destroying), causes disease; infection can lead to amoebic dysentery or amoebic liver abscess (Ramamurti, 1973).

1.2.6 Life Cycle of *Entamoeba histolytica*

The uninucleate trophozoite of *Entamoeba histolytica* inhabits the colon and rectum and at times, the lower end of the small intestine of the humans and other primates (Figure: 3.2). The motile trophozoite measures an average 25μm in diameter (range 15 to 60 μm) and is typically monopodial, producing one large, finger-like pseudopodium at a time (Figure: 3.1a). The single pseudopodium erupts and is withdrawn so rapidly that, in prepared slides, trophozoites with pseudopodia extended are rarely seen. The cytoplasm is differentiated into two zones: a clear, refractile ectoplasm and a finely granular endoplasm in which food vacuoles occur. Such vacuoles may contain host erythrocytes, leukocytes, and epithelial cells, as well as bacteria and other intestinal material. Trophozoites proliferate mitotically (binary fission) within the host's gut (Burton, 2005).

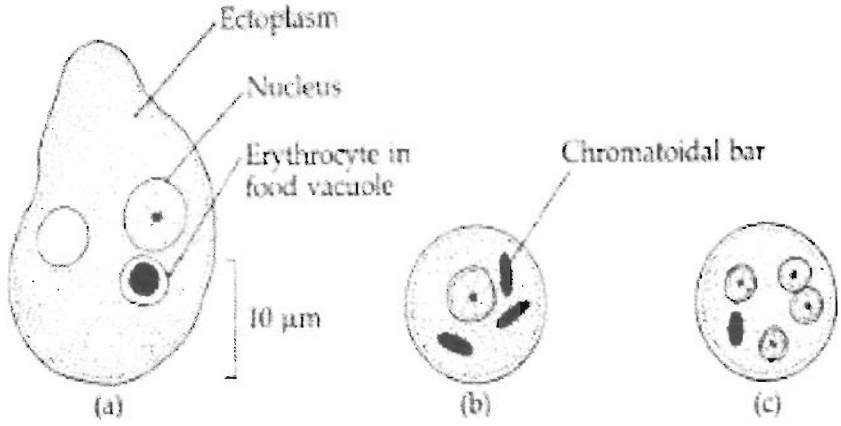

Figure 3.1: *Entamoeba histolytica* (a) Trophozite. (b) Early cysts with chromatoidal bars. (c) Later cyst (Burton 2005).

The nucleus is of special importance in differentiating *E. histolytica* from most of the other intestinal amoebae. In saline preparations, the nucleus has a barely discernible nuclear envelope. However, in stained preparations, the vesicular nucleus is clearly visible. Ideally, it has a well-defined envelope, lined on the inner surface with fine peripheral chromatin granules and a minute, centrally located endosome. Unfortunately, this "ideal" morphology is not confined to *E. histolytica*. Often, other species of *Entamoeba*, notably *E. dispar*, show similar nuclear morphologies (Burton, 2005).

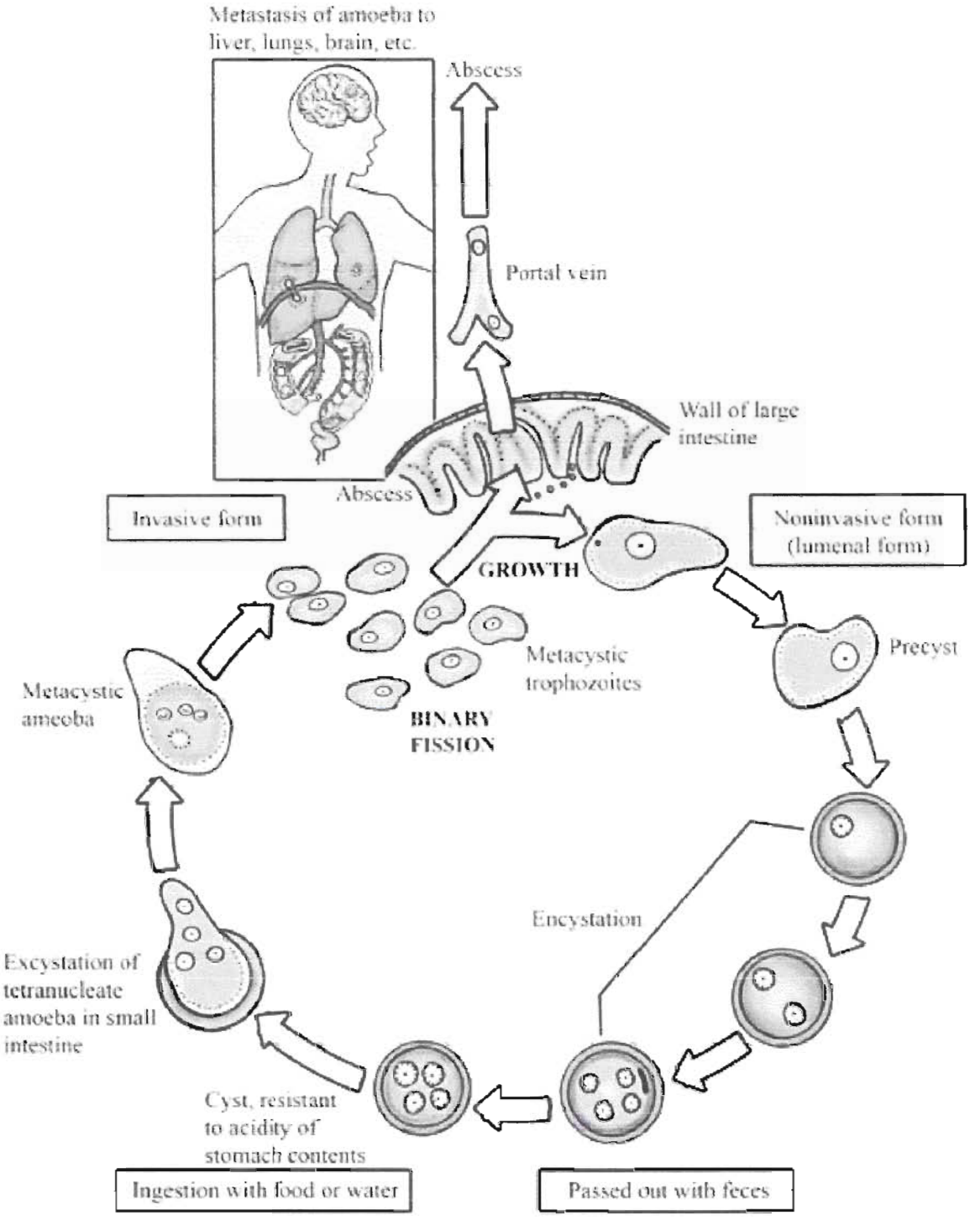

Figure 3.2: Life cycle of *Entamoeba histolytica* (Burton, 2005).

Under certain adverse environmental and/or physiological circumstances, throphozoites assume precystic characteristics by becoming more spherical and as food vacuoles are extruded, shrinking in size. Pseudopodia, if formed, are sluggishly extended and there appears to be no progressive movement. Encystation begins with the secretion by the precyst

trophozoite of a thin, surrounding hyaline membrane to form a cyst wall. At this stage, the cyst is usually spherical, an average 12μm in diameter (range 10 to 20μm) with a single nucleus. At times, glycogen masses and chromatoidal bars may be observed (Figure: 3.1b). The latter structures are considered to be deposits of nucleic acids such as RNA and may vary in shape but always have smoothly rounded ends in *E. histolytica.* This characteristic distinguishes *E. histolytica* cysts from those of *E. coli,* in which the chromatoidal bars have jagged or splintered ends. The nucleus undergoes two mitotic divisions to produce four vesicular nuclei in the mature cyst of *E. histolytica* (Figure: 4.1c). Such cysts represent the infective from and pass out of the host in feces, after which the glycogen and chromatoidal substances are slowly metabolized and disappear (Burton, 2005).

Cysts of *E. histolytica* are highly resistant to desiccation and even to certain chemicals. Cysts in water can survive for a month, while those in feces on dry hand can survive for more than 12 days; they tolerate temperatures up to a thermal death point of 50°C (Burton, 2005).

When food or water contaminated with *E. histolytica* cysts is ingested by a host, the cysts pass through the stomach to the ileum, where encystation occurs. The neutral or slightly alkaline environment afforded by the small intestine is apparently requisite for this phenomenon. However, *in vitro* studies suggest that excystation does not occur immediately; cysts placed in fresh culture (Burton, 2005).

Second only to the liver in frequency as an extra intestinal site are the lung. Pulmonary Amoebiasis is relatively rare, however and when seen is probably a direct result of hepatic infection. Unlikely most amoebic abscesses which are commonly bacteriological sterile, the pulmonary abscess is often vulnerable to secondary bacterial infections (Burton, 2005).

Immunity to infection with *E. histolytica* is associated with a mucosal IgA response against the carbohydrate-recognition domain of the Gal/GalNAclectin. Over a one-year period, children with this response had 86 percent fewer new infections than children without this response. Cell-mediated responses have been described in patients with amebic liver abscess, characterized by lymphocyte proliferation and lymphokine secretion that is amebicidal in vitro. One study found that in patients with liver abscess, the prevalence of the class II MHC haplotype HLA-DR3 is increased by a factor of more than three, suggesting a role of CD4+ T-cell function in the outcome of the disease. It is noteworthy, however, that the acquired immunodeficiency syndrome pandemic has not led to increases in invasive amebiasis,

although asymptomatic intestinal colonization is undoubtedly common. In fact, in the murine model of amebic colitis, the depletion of CD4+ T cells decreases the severity of the disease (Haque, 2003).

