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Acanthamoeba castellanii as a host and model to study bacterial virulence

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To my parents

ABSTRACT

Vibrio cholerae is the causative agent of cholera, a waterborne diarrheal disease and it is believed to be strictly extracellular bacterium. Cholera remains as a global public health threat affecting most of the developing world. In endemic areas, women and children are among the high risk. Recently outbreaks are reported from many countries. *V. cholerae* O1 and O139 have been associated with epidemic and pandemic outbreaks. Shigellosis is a global human health problem, especially in developing countries, with substandard hygiene and unsafe water supplies. The morbidity and mortality due to shigellosis are especially high among children in developing countries. *Acanthamoebae* are ubiquitous free-living amoebae that are distributed worldwide, living in diverse environments. *Acanthamoeba* has the ability to act as a host for bacterial pathogens. The aim of this thesis is to study the interaction of free-living amoebae *A. castellanii* and waterborne bacteria of *V. cholerae* and *Shigella* species, and the possibility to use the amoebae as model to study bacterial virulence.

V. cholerae and *Shigella* were co-cultivated with *A. castellanii* and the interaction was studied by cell count, viable count, fluorescence microscopy, electron microscopy and flow cytometry analysis.

The results showed that *V. cholerae* grew and survived intracellularly in the cytoplasm of trophozoites, and that the bacteria were found in the cysts of *A. castellanii*. *V. cholerae* O139 MO10 did not inhibit growth of the amoeba instead enhanced growth and survival of *V. cholerae* O139 MO10 occurred. The wild type *V. cholerae* O139 MO10 and its capsule mutant or capsule and LPS double mutant grew inside *A. castellanii* indicting no special role of those molecules in the interaction.

The co-cultivation of *A. castellanii* with *Shigella* showed that *S. dysenteriae* or *S. sonnei* grew and survived in the presence of amoebae for more than three weeks. Gentamicin assay showed that *Shigella* were viable inside the *A. castellanii* which confirmed by electron microscopy which disclosed the *Shigella* localized in the cytoplasm of the *A. castellanii*. *S. flexneri* (wild type) and mutant resulted in the cell death at 37°C and inhibition at 30°C. IpaB was found to play an important role in killing *A. castellanii*.

In conclusion the interaction showed a facultative intracellular behavior of *V. cholerae* and a possible role of *A. castellanii* as an environmental reservoir of *V. cholerae* species. Growth and survival of both amoebae and bacteria in recultivation of *A. castellanii* harboring intracellular *V. cholerae* indicate endosymbionts-host relation between these microorganisms. Neither the capsule nor the LPS O side chain of *V. cholerae* O139 was found to play any important role in the interaction with *A. castellanii* disclosing the ability of *V. cholerae* to multiply and survive inside *A. castellanii* as well as the role of *A. castellanii* as environmental hosts for *V. cholerae*.

The relationship between, *S. dysenteriae, S. sonnei* with *A. castellanii* is symbiotic, and amoeba may act as a reservoir for *Shigella* in environmental water. The interaction showed that wild type *S. flexneri*, IpaB mutant and virulence plasmid-cured kill *A. castellanii* by inducing necrosis.

This thesis shows that *A. castellanii* may act as reservoir for waterborne bacteria and the characteristics of *Acanthamoeba* makes it suitable non mammalian cell to be used in the investigation of host bacterial interaction.

Key words: *V. cholerae*, *Shigella*, *A. castellanii*, environmental reservoir

LIST OF PUBLICATIONS

I. Abd, H., **Saeed, A.**, Weintraub, A., Nair, G. B. & Sandstrom, G. (2007). ***Vibrio cholerae* O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii***. *FEMS. microbiology ecology* **60**, 33-39.

II. **Saeed A**, Abd H, Edvinsson B, & Sandstrom G (2007). ***Vibrio cholerae–Acanthamoeba castellanii* interaction showing Endosymbiont–Host relation**. Symbiosis **44**:153–158.

III. Abd, H., **A. Saeed**, Weintraub, A., & Sandstrom, G. (2009). ***Vibrio cholerae* O139 requires neither capsule nor LPS O side chain to grow inside *Acanthamoeba castellanii***. *J Med Microbiol* **58**(Pt 1): 125-31.

IV. **Saeed,A** .,Abd,H.,Edvinsson,B.& Sandstrom, G (2008). ***Acanthamoeba castellanii* an environmental host for *Shigella dysenteriae* and *Shigella sonnei***. *Archives of microbiology* **191**(1):83-8.

V. **Saeed,A** .,Abd,H.& Sandstrom, G (2009) **Role of type III secretion system on interaction between *Shigella flexneri* and *Acanthamoeba castellanii***. Submitted *J Med Microbiol*.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AK	*Acanthamoeba* keratitis
AMP	Adenosine mono phosphate
ATCC	American type culture collection
CFU	Colony-forming units
Ct	Cholera toxin
CNS	Central nervous system
GAE	Granulomatous amebic encephalitis
GFP	Green fluorescent protein
Ipa	Invasion plasmid antigen
Ipg	Invasion plasmid gene
LB	Luria-Bertani
LLAP	*Legionella*-like amoeba pathogens
LPS	Lipopolysaccharide
Mxi	Membrane expression of invasion plasmid antigens
PBS	Phosphate buffered saline
rRNA	Ribosomal ribonucleic acid
Spa	Surface presentation of antigens
Spp	Species
TCBS	Thiosulfate-citrate-bile-sucrose
TTSA	Type III secretion apparatus
TTSS	Type III secretion system
TCP	Toxin co-regulated pilus
VBNC	Viable but non-cultivable

1 Introduction

The discovery of the microscope in 1600s by Antonio van Leeuwenhoek, enable us to study the Protozoa. In the summer of 1674 Leeuwenhoek used the microscope to take a look at drop of water; he became the fist person to observe the free-living Protozoa. The largest single cell nonphotosynthetic animals that have no walls, but it took some long time around 80 years until Rosek von Rosenhof and for the first time described a free living amoeba as *Chaos proteus*.

The term amoebae are the largest diverse group of organisms protests (Fig 1), and have been studied for more than 400 years. These organisms have a common amoeboid motion; they have been classified into several different groups. These include parasitic organisms such as *Entamoeba spp*. which were discovered in 1873. In the 1899 Schardinger discovered the free-living amoebae *Naegleria*, and named it *Amoeba gruberi*. In 1912, Alexeieff proposed the name *Naegleria*, and much later in 1970 Carter identified *Naegleria fowleri* as the agent causing the fatal human infections (De Jonckheere, 1991; Khan, 2006).

In 1930, *Acanthamoebae* were discovered as eukaryotic cell culture contaminants and were classified as the genus *Acanthamoeba* (Khan, 2006). *Balamuthia mandrillaris* was discovered in 1986 from the brain of monkey. Over the years, free living amoebae have gained increasing attention and interest from the researchers around the world due to their multifunction (Khan, 2006).

1.1 *Acanthamoeba spp.*

In 1930 Castellani discovered amoebae in a culture of the *Cryptococcus pararoseus*. They were round or oval in shape with diameter ranging from 25 – 40 µm with the presence of pseudopodia. The encysted form of these amoebae characterized with double cell walls with a diameter ranging from 12- 20 µm. They were placed in the genus *Hartmannella* group, and given the name of *Hartmannella castellanii.* In 1931 Volkonsky subdivided the *Hartmannella* into three genera accordingly to the morphology of the cysts, amoebae characterized by round smooth walled cysts (*Hartmannella*), amoebae characterized by nuclear division in the cysts, and amoebae characterized by the appearance of pointed spindles at mitosis doubled walled cysts and an irregular outer layer (Khan, 2006; Marciano-Cabral & Cabral, 2003).

Researchers between 1950 and 1970 argued that the classification of amoeba by morphology and the cysts shape was of limited phylogentic and not relevant, cause the morphology of the cysts depends on many other factors.

In 1975 researchers (Sawyer & Griffin, 1975) described the family *Acanthamoebidae*. The prefix *acanth* (Greek for spikes) was added to the term *amoebae* to indicate the presence of spine like structures (acanthopodia) on the surface of these organisms.

After the discovery of *Acanthamoebae,* they were ignored for nearly the next three decades. Culberston et al (Culbertson *et al.*, 1958; Culbertson *et al.*, 1959) showed for the first time the pathological affect of these organisms by showing the ability of producing cytopathic effects on monkey kidney cells, and to kill *in vivo*. The first case of Granulomatous Acanthamoeba Encephalitis (GAE) in human was diagnosed by Jager & Stamm (Jager & Stamm, 1972). Nagington (Naginton *et al.*, 1974) reported the first cases of *Acanthamoeba* keratitis (AK).