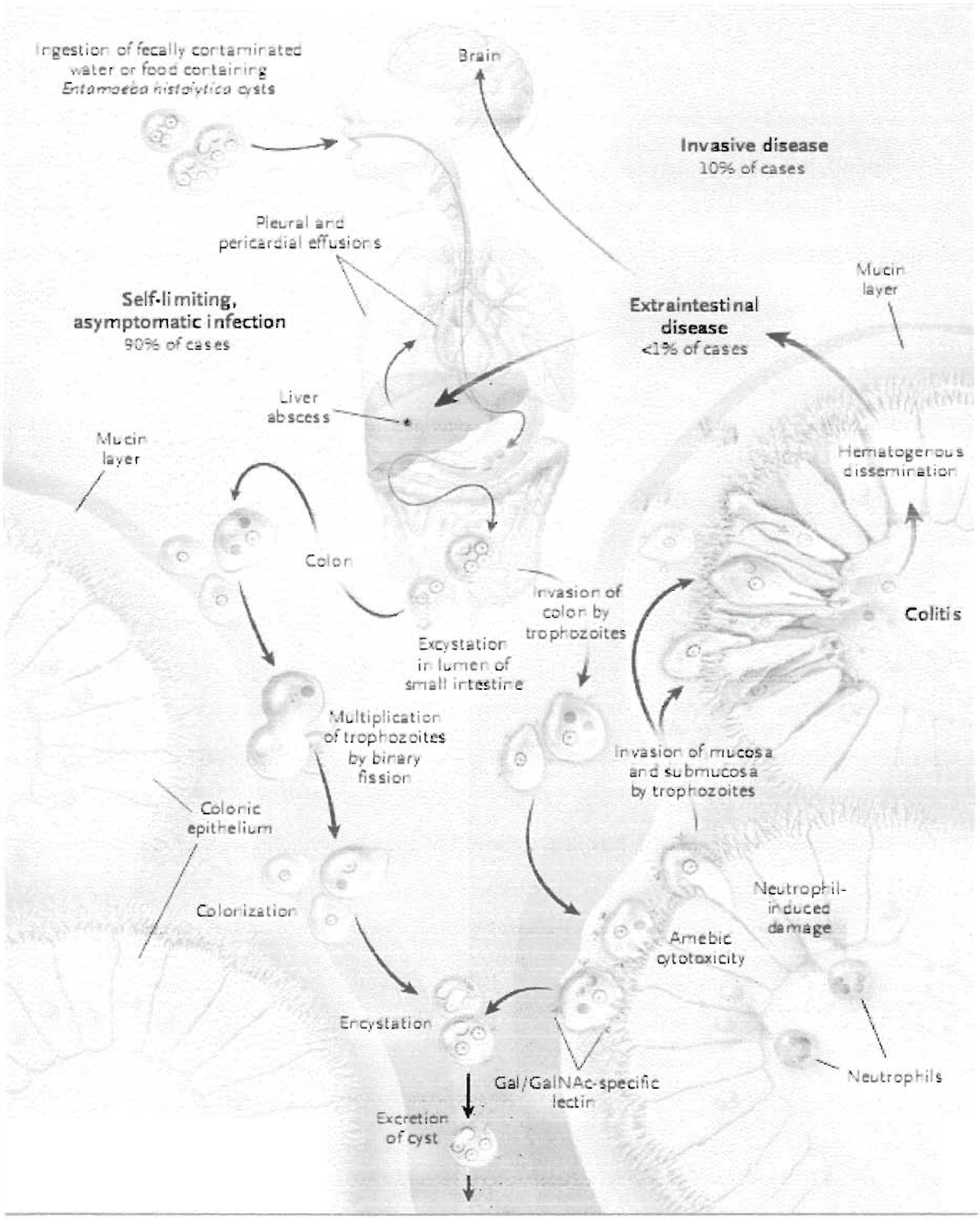

Figure 4: Pictorial Presentation of Life Cycle (Haque, 2003).

1.2.7 Incidence with *Entamoeba histolytica*

Infections with *Entamoeba histolytica* occur worldwide, and it has been suggested that 12% of the world's population is infected with this organism. About 10% of those infected every year have clinical symptoms; 80-98% of these patients have symptoms related to the intestinal mucosa and in the remaining 2-20%, the amoeba invades beyond the intestinal mucosa. Once the infection is cleared, recurrence of invasive colitis or amoebic abscess is unusual. With the exceptions of malaria-causing plasmodia and Schistosomes, Entimoeba histolytica causes more deaths than any other parasites. Groups at high risk for amoebas in developed countries include travelers, immigrants, migrant workers, immune compromised individuals, institutionalized individuals, and sexually active male homosexuals (Marshall, 1997).

1.2.8 Symptoms

The incubation period may vary from a few days to months, depending on the area of endemicity. Normally, the time frame ranges from 1-4 months. *Entamoeba histolytica* is unique among the intestinal amoeba because it is able to invade tissues. The presentation of disease may range from a symptomatic infection to a disseminated fatal disease. The 4 major intestinal syndromes caused by infection are asymptomatic colonization; acute amebic colitis, which usually presents with lower abdominal pain, frequent bloody stools over a period of several weeks, and fever, fulminant colitis, which occurs most often in children who present with defuse abdominal pain, profuse bloody diarrhea and fever; and ameboma, which as a completely asymptomatic lesion or as tender mass accompanied by symptomatic dysentery (Marshall, 1997).

1.3 Amoebiasis

Amoebiasis is one of the most common health problems in the world. It is estimated that annually about 480 million people develop clinical Amoebiasis and at least 40,000 die (Walsh, 1998).

Invasive Amoebiasis generally induces a systemic humoral immune response demonstrable about 1 week after the onset of symptoms (Ortiz, 1990; Ortiz, 1980).

Anti-amoebic antibodies persist even after invasive amoebiasis has healed or after subclinical amoebic infection has disappeared (Knobloch, 1983).

Amoebiasis is an infectious disease caused by a one-celled parasite called *Entamoeba histolytica*, which causes both intestinal and extraintestinal infections. Two species of Entamoeba are morphologically indistinguishable: *Entamoeba histolytica* is pathogenic and *Entamoeba dispar* harmlessly colonizes the colon. Amoebas adhere to and kill the cells of the colon and cause dysentery with blood and mucus in the stool. Amoebas also secrete substances called proteases that degrade lining of the colon and permit invasion into the bowel wall and beyond. Amoebas can spread via the circulation to the liver and cause liver abscesses. The infection may spread further by direct extension from the liver or through the bloodstream to the lungs, brain, and other organs (Kumar, 1999).

Amoebiasis can affect people living anywhere in the world, but is most common where living conditions are crowded or there is poor hygiene and sanitation. Amoebiasis is common in parts of Africa, Central America, South America, India and Southeast Asia. About 500 million people worldwide are believed to carry *Entamoeba histolytica* in their intestines. Amoebiasis is believed to cause between 40,000 and 100,000 deaths worldwide each year (Zaki, 2006; Bansal, 2004).

1.3.2 Pathogenesis

Cysts of *E. histolytica* are ingested in water or uncooked food contaminated by human faces. Lettuce is a common vehicle of infection. In the colon vegetative trophozoite forms emerge from the cysts. The parasite may invade the mucous membrane of the large bowl, producing lesions that are maximal in the caecum but found as far down as the anal canal. These are

flask-shaped ulcers varying greatly in size and surrounded by healthy mucosa. A mass in the rectum or a filling defect in the colon on radiography is a complication. This responds well to anti-amoebic treatment so should differentiated from colonic carcinoma (Niclolas, 2006).

Amoebic ulcers may cause severe haemorrhage but rarely perforate the wall. Cutaneous amoebiasis causes progressive genital, perianal or peri-abdominal surgical wound ulceration (Niclolas, 2006).

Ingestion of the quadrinucleate cyst of *E. histolytica* from fecally contaminated food or water initiates infection. This is a daily occurrence among the poor in developing countries and is a threat to inhabitants of developed countries, as the epidemic linked to contaminated municipal water supplies in Tbilisi, Republic of Georgia, demonstrates. Excystation in the intestinal lumen produces trophozoites that use the galactose and N-acetyl-d-galactosamine (Gal/GalNAc) specific lectin to adhere to colonic mucins and thereby colonize the large intestine. The reproduction of trophozoites has no sexual cycle, and the overall population of *E. histolytica* appears to be clonal. Aggregation of amebae in the mucin layer most likely triggers encystation by means of the Gal/Gal-NAc-specific lectin. Cysts excreted in stool perpetuate the life cycle by further fecal–oral spread (Haque, 2003).

Colitis results when the trophozoite penetrates the intestinal mucous layer, which otherwise acts as a barrier to invasion by inhibiting amebic adherence to the underlying epithelium and by slowing trophozoite motility. Invasion is mediated by the killing of epithelial cells, neutrophils, and lymphocytes by trophozoites, which occur only after the parasite lectin engages host N-acetyl-d-galactos-amine on O-linked cell-surface oligosaccharides. The interaction of the lectin with glycoconjugates is stereospecific and multivalent. The identity of the high-affinity intestinal epithelial-cell receptor is unknown. Secretion by the ameba of amoebapore, a 5-kD pore-forming protein, may contribute to killing. Activation of human caspase 3, a distal effectors molecule in the apoptotic pathway, occurs rapidly after amebic contact, and caspases are required for cell killing in vitro and for the formation of amebic liver abscesses in vivo (Haque, 2003).

Interaction of the parasite with the intestinal epithelium causes an inflammatory response marked by the activation of nuclear factor kB and the secretion of lymphokines. The development of this epithelial response may depend on trophozoite virulence factors such as cysteine proteinase and leads to intestinal abnormalities through neutrophil-mediated damage.

Neutrophils can also be protective, however, in that activation of neutrophils or macrophages by tumor necrosis factor a or interferon g kills amebae in vitro and limits the size of amebic liver abscesses. In contrast to the intense inflammatory response typical of early invasive amebiasis, inflammation surrounding well-established colonic ulcers and liver abscesses is minimal, given the degree of tissue damage (Haque, 2003).

During chronic infection, *E. histolytica* evades the host immune response in several ways. The Gal/GalNAc-specific lectin has sequence similarity and antigenic cross-reactivity to CD59, a human leukocyte antigen that prevents the assembly of the complement C5b–C9 membrane attack complex. Amebic cysteine proteinases rapidly degrade the complement anaphylatoxins C3a and C5a. The cysteine proteinases also degrade secretory IgA and serum IgG, possibly protecting amebae from opsonization. Finally, amebae appear to suppress both the macrophage respiratory burst and antigen presentation by class II major-histocompatibility-complex (MHC) molecules (Haque, 2003).

1.3.3 Diseases and Symptoms

Amoebiasis is first and foremost an intestinal disease. Contamination occurs via the digestive tract, the colon being the seat of infection.

Amoebic liver disease: This is the most common form of invasive Amoebiasis. This can be found in all age groups, but are 10 times more frequent in adults than in children. Fever is present in most cases; it varies between 38-40^{O}C, Anorexia, Weight loss, Vomiting and Fatigue may all be present as symptom (Gordon, 2009).

Peritoneal amoebiasis: This is caused by the rupture of a hepatic liver abscess or, less frequently, by perforation of the ceaecum. It is characterized by sudden increase in abdominal pain (Gordon, 2009).

Pericardial amoebiasis: Pericardial involvement is the most serious complication of an amoebic liver abscess. It occurs in less than 1% of all amoebic liver abscesses, especially of the left lobe (Gordon, 2009).

Pleuropulmonary amoebiasis: Invasion of the pleural cavity is most commonly due to extension from a liver abscess and occurs in <1% of those with amoebic dysentery. The first

clinical symptoms are those of the liver abscess, followed by severe pain in the lower chest, often radiating to the right shoulder, there may be non productive cough (Gordon, 2009).

Cerebral amoebiasis: Symptoms depend on the site and size of the lesion as many as 50% of patients may have abrupt onset of symptoms and die from cerebellar involvement or rupture within 12-72hours (Gordon, 2009).