In 1954 *Acanthamoebae* were first thought to be infected with bacteria (Drozanski, 1956) and in 1975 showed to harbour bacteria as endosymbionts (Proca-Ciobanu *et al.*, 1975). During the last decades, *Acanthamoebae* species have become interesting and important microbes. They are now well known as human pathogens causing serious and life-threatening infections. They play an important role in ecosystems and acts as a reservoir and a carrier for prokaryotes.

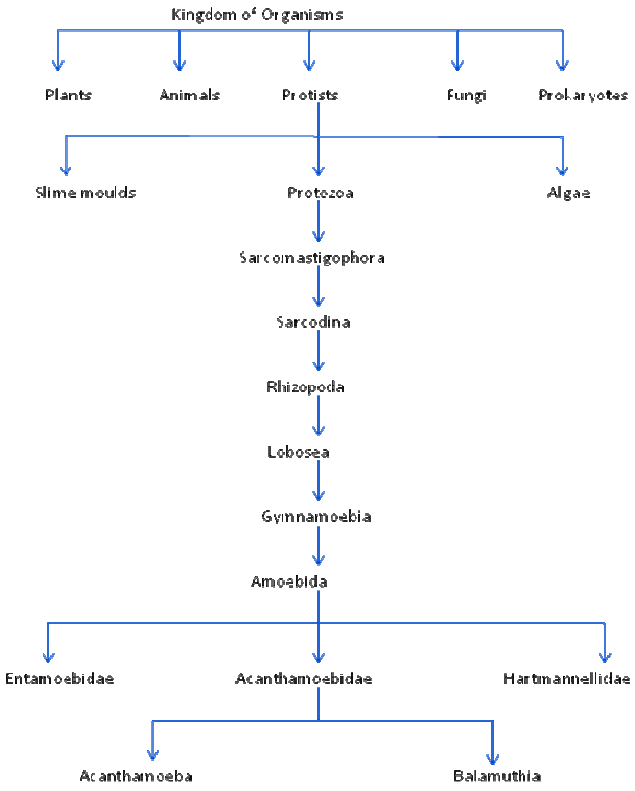

Figure 1 Classification of free-living amoebae.

Acanthamoeabae are ubiquitous free living amoebae that are distributed worldwide. They are among the most prevalent protozoa found in the environment (Mergeryan, 1991; Page, 1967; Rivera *et al.*, 1989; Rivera *et al.*, 1991; Rodriguez-Zaragoza, 1994). They have the ability to survive in the variety of environmental conditions and have been isolated from public water supplies, swimming pools, bottled water, seawater, pond water, stagnant water, freshwater lakes, salt water lakes, river water, distilled water bottles, ventilation ducts, the water-air interface, air-conditions units, sewage, compost, sediments, soil, beaches, vegetables, air, surgical instruments, contact lenses, dental treatment units, hospital and dialysis units, yeast , mammalian cell culture (Barbeau & Buhler, 2001; Casemore, 1977; De Jonckheere, 1991; Jahnes & Fullmer, 1957; Kingston & Warhurst, 1969; Mergeryan, 1991; Michel *et al.*, 2001; Paszko-Kolva *et al.*, 1991; Rivera *et al.*, 1989; Szenasi *et al.*, 1998). *Acanthamoeba* spp. also have been isolated from nasal mucosa and throats of apparently healthy humans, and also from infected brain and lung tissue, from skin lesions of immunocompromised patients and from corneal tissue of patient with AK (De Jonckheere, 1991; Dykova *et al.*, 1999; Lalitha *et al.*, 1985; Madrigal Sesma, 1988; Martinez & Visvesvara, 1997; Newsome *et al.*, 1992; Victoria & Korn, 1975).

Acanthamoeaba life cycle consists of two stages: a vegetative, dividing trophozoite stage and a dormant, protective cyst stage. The trophozoites normally varies in size ranging from 25 -40 µm in diameter. The trophozoites show spine-like structures on their surface known as acanthopodia. The acanthopodia is most important functions for adhesion to surfaces, cellular movements and in capturing prey. The trophozoites normally possess a single nucleus that is approximately one-sixth the size of the trophozoite. During the trophozoite stage, *Acanthamoeba* actively feed on bacteria, algae, yeasts or small organic particles and many food vacuoles can be seen in the cytoplasm of the cell. The *Acanthamoeba* divide asexual by binary fission. *Acanthamoeba* can be maintained in the trophozoite stage with enough food supply, natural pH, appropriate temperature (30ºC) and osmolarity between 50- 80 mOsmol. Harsh conditions such as lack of food, increases osmolarity or hypo-osmolarity, extremes in pH and temperatures induce the transformation of trophozoites into cyst stage. In a simple terms, the trophozoite becomes metabolically inactive and encloses itself within a resistant shell to help the amoeba survives in proper conditions, cellular levels of RNA, proteins, triacylglycerides and glycogen decline substantially during the encystment process, resulting in a decrease of cellular volume and dry weigh (Weisman, 1976).

The doubled walled wrinkled cyst composed of an ectocyst and endocyst. The cysts are normally ranging between 12- 20 µm in diameter. They are airborne, which may help the spreading of *Acanthamoebae* in the environment and/or carry these pathogens to the susceptible hosts. The *Acanthamoeba* cyst can remain viable for several years. The walls of the cysts contain cellulose that accounts for 10 % of the total dry weight of the cyst (Tomlinson & Jones, 1962) which gives the cyst the hard form. However cyst wall composition varies between isolates. In the cyst they are pores which are used to monitor environmental changes known as ostioles. The trophozoites emerge from the cysts under favorable conditions leaving behind the outer shell. The encystment and excystment processes require active macromolecule synthesis and can be blocked by cycloheximide (Khunkitti *et al.*, 1998; Lloyd *et al.*, 2001; Turner *et al.*, 2000).

Acanthamoebae feed on microorganisms present on the surface, in diverse environments (Brown & Barker, 1999) and even at the air-water interface (Preston *et al.*, 2001). The spiny structures or acanthopodia that arise from the surface of *Acanthamoeba* tophozoites may be used to capture food particles, which usually are bacteria (Weekers *et al.*, 1993), but algae, yeast (Allen & Dawidowicz, 1990) and other protists are also grazed upon. Food uptake in *Acanthamoeba* occurs by pinocytosis and phagocytosis. Phagocytosis is a receptor-dependent process, while pinocytosis uses a nonspecific process through membrance invaginations and is used to take up large volumes of solutes/food particles (Bowers & Olszewski, 1972). *Acanthamoeba* uses both specific phagocytosis and nonspecific pinocytosis for the uptake of food particles and large volumes of solutes (Allen & Dawidowicz, 1990; Alsam *et al.*, 2005; Bowers & Olszewski, 1972). Solutes of varying molecular weights, including albumin, glucose and leucine enter amoebae at the same rate but how amoebae choose between pinocytosis and phagocytosis is still unclear and the differences between pathogenic and non pathogenic amoeba need further analysis (Alsam *et al.*, 2005). The particle uptake into a vacuole, *Acanthamoeba* exhibits the ability to differentiate between digestible and non digestible particles. Bowers & Olszewski (Bowers & Olszewski, 1983) have shown that the origin of the vacuole depends on the nature of the particles, latex beads and versus food particles. The vacuoles which containing food particles are retained and digested, whereas latex beads are exchanged upon present of a new particle. Overall, the particle uptake in *Acanthamoeba* is a complex process that may play a significant role both in food uptake and pathogenesis of the *Acanthamoeba*.

After the discovery of *Acanthamoeba*, several isolates belonging to the genus with different morphology were isolated and has been given names based on the source