Genitourinary amoebiasis: Those people having renal amoebiasis usually respond well to aspirating and medical therapy. Genital lesions also occur infrequently and are usually caused by fisulates from a liver abscess or rectocolitis. Typically lesions are painful punched-out ulcers with profuse discharge (Gordon, 2009).

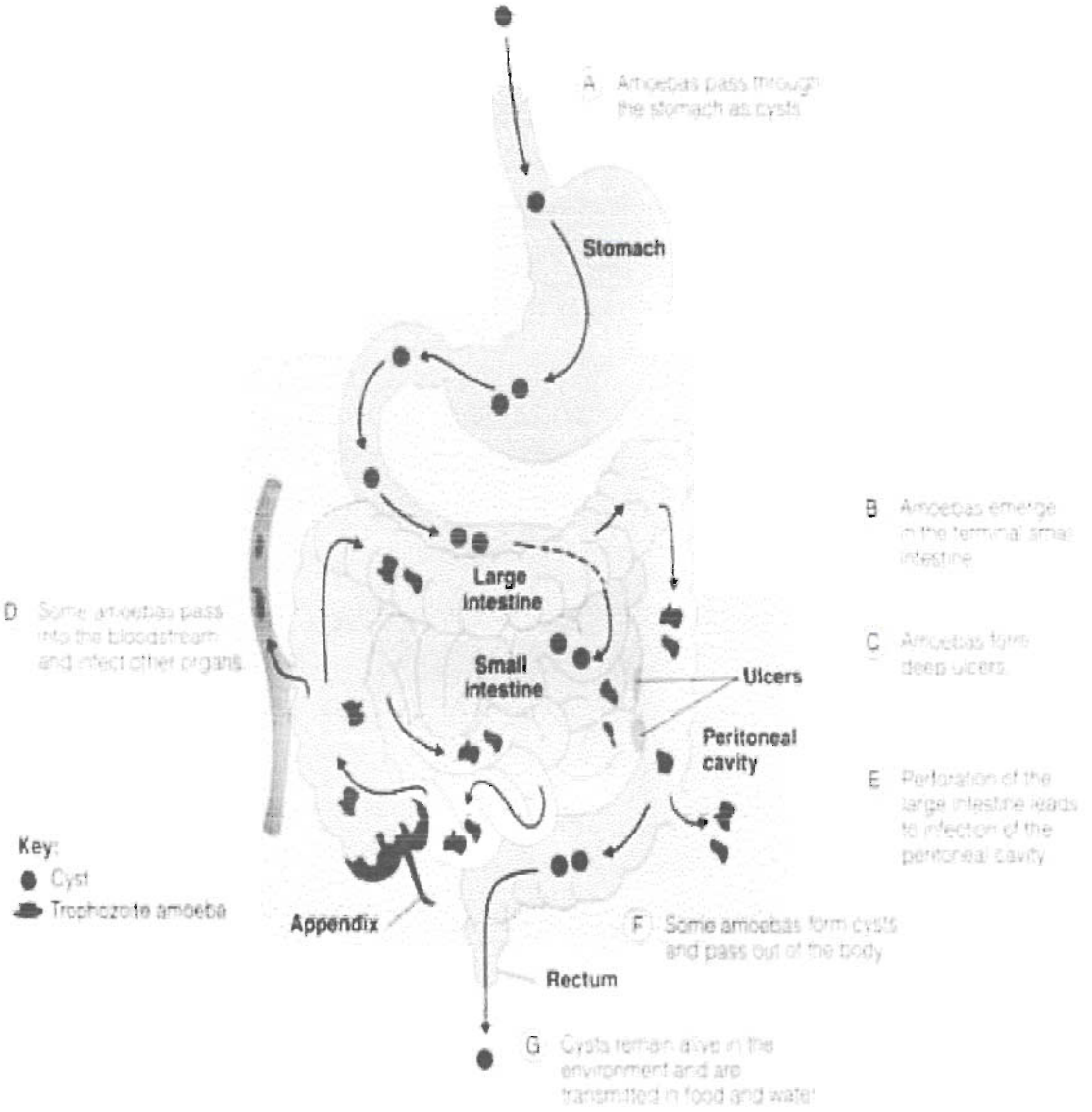

Figure 5: The course of Amoebiasis due to *Entamoeba histolytica* (Jeffrey, 2001).

1.3.4 Diagnosis

Clinically, it is desirable to definitively distinguish *E. histolytica* from *E. dispar* and *E. moshkovskii* because, of the 3, it is the only proven human pathogen. The diagnosis of invasive amebiasis is usually suggested by the patient's presenting symptoms, exposure history, and radiologic findings but should be confirmed with microbiological laboratory results. Many laboratory methods exist for identification of *E histolytica*, *E dispar*, and/or *E moshkovskii*, and the clinician should be aware that tests vary considerably in price, sensitivity, specificity, and the ability to definitively differentiate among the 3 species (Pritt, 2008).

Light microscopic examination of fecal specimens is often the first step in diagnosis; the characteristic trophozoites and cysts can often be identified through direct, concentrated, and/or permanently stained smears (Figure 6) (Pritt, 2008).

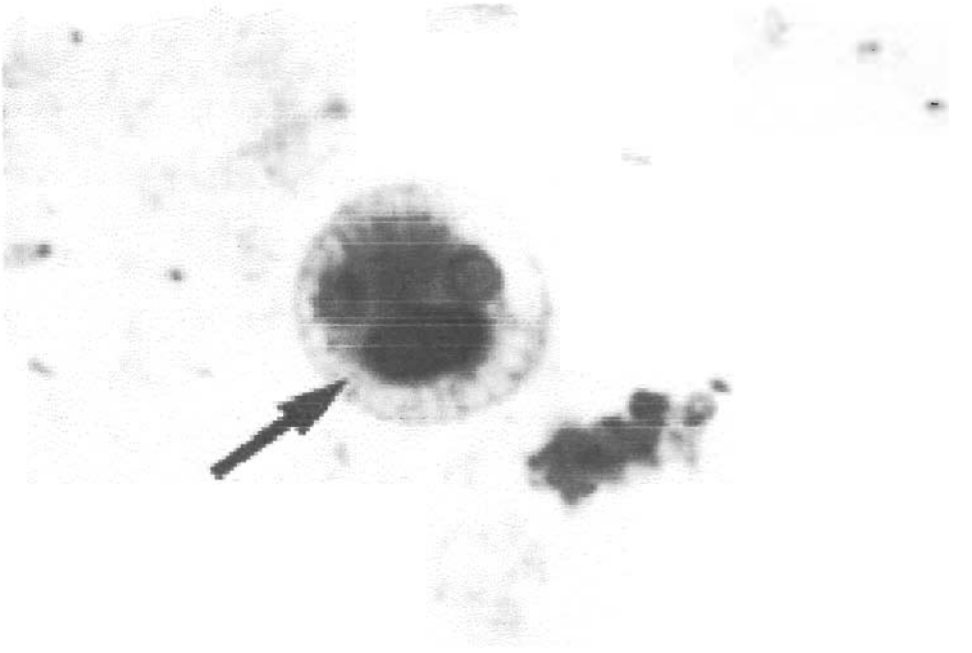

Figure 6: Classic cyst morphology (Pritt, 2008).

Because organisms may appear intermittently, current recommendations call for submission of 3 stool specimens on different days during a period of 10 days. As mentioned previously, stool specimens from patients with disseminated disease may not contain cysts and trophozoites, despite repeated examinations (Pritt, 2008).

If stool cannot be examined in the fresh state (within 15 minutes) for motile trophozoites, then it should be placed immediately in an appropriate fixative to prevent deterioration of organisms. Unfortunately, microscopy alone cannot differentiate *E histolytica* from *E dispar* and *Emoshkovskii*; additional tests are required for definitive speciation. The rare exception is when trophozoites containing ingested red blood cells are identified; they are strongly (but not definitively) indicative of invasive amebiasis. Trophozoites may also be identified in intestinal biopsy specimens, scrapings, or aspirates, allowing a diagnosis of amebiasis to be made if mucosal invasion and ulceration are also observed (Pritt, 2008).

Serologic tests detect the presence of species-specific antibodies in the patient's serum. They are particularly useful in nonendemic countries where prevalence is low and have a good sensitivity and specificity for detecting invasive intestinal disease. They are also the test of choice for ALA because titers are typically high and test sensitivities and specificities exceed 95% with most assays (Pritt, 2008).

Fecal antigen detection tests use specific monoclonal or polyclonal antibodies to detect E histolytica antigens. They are rapid, highly sensitive, and widely used in the diagnostic laboratory. Antigen tests are useful for confirming microscopic findings and providing a diagnosis in patients with negative fecal smear results (Pritt, 2008).

The highest sensitivity and specificity for the diagnosis of *E histolytica* are offered by DNA-based tests. Many assays are available, including conventional and real-time polymerase chain reaction formats; however, they are currently used primarily by research and reference laboratories. Like most molecular amplification assays, they remain impractical for resource-limited settings because of their equipment, personnel, and facility requirements (Pritt, 2008).

1.3.5 Treatment

WHO/PAHO recommendations state that, when possible, *E histolytica* should be differentiated from morphologically similar species and treated appropriately. Given the small but substantial risk of invasive disease and the potential to transmit the infection to others, WHO/PAHO recommends treating all cases of proven *E histolytica*, regardless of symptoms. If *E dispar* is the only species identified, then no treatment should be given and other causes should be sought as appropriate (Pritt, 2008).

The medications recommended to treat confirmed amebiasis vary with clinical manifestation. Asymptomatic intestinal infection with E histolytica should be treated with luminal amebicides, such as paromomycin and diloxanide furoate. These medications will eradicate the luminal amebae and prevent subsequent tissue invasion and spread of the infection through cysts. Paromomycin, more widely available in the United States, has the advantage of not being absorbed in the bowel. Abdominal cramps and nausea are the most commonly reported adverse effects. A 10-day course at 30 mg/kg per day (divided into 3 daily doses) is typical. Some recommend follow-up stool examination to confirm eradication of cysts (Pritt, 2008).

Like amebic colitis, ALA typically responds well to a 5- to 10-day course of metronidazole, which should also be followed with a luminal amebicide (Pritt, 2008).

Amoebae rarely disseminate beyond the portal circulation. Given the small number of cases, no definitive treatment guidelines are available for management of extra intestinal, extrahepatic disease. As mentioned previously, infections with *E dispar* do not require treatment. Less is known about *E moshkovskii*, but it is likely that this infection also would not require treatment in most cases (Pritt, 2008).

Table 2: Drug Therapy for the Treatment of Amoebiasis.

Drug	Adult Dosage	Pediatric Dosage	Side Effects
Amebic liver abscess			
		give	
Metronidazole	750 mg orally 3 times a day for 7–10 days	35–50 mg/kg of body weight/ day in 3 divided doses for 7–10 days	Primarily gastrointestinal: anorexia, nausea, vomiting, diarrhea, abdominal discomfort, or unpleasant metallic taste; disulfuram-like intolerance reaction with alcohol; rarely, neurotoxicity, including seizures, peripheral neuropathy, dizziness, confusion, irritability
		or	
Tinidazole†	800 mg orally 3 times a day for 5 days	60 mg/kg/day (maximum, 2 g) for 5 days	Primarily gastrointestinal and disulfuram-like intolerance reaction as for metronidazole
		followed by a luminal agent	
Paromomycin	25–35 mg/kg/day in 3 divided doses for 7 days	25–35 mg/kg/day in 3 divided doses for 7 days	Primarily gastrointestinal: diarrhea, gastrointestinal upset
		or second-line agent	
Diloxanide furoate‡	500 mg orally 3 times a day for 10 days	20 mg/kg/day in 3 divided doses for 10 days	Primarily gastrointestinal: flatulence, nausea, vomiting, pruritus, urticaria
Amebic colitis			
		give	
Metronidazole	750 mg orally 3 times a day for 7–10 days	35–50 mg/kg/day in 3 divided doses for 7–10 days	As for amebic liver abscess
		followed by a luminal agent (as for amebic liver abscess)	
Asymptomatic intestinal colonization			
		give	
Paromomycin	25–35 mg/kg/day in 3 divided doses for 7 days	25–35 mg/kg/day in 3 divided doses for 7 days	Primarily gastrointestinal: diarrhea, gastrointestinal upset
		or second-line agent	
Diloxanide furoate‡	500 mg orally 3 times a day for 10 days	20 mg/kg/day in 3 divided doses for 10 days	Primarily gastrointestinal: flatulence, nausea, vomiting, pruritus, urticaria

* The information is updated annually by the *Medical Letter on Drugs and Therapeutics* at http://www.medletter.com/html/prm.htm#Parasitic.
† This drug is not yet available in the United States.
‡ This drug is not available in the United States.

1.3.6 Prevention

Food and water contaminated with feces containing the cysts of *E. histolytica* are the most common vehicles for transmission. Prevention, therefore, depends on interruption of the contamination-in gestation cycle. One such measure is the boiling or iodination of drinking water in endemic areas. In many areas, fruits and vegetables become contaminated when human excrement is used as fertilizer. Transmission of *E. histolytica* by infected food handlers can be controlled by local ordinances requiring periodic physical examinations, including stool examination, for all food handlers (Burton, 2005).

1.3.7 Nature of the Disease

Infections can sometimes last for years. Symptoms take from a few days to a few weeks to develop and manifest themselves, but usually it is about two to four weeks. Symptoms can range from mild diarrhea to dysentery with blood and mucus. The blood comes from amoebae invading the lining of the intestine. In about 10% of invasive cases the amoebae enter the bloodstream and may travel to other organs in the body. Most commonly this means the liver, as this is where blood from the intestine reaches first, but they can end up almost anywhere (David, 2003).

Onset time is highly variable and the average asymptomatic infection persists for over a year. It is theorized that the absence of symptoms or their intensity may vary with such factors as strain of amoeba, immune response of the host, and perhaps associated bacteria and viruses (David, 2003).

In asymptomatic infections the amoeba lives by eating and digesting bacteria and food particles in the gut, a part of the gastrointestinal tract.It does not usually come in contact with the intestine itself due to the protective layer of mucus that lines the gut. Disease occurs when amoeba comes in contact with the cells lining the intestine. It then secretes the same substances it uses to digest bacteria, which include enzymes that destroy cell membranes and proteins. This process can lead to penetration and digestion of human tissues, resulting first in flask-shaped ulcers in the intestine. *Entamoeba histolytica* ingests the destroyed cells by phagocytosis and is often seen with red blood cells inside when viewed in stool samples.

Especially in Latin America, a granulomatous mass (known as an amoeboma) may form in the wall of the ascending colon or rectum due to long-lasting immunological cellular response, and is sometimes confused with cancer (David, 2003).

AIM & OBJECTIVE

2.1 Aim of the Study

In the perfect world amoebiasis would be prevented eradicating fecal contamination of food and water. However, providing safe food and water for the children in developing countries like Bangladesh would require massive societal changes and monetary investments.

An effective herbal agent would be much less costly, and there are several reasons to indicate that an herbal agent is a desirable and feasible.

There are not so many researches carried out to find out the herbal agent to treat this disease and not all the plants have anti-amoebic properties. Natural agent would have fewer side effects than the synthetic agent but more effective than the synthetic agent.

Therefore, the present study was aimed to find out the *in vitro* sensitivity of clinical isolates of *Entamoeba histolytica* to carica papaya seed extract.

Determinations of the impact of Carica papaya seed extract on viable and non viable counts of *Entamoeba histolytica* are also a major objective of this study.

2.2 Significance of the study

About 90 percent of infections are asymptomatic and the remaining 10 percent produces a spectrum varying from dysentery to amoebic liver abscess. In Bangladesh there are some pharmaceutical companies making the antiamoebics. The effects and the side effects of those drugs are known to be costly for the people who are living under poverty line in our country.

Some of the synthetic drugs have lower efficacy, which can results the patients uncured or resistant to the drug forever. Indiscriminate use of drugs and different efficacy of a single drug treated with a patient at different times may lead to the physiological problems.

Extract of Carica papaya seed is a potential antiamoebic preparation obtained from natural source. From some study it is observed that by using the seed extract the incidence of amoebic dysentery reduces effectively. It is found that the seed extract of Carica papaya is safer and less toxic than synthetic drugs in many ways because it is obtained from plant source. Researches on the herbal medicine have huge impact as it for less expensive, less side effects, more efficiency and even the rural people can easily get the treatment.

MATERIALS AND METHOD

Materials and Method

3.1 Research design

In vitro sensitivity of Carica papaya extract against clinical isolates of *Entamoeba histolytica*.

3.2 Clinical isolates

Clinical isolates from patients attending the Out Patient Departments of ICDDR,B hospital, attached to the Parasitology Laboratory, ICDDRB, Dhaka, Bangladesh, identified earlier as either *Entamoeba histolytica* by Techlab ELISA were used in the present study. These have been cultured in Robinson's medium (Robinson GL, 1968).

3.3 Preparation of culture media

Clinical isolates are cultured in first xenic media then it is axenically process to axenic or pure culture. Mentioned below these are the processes:

3.3.2 Xenic Culture Media

Preparation of Rice Starch: Purified rice starch is important for growth of *E. histolytica* in all the following media. To prepare (18), 500 mg of powdered rice starch is placed into each of several culture tubes (16 by 125 mm) and is heated at 150°C, with loose caps, in a dry oven for about 3 hours. Sterilization of the rice starch prevents alteration of the bacterial flora when it is added to the culture and is thus recommended.

After cooling, the caps are tighten and are stored at room temperature. To prepare for use, 9.5 ml of sterile distilled water or phosphate-buffered saline (PBS) is added to one tube. The tubes are vortexed for resuspension. 1 ml of the resuspended starch is distributed to each of 10 tubes containing 9 ml of sterile water or PBS, and they are refrigerated.

The final concentration of diluted rice starch is 5 mg/ml. Before use, the rice is resuspend by vortexing or vigorous shaking. The desired volume is taken into culture tubes with medium, making sure that the stock rice stays in suspension. Different isolates require various amounts of rice starch, but 0.2 ml (1 mg) is often a suitable amount to add per culture tube.

Entamoeba will not ingest all forms of rice. Most important is the size of the rice particle, as it must be within the ameba's ability to phagocytize it.

3.3.3 Diphasic media

Robinson's medium: Robinson's medium (54) is a complex medium that has nevertheless found widespread use for the isolation of enteric amebae. Robinson's medium is prepared with the six following stock solutions.

(a) 0.5% erythromycin 0.5% erythromycin is prepared in distilled water and filtered and sterilized. Then it is refrigerated.

(b) 20% Bacto Peptone. 20% Bacto Peptone is prepared in distilled water. Then it is autoclaved and refrigerated.

(c) 10Xphthalate solution stock. 10 X phthalate solution stock is prepared by mixing 102 g of potassium hydrogen phthalate and 50 ml of 40% sodium hydroxide. The solution is made to 1 liter at pH is adjusted to 6.3. Then the solution is autoclaved for 15 min at 121°C under a pressure of 15 lb/in2. The solution is stored at room temperature. It is diluted 1:10 with sterile water before use.

A stock of phthalate-Bacto Peptone can be made by adding 1.25 ml of 20% Bacto Peptone per 100 ml of 1 X phthalate solution. The solution is refrigerated.

(d) 10X R medium stock. 10X R medium stock, is prepared by dissolving the following in distilled water: 25.0 g of sodium chloride; 10.0 g of citric acid; 25.0 g of potassium phosphate, monobasic; 5.0 g of ammonium sulfate; 0.25 g of magnesium sulfate. 7H2O; and 20 ml of 85% lactic acid solution. It is made to 500 ml.

Stock is diluted to 1:10, pH is adjusted to 7.0. The solution is autoclaved for 15min at 121°C under a pressure of 15 lb/in^2 in 20-ml amounts.

(e) BR medium. To prepare BR medium, inoculate 1×R medium with a standard *E. coli* strain such as O111. Incubate at 37°C for 48 h and store at room temperature (good for several months).

(f) BRS medium. To prepare BRS medium, add an equal volume of heat-inactivated bovine serum to BR medium and incubate at 37°C for 24 h. Store at room temperature (good for several months).

To prepare agar slants, many people use screw-cap glass bijou bottles (total volume, 7 ml), but we have also used standard culture tubes with good success. Autoclave a solution of 1.5% Noble agar in 0.7% sodium chloride–distilled water for 15 min at 121°C under a pressure of 15 lb/in2. Dispense in 5-ml (tube) or 3-ml (bottle) amounts, reautoclave, and slant until cool and set. For slants in tubes, use an angle that produces a 12- to 15-mm (ca. 0.5-in.) butt. When cool, tighten lids and store at room temperature or refrigerated.

To one tube or bottle add the following: 3 ml of 1×phthalate-Bacto Peptone, 1 ml of BRS medium, and 50μl of erythromycin. This must be done on the same day as inoculation.

Note that although erythromycin is added to Robinson's medium at every subculture, this does not lead to a monoxenic culture as occasionally stated. Additional antibiotic treatment would be needed for this to occur.

3.3.4 Axenic Culture Media

One constant problem facing those who rely on axenic cultures is the fastidiousness of these organisms. Although the others are also affected to a significant degree, this is especially true of *E. histolytica*. Lot-to-lot variations in several components of the axenic culture media in particular can have profound effects on the ability of a medium to support growth of the organisms; some lots may even be toxic. Trypticase (casein digest peptone), yeast extract, and serum are the medium components most commonly affected, but the quality of the distilled water and even the type of glass used in making the culture tubes can cause problems (18) (screw-cap borosilicate glass tubes should be used when possible). For this reason, we highly recommend that those wishing to undertake axenic cultivation of these organisms test the ability of each new lot of reagent to support growth before starting to use it.

***E. histolytica*. LYI-S-2:** In the course of developing YI-S, several combinations of liver digest and yeast extract were studied. One of these, designated LYI-S-2 (containing liver digest, yeast extract, iron, and serum), was found to result in growth equal to that in TYI S-33. Intent on producing a medium with as few biological ingredients as possible, the medium containing only yeast extract, YI-S, was extensively tested and published. No difference in

the ability of YI-S and LYI-S-2 to support growth of *E. histolytica* was observed (unpublished results). After publication of YI-S, further testing within our laboratory and by others disclosed the fact that some lots of yeast extract would not support any growth of the ameba while with others growth was very poor. In the case of the latter it was found that substitution of a small amount of liver digest for an equal amount of yeast extract enhanced growth considerably. LYI-S-2 is recommended when a given lot of yeast extract will support some growth, though poorly, of *E. histolytica*. LYI-S-2 is identical to YI-S except that weight for weight it contains 0.5% neutralized liver digest and only 2.5% yeast extract. It has been used in the long-term cultivation of several isolates of *E. histolytica* and a number of other *Entamoeba* species, with yields similar to those observed with the more widely used TYI-S-33 and YI-S (unpublished data).

3.4 Establishment of Cultures

It is very important to remember that a negative culture result does not mean that the patient is uninfected. None of the organisms being considered here produce cultures 100% of the time from microscopy-positive samples, and in the case of *E. histolytica* the success rate appears to be between 50 and 70% in most laboratories, based on personal communications. It is also important to remember that what grows in culture is not necessarily the organism seen by microscopy.

General considerations: *E. histolytica* needs to be established in xenic culture. The most-common source of material will be stool samples, and this is what is assumed below. In rare instances rectal biopsy specimens or liver abscess aspirates have been the starting point for cultures. In the latter case, since the abscess is sterile, addition of a bacterial flora is necessary before inoculation of amebae into xenic culture. Such material has also been used for the direct establishment of *E. histolytica* into monoxenic cultures with either a bacterium (28) or a trypanosomatid (69) as the associate. Unless a stool sample is from a patient with dysentery, it is likely that the amebae will be in the encysted form. This allows for several approaches to the establishment of cultures.

Elimination of unwanted organisms: One of the banes of xenic cultivation is the likelihood of unwanted organisms overgrowing the desired ameba. The most-frequent source of this problem is *B. hominis*, which may be the most-common parasitic infection of humans. This organism is often missed on stool examination but grows luxuriantly in all the media used to

cultivate xenic *Entamoeba*. Some authors control the growth of *B.hominis* with acriflavin as first described by Dobell and Laidlaw (25), but this also has an adverse effect on the bacterial flora and, directly or indirectly, on the ameba of interest. We have successfully used two methods to eliminate *B. hominis* from *Entamoeba* cultures.

The first method also was described by Dobell and Laidlaw in 1926 (25). In this method, cysts are treated with 0.1 N hydrochloric acid at room temperature for 10 min, washed thoroughly with distilled water, and reinoculated into culture medium to which a suitable bacterial flora has been added. The acid kills the bacteria, any fungi, *B. hominis*, intestinal trichomonads, and any nonencysted amebae while leaving the cysts intact and viable. We have found that it is not necessary in most cases for the cysts to be mature. We do not know whether the cysts complete their maturation upon inoculation or whether immature cysts respond to the stimulus and excyst directly. The cysts used can be either from stool or cultures; *Entamoeba* cultures in LE medium in particular frequently produce small numbers of cysts spontaneously. The bacterial flora used in the above method is separated from another xenic culture by inoculating into culture medium, without rice starch, a small amount of supernatant from an established culture, subculturing twice, and refrigerating the flora for 48 h.

The successful separation of the flora can be checked by inoculating a substantial volume into fresh medium with rice starch and checking for amebal growth. The flora can be stored at 4°C indefinitely. The second method is that of Smedley (60) and is used when *B. hominis* appears in cultures after inoculation. It does not rely on cysts being present and so has advantages over the method of Dobell and Laidlaw in that respect. However, the method may need to be repeated a couple of times before the *B. hominis* is completely eliminated. Cultures are pelleted, and the pellet, which contains a mixture of all the organisms present, is resuspended in distilled water at room temperature for 15 min.

The material is then repelleted and inoculated into fresh culture medium. Perhaps surprisingly, many *Entamoeba* trophozoites survive this treatment while *B. hominis* generally does not. A few cells or cysts of *B. hominis* may survive and start to grow, and the procedure will then need to be repeated. The advantages of Smedley's method are its simplicity and the fact that no separate bacterial flora is needed. Other unwanted organisms such as fungi and trichomonads will usually disappear from xenic cultures after several passages. However, occasional instances of balanced mixed cultures are known.

Isolation: In our experience LE medium has proven to be the best medium for primary isolation of *Entamoeba* species from stool, although we have limited experience with Robinson's medium, which is widely used by others for this purpose. TYSGM-9 can also be used for isolation, but its primary utility is in generating large numbers of amebae from established cultures. The numbers of amebae obtained from the two diphasic media are generally low in comparison with TYSGM-9, but their success in primary isolation of amebae from microscopically positive stool is higher (58; unpublished observations). In all cases, rice starch is added to the medium before inoculation, as are the antibiotics when needed. Material for inoculation of xenic cultures can be prepared in several ways (18).

Most commonly, stool samples are emulsified in saline and passed through a mesh to remove most of the larger particulates from the material before addition to the culture medium. However, small-pea-size pieces of fecal material can be added directly to the medium. It is always a good idea to include portions of the stool that appear mucoid or bloody if these are present. Stool fractionation by flotation in zinc sulfate (1) or sucrose (68) is also used, as this reduces the amount of debris while concentrating the cysts present in the sample. However, zinc sulfate shrinks the cysts and may damage them, resulting in lower isolation rates. Cyst purification on Percoll gradients (2) is very successful when using culture material as a starting point and could well be adapted to isolation from stool.

We routinely use more than one medium, if available, and set up duplicate cultures in which one has antibiotics added and the other does not. Penicillin streptomycin and erythromycin are the antibiotics of choice, as they appear to have little direct effect on the amebae. However, the widespread occurrence of antibiotic resistance in bacteria makes it impossible to generalize about the amount and type of antibiotics necessary to control the growth and rice-splitting activity of human bacterial flora. Culture tubes, containing medium and rice starch, to which stool-derived material has been added, are incubated vertically at 35.5°C for 48 h before examination (Fig. 1). A drop of sediment can be extracted from the tube for examination on a microscope slide. Alternatively, cultures can be examined in situ by slanting the tubes and using an inverted microscope. Amebae can be observed adhering to the walls of the glass culture tubes above the fecal material and above the slant in diphasic media. In situ examination is much easier in monophasic medium due to its relative clarity. If no growth is observed at 48 h, a blind passage should be made.

Most of the liquid overlying the sediment is discarded to leave less than 1 ml in the tube. The sediment is resuspended in the remaining fluid and transferred to a fresh culture tube with medium and rice starch (and antibiotic if appropriate). After incubation for an additional 48 h the culture is reexamined as described above. If no amebae are seen a further 48-h incubation is warranted, and this is followed by reexamination. If there are still no amebae seen, the culture is discarded as negative. If cultures are positive for amebae, it is usually helpful to centrifuge the cultures in a swinging-bucket rotor (275 × *g*, 3 min) and divide the pellet among the recipient tubes. This can be done by chilling the culture tubes for 5 min in an ice-water bath, inverting several times to detach adherent amebae, and transferring the liquid phase to an empty culture tube before centrifugation. Cultures in LE medium can also be pelleted in situ, but in our experience the agar slant of Robinson's medium is not as conducive to this approach. As growth improves, centrifugation is no longer necessary as measured inocula (<2 ml) can be transferred to the fresh medium.

Axenization: As previously mentioned, axenization of *E. histolytica* is a long and laborious procedure involving gradual adaptation of the parasite to a new way of life. A brief overview of the method is given here. A more complete description can be found in the works of Diamond (16, 18). The first step is to grow the organisms in monoxenic culture and is achieved by washing the xenic amebae in PBS to remove as many bacteria as possible and then placing the trophozoites in a rich medium with their new food organism and antibiotics. The medium can be a specialized monoxenic culture medium as described previously (16), but we have also had success initiating such cultures using one of the axenic media such as TYI-S-33.

The monoxenic associate we have used most frequently is *C. fasciculate* ReF-1:PRR (15) (ATCC 50083). This insect flagellate is grown as a stock culture at room temperature and added to the monoxenic culture of amebae at each subculture, as *Crithidia* does not grow at the incubation temperature of the amebae; the amount added varies. *T. cruzi* Culbertson (ATCC 30013) has also been used successfully as the associated organism but is not recommended due to the potential for infection, even though this strain is of very low virulence. The antibiotics added vary both in type and amount depending on the sensitivities of the flora in which the amebae were growing. We have used a cocktail of rifampin, amikacin, oxytetracycline, and cefotaxime with good success. Except for the first agent, these have little effect on the amebae.

The initial concentration is often as high as 0.1 mg/ml of culture medium. After 24 h, the cells are pelleted by centrifugation and the medium is replaced. As the ameba cell numbers increase, the cell pellet can be divided between two tubes. By reducing the antibiotic concentration gradually in one of a pair of tubes to test for bacterial growth, sterility can be achieved gradually while at the same time the numbers of amebae are increasing. At least two subcultures in the absence of antibiotics should be performed before the cultures can be considered free of bacteria. This can be verified using standard aerobic and anaerobic testing procedures for bacteria, including mycoplasmas, and fungi (18). Established monoxenic cultures, those in which growth is reproducible and bacteria are absent, are then used to initiate axenic cultures.

This uses the same medium but with no *Crithidia* added. After a few subcultures the flagellates disappear as a result of dilution and ingestion. It is often helpful, although not always necessary, to add a small amount of Noble agar to the tubes at each subculture (0.01%, wt/vol; for example, adding 0.25 ml of a melted 0.5% stock agar solution to 13 ml of medium in a tube). It appears to form a substrate for the amebae. In addition, the tubes should be incubated vertically rather than at 5° to the horizontal, as this appears less "stressful" to the cells. Often the culture will flourish initially and then numbers will crash. It is at this crisis point that the cultures are most vulnerable. As long as a few live cells persist, it is worth continuing to replace the medium every few days. With luck, the numbers will gradually start to increase again, and eventually addition of the agar will no longer be needed. When established, the axenic cultures can be incubated at 5° to the horizontal and eventually should reach cell concentrations of 150,000 to 300,000 per ml on a twice-per-week subculture schedule.

Principles of inducing encystment of *E. histolytica*: The methods of inducing encystment of *E. histolytica* are based on Dobell and Laidlaw's discovery that ". . . .cyst production may sometimes be temporarily increased by cultivating the amoebae in starch-free media for one or two generations, and then transferring them to media containing this substance: but the results are uncertain, and the number of cysts produced in any culture cannot be predicted" (25). Each point these authors make holds true to this day, and anyone attempting to induce encystment must bear these points in mind at all times. To date, cysts of this species have been induced only in xenic culture.

No one as far as we can determine has published a detailed account of the process of inducing encystation. Here we present a protocol used for many years in the NIH Laboratory of Parasitic Diseases but never previously published in detail. Three things are of special concern in obtaining cysts: the media, bacterial flora, and rice starch. Some media are better than others for this purpose. LE medium is the one with which we and others have had excellent results (see above). The accompanying bacterial flora present in a xenic culture plays an important role in the process of encystment. Here again some are better than others. It is good practice for those requiring a steady source of large numbers of cysts to isolate and maintain the bacterial flora of a xenic culture in which cysts regularly form spontaneously. A technique for doing this is presented above (under "Elimination of unwanted organisms").

3.5.1 Protocol: encystment

(a) Day 1. Begin the process with three amebae-rich 48-h cultures in LE medium. Harvest them by chilling the culture tubes for 5 min in an ice water bath, invert the tubes 10 times to mix contents and free amebae adhering to the glass and egg slant, and centrifuge for 3 min at 275 × *g*. Remove and discard all but 1 ml of the spent overlay. Resuspend pelleted amebae, pool, and transfer equal amounts to six tubes of LE medium without rice. Incubate the cultures in an upright position for 72 h.

(b) Day 4. Harvest each of the six cultures: Chill, remove and discard all but 1 ml of overlay. Mix remaining overlay of each culture and transfer equal amounts to two tubes of LE medium without rice. There will now be 12 cultures. Incubate 48 h.

(c) Day 6. Harvest the 12 cultures and subculture as on day 4. Incubate the 24 cultures for 48 h.

(d) Day 8. Carefully remove the overlay from each culture, leaving only enough to cover the sediment at the interface of the egg slant and overlay. Collect the sediments from three cultures and transfer to one tube of medium to which rice has been added. Repeat with the remaining cultures. Incubate the resulting eight cultures for 48 h.

(e) Day 10. Remove a small drop of sediment from each culture, stain with Lugol's iodine solution, and search for presence of quadrinucleate cysts. If found, harvest cultures as on day 1. Remove overlay, leaving only the sediment. Pool sediments and wash two times with

distilled water. Cysts will remain viable from 10 to 14 days when stored at 4°C. If cysts are not found, incubate an additional 24 h.

3.5.2 Protocol: excystment of cysts induced in vitro:

Inducing *E. histolytica* to excyst is relatively easy compared to getting the ameba to encyst. How this is accomplished depends on the goal. If the goal is to propagate the amebae in a xenic environment, then the medium in which the cysts were induced is used, in this case LE medium. If the goal is to excyst them in a bacteria-free environment, any of the monophasic liquid media devised for axenic culture can be used. In the latter case freshly prepared medium must be used. While the amebae will excyst in the axenic media, no one, as yet, has been able get them to encyst in this environment. Best results are obtained when at least 50% of the cysts produced are in the quadrinucleate stage. Usually no more than 25% of the cysts can be expected to excyst. To induce excystment, the cysts are first treated to remove unwanted organisms as recommended above. They are then placed in a tube of LE medium inoculated with a suitable bacterial flora for xenic growth or in a medium capable of sustaining axenic growth. Upon incubation most of the cysts capable of undergoing excystation will have done so by the end of 6 h.

3.6 Rexenization of axenically cultivated *E. histolytica*:

Occasions will arise when it is desirable to return axenized amebae to the xenic state. The following protocol has worked well in our hands.

(i). Inoculate three tubes of LE medium with a bacterial flora known to support xenic growth.

(ii). Chill a 72-h culture of axenically cultivated amebae in an ice-water bath for 5 min. Invert culture tube 10 times to dislodge amebae from glass surfaces. Centrifuge 3 min at 275 × *g*. Remove supernatant and discard.

(iii). Resuspend amebae in 1 ml of fresh medium for axenic culture, count cells, and inoculate the tubes of LE medium with 1 ×105, 2 ×105, and 4 ×105 amebae, respectively. **(iv).** Incubate 48 h. Remove all but approximately 1 ml of overlay. Resuspend the sediment located at the interface of the slant and overlay. Examine a drop with a microscope. The majority of inoculated amebae will have died. Select the best of the three cultures and subculture.

(v). Subculture. The number of amebae transferred can be determined only by trial and error. In the early stages of establishing the culture, transfer one-half of the material from the old culture to each of two tubes of fresh medium (do not add additional bacteria after the initial inoculation of the medium). Later, as amebic growth improves, transfer smaller portions, e.g., one-third to one-fourth. Do not reduce the inoculum further. If only a few amebae are present, transfer all the sediment to a fresh tube of medium. Subculture three times per week as noted under "Maintenance of Cultures."

3.7 Maintenance of cultures

Established cultures of all parasites are handled in essentially the same way. Xenic cultures of *E. histolytica* are routinely passaged at 48- to 72-h intervals; usually a

Sunday-Tuesday-Thursday schedule is convenient. Occasionally cultures of these organisms will be found that do better with twice-weekly subculture. The inoculum size for the longer incubation period should be smaller than that for shorter incubations. However, variation among isolates and flora means that no generalities can be made regarding the size of inocula or the amount of rice and antibiotics to be added to the medium for optimal growth. It is very much a case of trial and error combined with experience in evaluating growth of cultures that leads to successful establishment of these parasites in xenic culture. It is recommended that xenic cultures be passaged using two or more inoculum sizes to ensure a successful subculture. A significant threshold effect can sometimes be encountered, in which a certain inoculum size gives rise to a healthy culture but an inoculum smaller by as little as 50 μl may result in no growth. Established axenic cultures of *E. histolytica* are passaged at 72- and 96-h intervals, with a Sunday-Thursday schedule being convenient. Visual inspection of every culture before subculture is recommended, since what appears to be a heavy culture may in fact contain many lysed cells, indicating that the inoculum previously used was too large. An increased inoculum volume may be warranted for the subsequent subculture to compensate for the dead amebae. Likewise, parallel duplicate cultures are recommended in case of inadvertent contamination or tube breakage. The unused culture can be kept at 33°C as a backup in case of problems. The method for subculturing many types of cultures is essentially the same. Cultures are chilled in an ice-water bath for 5 min (xenic cultures and axenic *E. histolytica*) to release trophozoites attached to the glass culture tube. Tubes are inverted several times to disperse the cells and a measured inoculum is passed aseptically to a

culture tube containing fresh medium. The tubes are capped tightly and incubated at 36 to 37°C, either vertically at 5° to the horizontal (established axenic cultures of *E. histolytica*).

3.8 Preparation of Carica Papaya seed extract

The plant materials were used in this study collected from an authentic source and identified it in Botany department of Dhaka University, whether it belonged to Carica papaya plant or not.

Then the dried seed (200g) was grounded and extracted with a Methanol: Ether (50:50) by maceration for 3days.The solvent was evaporated using rotary evaporator. The residue of extract was stored at 4^0 c.

Finally, the mixture was filtered by folding clothes through funnel. The resulting filtrate was concentrated by rotary evaporator in 40^0C under vacuum until it became dry. Polar compounds in the maceration were mostly eluted with methanol.

3.9 Preparation of different concentration of Carica Papaya seed extract

Dilution was done from initial concentration to make the following concentration:

Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7
0.12 mg/ml	0.24 mg/ml	0.49 mg/ml	0.98 mg/ml	1.97 mg/ml	3.95 mg/ml	7.89 mg/ml

3.10 Measurement of amoebicidal activity

The extract of the seeds of Carica papaya has a good amoebicidal activity according to the study. The isolates were treated with the seed extract at different concentrations. A control group was made to measure the change in the viable counts and was put into the ELISA plate. Each well of an ELISA plate now contained different concentration of extract and same amounts of parasite(*Entamoeba histolytica*).After that the preparation was put in the incubator and was incubated for a definite period of time (24 hours and 48 hours).Finally the viable counts of *Entamoeba histolytica* was counted and recorded in a table or graphs.

RESULTS

Results

4.1 Viable count of *Entamoeba histolytica* after 24 hours of incubation with different concentration of Carica papaya seed extract.

The viable count of *Entamoeba histolytica* after treating with of carica papaya seed extract for 24 hours at different concentrations.

Concentration of carica papaya seed extract (mg/ml)	Initial count of *Entamoeba histolytica* (per ml)	Viable count of *Entamoeba histolytica* after 24 hours (per ml)	Percentage (%)
0.12	$1.94*10^6$	$19.25*10^4$	9.92 %
0.24	$1.94*10^6$	$16.75*10^4$	8.63 %
0.49	$1.94*10^6$	$12.75*10^4$	6.57 %
0.98	$1.94*10^6$	$10.25*10^4$	5.28 %
1.97	$1.94*10^6$	$9.25*10^4$	4.77 %
3.95	$1.94*10^6$	$7.25*10^4$	3.74 %
7.89	$1.94*10^6$	0	0 %
Control	$1.94*10^6$	$33.25*10^4$	17.14 %

Table 3: Viable count of *Entamoeba histolytica* after 24 hours

Table 3 shows that the initial count of *Entamoeba histolytica* was $1.94*10^6$ mg/ml. After 24 hours the viable count was $19.25*10^4$ at the concentration of 0.12 mg/ml of Carica papaya seed extract, viable count of *Entamoeba histolytica* was decreased to $7.25*10^4$ when the concentration of papaya increased to 3.95 mg/ml and there is not any *Entamoeba histolytica* present in 7.89 mg/ml concentrated papaya extract.

Although there is not carica papaya seed extract present in control but there is also decrease of viable Entamoeba histolytica.

4.2 Viable count of *Entamoeba histolytica* after 48 hours of incubation with different concentration of papaya extract.

The viable count of *Entamoeba histolytica* after treating with Carica papaya seed extract for 48 hours at different concentrations.

Concentration of carica papaya seed extract (mg/ml)	Initial count of *Entamoeba histolytica* (per ml)	Viable count of *Entamoeba histolytica* after 48 hours (per ml)	Percentage (%)
0.12	$1.94*10^6$	$4.25*10^4$	2.19 %
0.24	$1.94*10^6$	$2.75*10^4$	1.42 %
0.49	$1.94*10^6$	$1.75*10^4$	0.90 %
0.98	$1.94*10^6$	0	0 %
1.97	$1.94*10^6$	0	0 %
3.95	$1.94*10^6$	0	0 %
7.89	$1.94*10^6$	0	0 %
Control	$1.94*10^6$	$32.00*10^4$	16.49 %

Table 4: Viable count of *Entamoeba histolytica* after 48 hours

Table 4 shows the non viable count of *Entamoeba histolytica* after treating with different concentration of carica papaya seed extract for 48 hours. The initial count of *Entamoeba histolytica* was $1.94*10^6$ mg/ml. After 48 hours the viable count was $4.25*10^4$ in 0.12 mg/ml concentrated papaya extract, viable count of *Entamoeba histolytica* was decreased to $1.75*10^4$ when the concentration of papaya increased to 0.49 mg/ml and there is not any *Entamoeba histolytica* present in 0.98 mg/ml, 1.97 mg/ml, 3.95 mg/ml and 7.89 mg/ml concentrated papaya extract.

Although there is not carica papaya seed extract present in control but after 48 hours the viable count of *Entamoeba histolytica* also decreased.

4.3 Viable counts of *Entamoeba histolytica* after 24 hours of incubation with different concentration of Carica papaya seed extract.

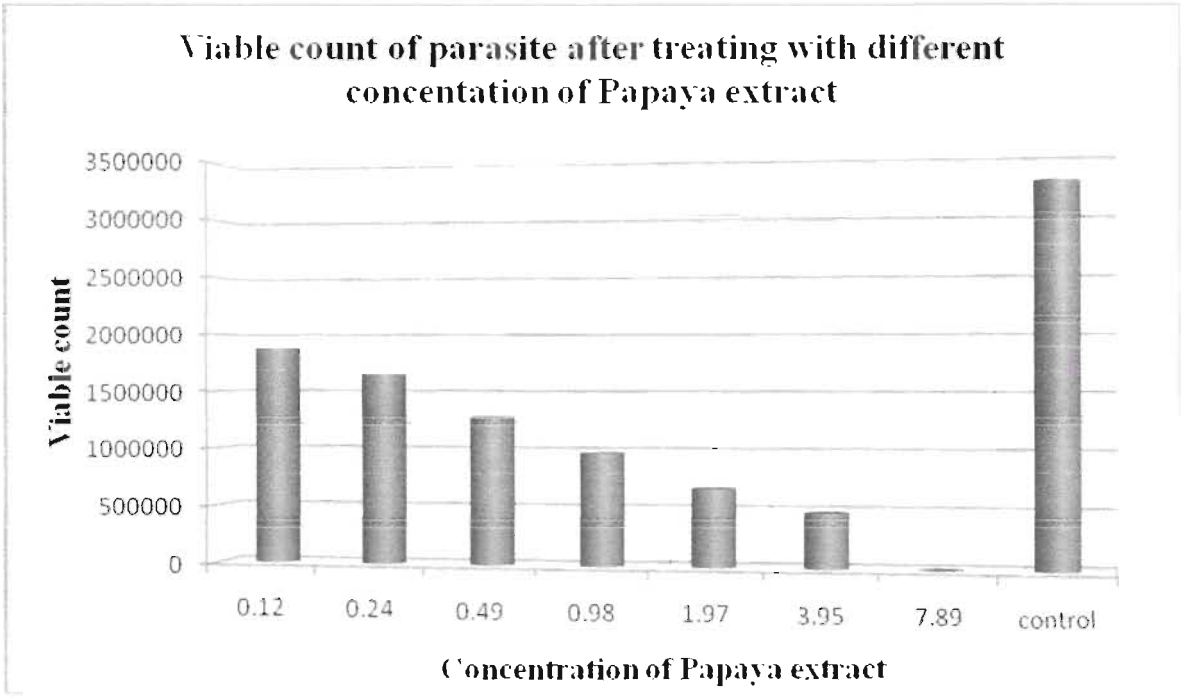

Figure 7: Viable count of *Entamoeba histolytica* after 48 hours.

Figure 7 shows the viable count of *Entamoeba histolytica* after 24 hours of incubation with different concentrated carica papaya seed extract but the initial concentration of the *Entamoeba histolytica* was same as $1.68*10^6$ mg/ml. the figure shows the lower number of *Entamoeba histolytica* count was found in the concentration of 7.89 mg/ml carica papaya seed extract and the highest number of *Entamoeba histolytica* found in the concentration 0.12 mg/ml papaya extract.

In the control the viable count of *Entamoeba histolytica* was the highest.

4.4 Viable counts of *Entamoeba histolytica* after 48 hours of incubation with different concentration of Carica papaya seed extract.

Figure 8 shows the viable count of *Entamoeba histolytica* after 48 hours of incubation with different concentrated carica papaya seed extract but the initial concentration of the *Entamoeba histolytica* was same as $1.68*10^6$ mg/ml. the figure shows the lower number of *Entamoeba histolytica* count was found in the concentrations of 1.97 mg/ml, 3.95mg/ml and 7.89 mg/ml papaya extracts and the highest number of *Entamoeba histolytica* found in the concentration 0.12 mg/ml papaya extract.

In the control the viable count of *Entamoeba histolytica* was the highest.

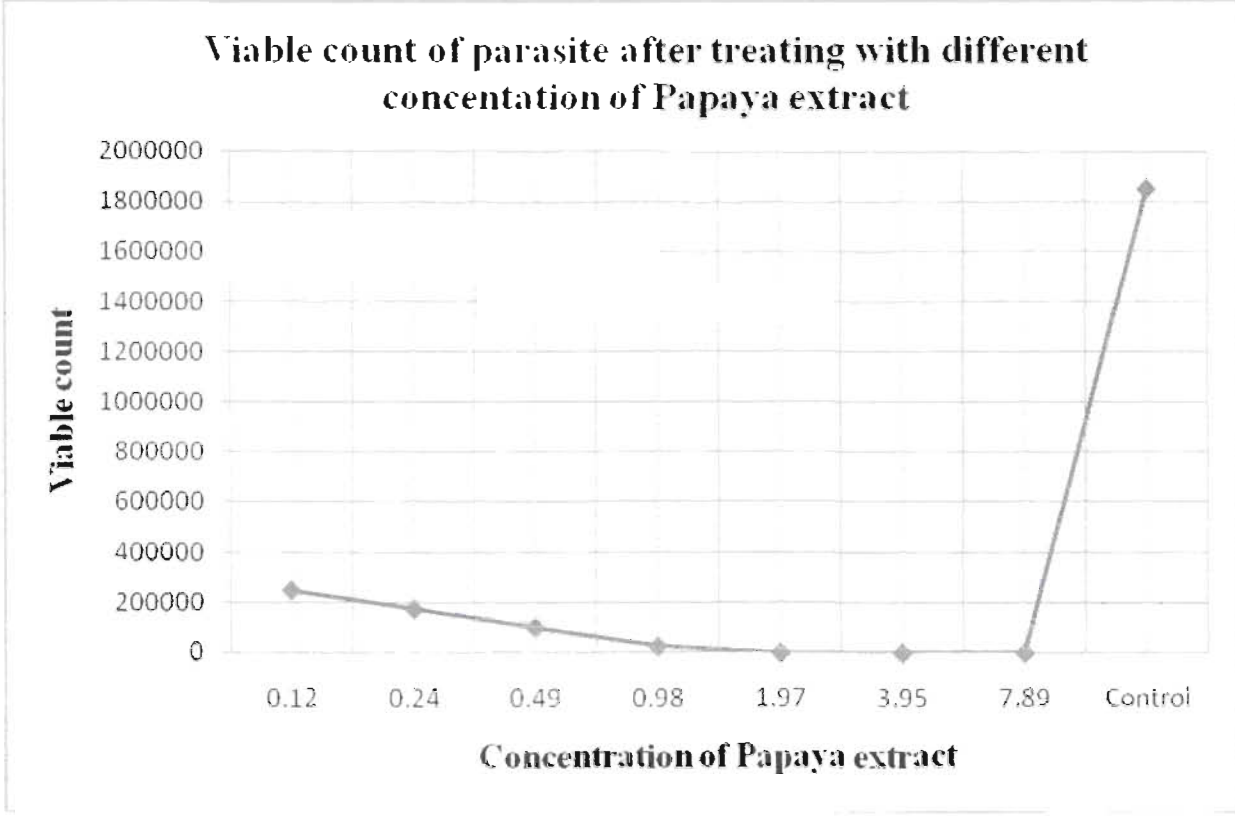

Figure 8: Viable count of *Entamoeba histolytica* after 48 hours.

4.5 Non viable counts of *Entamoeba histolytica* after 24 hours incubation with different concentration of Carica papaya seed extract.

Figure 9 shows the non viable count of *Entamoeba histolytica* after 24 hours of incubation with different concentrated carica papaya seed extract but the initial concentration of the *Entamoeba histolytica* was same as $2.00*10^6$ mg/ml. Non viable count of *Entamoeba histolytica* was increasing with the concentration of the papaya extract.

The lower number of non viable *Entamoeba histolytica* count was found in the control and highest in the 7.89 mg/ml papaya extract.

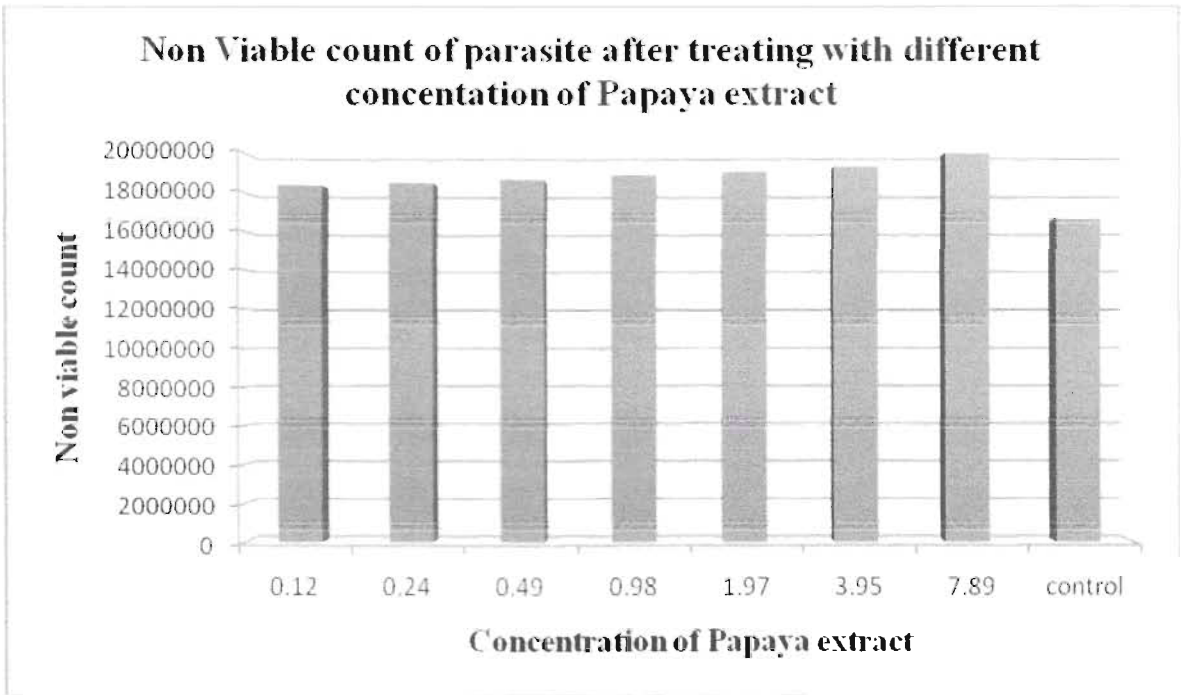

Figure 9: Non viable count of *Entamoeba histolytica* after 24 hours.

4.6 Non viable counts of *Entamoeba histolytica* after 48 hours of incubation with different concentration of Carica papaya seed extract.

Figure 10 shows the non viable count of *Entamoeba histolytica* after 48 hours of incubation with different concentrated carica papaya seed extract but the initial concentration of the *Entamoeba histolytica* was same as $2.00*10^6$ mg/ml. Non viable count of *Entamoeba histolytica* was increasing with the concentration of the carica papaya seed extract and when the concentration reached to 0.98 mg/ml then it becomes constant.

The lower number of non viable *Entamoeba histolytica* count was found in the control and highest in the 0.98 mg/ml, 1.97 mg/ml, 3.95 mg/ml and 7.89 mg/ml papaya extracts.

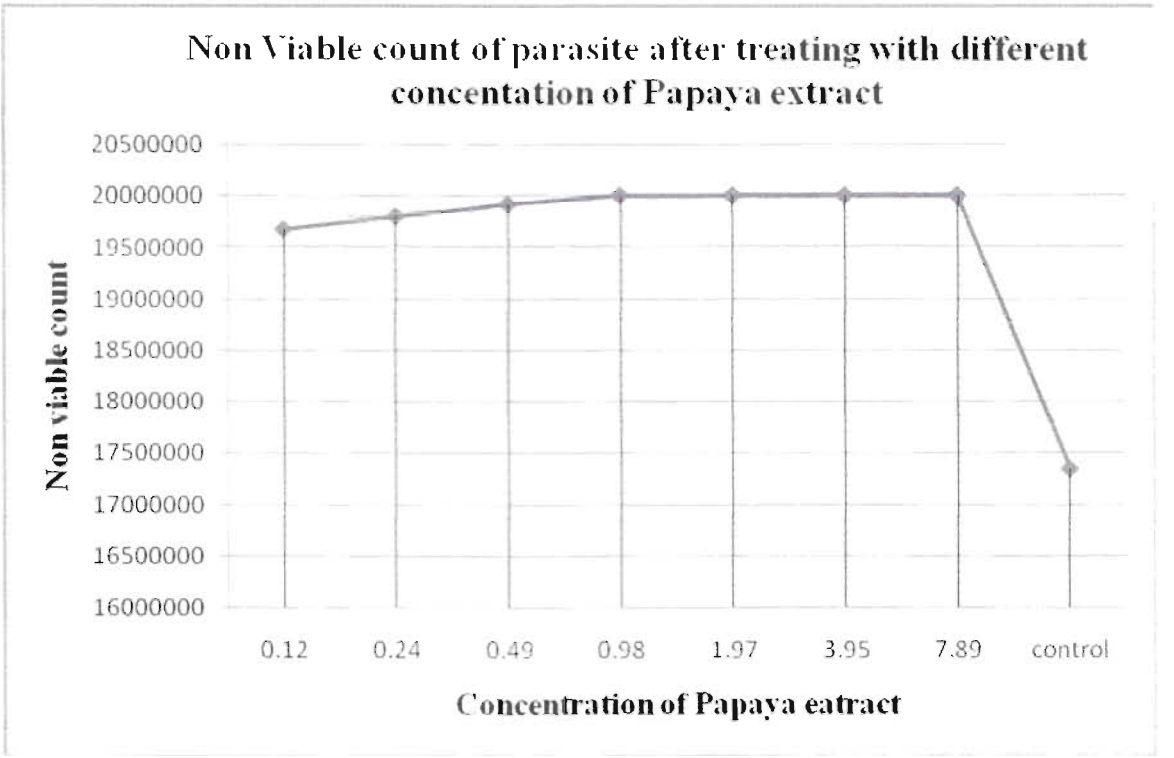

Figure 10: Non viable count of *Entamoeba histolytica* after 48 hours.

DISCUSSION

Discussion

The result of the study show that after 24 hours of incubation concentration of 7.89mg/ml and after 48 hours of incubation concentrations of 0.98mg/ml, 1.97mg/ml, 3.95mg/ml, 7.89mg/ml no presence of the clinical isolates of *Entamoeba histolytica.*

Amoebiasis is one of the most common health problems in the world. It is estimated that annually about 480 million people develop clinical Amoebiasis and at least 40,000 die (Walsh, 1998).

Invasive Amoebiasis generally induces a systemic humoral immune response demonstrable about 1 week after the onset of symptoms (Ortiz, 1990; Ortiz, 1980).

Anti-amoebic antibodies persist even after invasive amoebiasis has healed or after subclinical amoebic infection has disappeared (Knobloch, 1983).

Amoebiasis is an infectious disease caused by *Entamoeba histolytica*, which causes both intestinal and extraintestinal infections. Two species of *Entamoeba* are morphologically indistinguishable: *Entamoeba histolytica* is pathogenic and *Entamoeba dispar* harmlessly colonizes the colon. Amoebas adhere to and kill the cells of the colon and cause dysentery with blood and mucus in the stool. Amoebas also secrete substances called proteases that degrade lining of the colon and permit invasion into the bowel wall and beyond. Amoebas can spread via the circulation to the liver and cause liver abscesses. The infection may spread further by direct extension from the liver or through the bloodstream to the lungs, brain, and other organs (Kumar, 1999).

Entamoeba histolytica-associated infection is a public health problem in many developing countries, where it is responsible for severe morbidity and mortality (Ghosh, 2000).

Nitroimidazoles are highly potent anti-protozoal agents with well-established indications. The drugs which are used in the amoebiasis are metronidazole, tinidazole, ornidazole, diloxanide furoate, emitine, chloroquine, secnidazole etc (Devendra Bansal, 2006).

Since the discovery of metronidazole, a number of this group, the treatment of amoebiasis has been revolutionized (Rossignol, 1984; Rosenblatt, 1987).

The medications recommended to treat confirmed amoebiasis vary with clinical manifestation. Asymptomatic intestinal infection with *E. histolytica* should be treated with

luminal amoebicides, such as paromomycin and diloxanide furoate. These medications will eradicate the luminal amoeba and prevent subsequent tissue invasion and spread of the infection through cysts. Paromomycin, more widely available in the United States, has the advantage of not being absorbed in the bowel. Abdominal cramps and nausea are the most commonly reported adverse effects. A 10-day course at 30 mg/kg per day (divided into 3 daily doses) is typical. Some recommend follow-up stool examination to confirm eradication of cysts (Pritt, 2008).

The plant materials of Carica papaya have many medicinal effects. Extracts of ripe and unripe papaya fruits and of the seeds are active against gram-positive bacteria. Papaya milk latex inhibits fungal growth, especially that of *Candida albicans* and thus would be useful in the treatment of skin eczema caused by this fungus (Giordani, 1991). Extracts of papaya seeds could be used as a contraceptive or anti fertility agent (Jaime, 2007). Extracts from fruits showed effective anti-microbial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas* sp. and Shigella sp (Jaime, 2007). Papain is the active principle in *C. papaya* that exerts the ulcer-protective effect (Chen, 1981). The fermented papaya preparation has the antioxidant properties (Mahmood, 2005). Aqueous extracts of *Carica papaya* leaves accelerates the progression of wound healing activity (Mahmood, 2005). The seeds and fruits demonstrated in vitro anti amoebic activity (Okeniyi, 2007; Jaime, 2007).

The fresh leaves and extract of the plant *Carica papaya* are a traditional herbal treatment in developing countries for burns and skin infection. It gives the protective action (Mahmood, 2005).

The smoke from dried papaya leaves relieves asthma attacks (Jaime, 2007). The dried leaf infusion is taken for stomach troubles in Ghana and it is used as a purgative (Jaime, 2007).

In our study the clinical isolates of *Entamoeba histolytica* was collected from stool of the patient then it was cultured in the International Center for Diarrheal Disease Research, Bangladesh (*icddr, b*). The clinical isolate was prepared in different concentrations.

Incubation of the clinical isolates was done for two periods 24 hours and 48 hours. After 24 hours and 48 hours of incubation of the clinical isolates with papaya extract show the

decrease of the viable count of the parasite present in the extract. Concentration of (0.12mg/ml) shows the highest number of the clinical isolate presence in both incubation periods $19.25*10^4$ for 24 hours and $4.25*10^4$ for 48 hours. When the concentration doubled (0.24mg/ml) those becomes $16.75*10^4$ for 24 hours and $2.75*10^4$ for 48 hours. Concentration of (0.49 mg/ml) viable count was $12.75*10^4$ after 24 hours of incubation and $1.75*10^4$ after 48 hours of incubation. Increasing the concentration of the papaya extract simultaneously decreases the viable count of the parasite. After 24 hours of the incubation highest concentration of the papaya extract (7.89 mg/ml) showed no presence of the viable count of parasite. After 48 hours of the incubation the concentrations of (0.98mg/ml), (1.97mg/ml), (3.95mg/ml) and (7.89mg/ml) showed no presence of the viable count of parasite. In both incubation periods, one control was also incubated that also shows the decrease of the viable count of the parasite.

As because the seed extract of carica papaya is obtained from natural source, the concentration needed for the inhibition of the *Entamoeba histolytica* is more than the traditional ant amoebic drugs.

The result of the different concentrations shows that increasing the concentration of the carica papaya seed extract and increasing the time of incubation periods simultaneously decreases the clinical isolates *Entamoeba histolytica*. After comparing results our study demonstrated that Carica papaya seed extract had sensitivity to the clinical isolates of *Entamoeba histolytica*. Our study also demonstrated that Carica papaya seed extract can be used for the *Entamoeba histolytica* associated amoebiasis treatment.

CONCLUSION

Conclusion

Papaya is widely used in hot climate countries as traditional remedy in wound treatment. Its therapeutical value has been proven in several animal and human trials (Starley et al. 1999). Papain and chymopapain are known to be effective in desloughing necrotic tissue, prevention of infection and the antimicrobial and antioxidant properties related to hydroxyl scavenging and iron chelating properties (Anuar et al. 2008). Moreover they decrease the risk of oxidative damage to tissue.

In this study our aim was to find out the sensitivity of carica papaya against *Entamoeba histolytica* and this study clearly demonstrated that *Entamoeba histolytica* was sensitive to Carica papaya seed extract, with different concentrations.

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