or any other criteria. To organize the increasing number of the isolates belonging to this genus, classification of the genus based on morphological characteristics of the cysts which were acceptable at that time. The genus *Acanthamoeba* divided into three morphological groups (I, II and III) based on cyst size and shape (Page, 1967). Group I were grouped on the basis of a large cyst in comparison to the other groups. Group II were characterized with wrinkled ectocyst and an endocyst which could be polygonal, triangular, or oval shaped. Group III were characterized with a typically thin, smooth ectocyst and round endocyst. It is very clear that classification of the *Acanthamoeba* spp. based on morphological features is unreliable because cyst morphology can change depending on environmental conditions (Armstrong, 2000; Daggett *et al.*, 1985; Sawyer & Griffin, 1975; Stratford & Griffith, 1978). Several studies discovered that the ionic strength of the growth medium could change the shape of the cyst walls (Sawyer & Buchanan, 1971). Furthermore the above classification had limited value when it comes to pathogenesis, cause same group may contain virulent, weakly virulent or avirulent. The discovery of the molecular techniques led to the pioneering work in classification of the genus *Acanthamoeba* based on the rRNA gene sequences (Gast, 2001; Stothard *et al.*, 1998). The new classification is a highly precise, reliable and informative classification. Each base presents a single character providing an accurate and diverse systematic. Based on the rRNA classification *Acanthamoebae* classified into 15 different genotypes T1 – T15 (Schuster & Visvesvara, 2004). Each genotype exhibits 5% or more sequence divergence between different genotypes. Maghsood et al (Maghsood *et al.*, 2005) proposed to subdivided T2 into two subgroup T2a and T2b, due to the sequence similarity of 4.9% between these two groups, which is very close to the cut-off limit of 5% between different genotypes.

1.2 *Acanthamoeba* in human Infections

The first suggestion that *Acanthamoebae* can cause a disease came in 1958 during polio-vaccine safety trail. Plaques appeared in cell cultures used to prepare vaccine and were thought to be virus induced because animals died from encephalitis following the inoculation of tissue culture fluid. However these plaques were found later to be caused by amoeba (Culbertson *et al.*, 1958; Culbertson *et al.*, 1959). *Acanthamoebae* cause two well-recognized diseases that cause serious health problems in human; a rare Granulomatous Amebic Encephalitis (GAE) involving the central nervous system (CNS) that is limited typically to immunocompromised patients which result in death, and a painful keratitis that can result in blindness. They have been associated with cutaneous lesions and sinusitis in AIDS patient and other

immunocompromised individuals (Dunand *et al.*, 1997; Friedland *et al.*, 1992; Marciano-Cabral & Cabral, 2003).

Acanthamoeba keratitis was discovered in 1974 (Naginton *et al.*, 1974) in the UK, It has been recognized as a significant ocular microbial infection. The main cause of *Acanthamoeba* keratitis is the use of contact lenses exposed to contaminated water, but the exact mechanisms associated with the process are not fully understood.

Acanthamoeba keratitis is a difficult infection to treat. Early diagnosis followed by aggressive treatment is essential for a successful prognosis (Perez-Santonja *et al.*, 2003). The recommended treatment includes a bigunaide (0.02 % polyhexamethylene biguanide, PHMB, or 0.02 % chlorhexidine digluconate,CHX) togther with a diamidine (0.1 % hexamidine isethionate). If bacteria are also associated and/or suspected with the infection, antibiotic are added to the treatment.

GAE is a rare infection but almost always a fatal one. The mechanisms associated with its pathogenesis are unclear, but pathologiclogical complications involving the CNS most likely include induction of the proinflammatory responses, invasion of the blood-brain barrier and the connective tissue, and neuronal damage leading to brain dysfunction. Routes of amoebae entry include the lower respiratory tract, leading to amoebae invasion of the intravascular space, followed by haematogenous spread, skin lesions may provide direct amoebae entry into the bloodstream, thus by passing the lower respiratory tract. Amoebae entry to the CNS most likely occurs at the sites of the blood-brain barrier. The cutaneous and respiratory infections can last for months but the involvement of the CNS can result in fatal consequences within days or weeks (Martinez & Visvesvara, 1997). There are no recommended treatments and the majority of cases due to GAE are identified post-mortem. This is due to low sensitivity of *Acanthamoeba* to many antiamoebic agents; the biggest problem is the inability of antiamoebic agents to cross the blood-brain barrier into the CNS. Current therapeutic agents include a combination of Ketoconazole, Fluconazole, Sulfadiazine, Pentamidine Isethionate, Amphotericin B,Aziththromycin, Itraconazole or Rifampin that may be affective against CNS infections due to free-living amoebae, but have sever side-effects. Recent studies have suggested that alkylphosphocholine compounds, such as Hexadecylophocholine, exhibit anti-*Acanthamoeba* properties as well as the ability to cross the blood-brain barrier and may therefore have a part in the treatment of GAE (Kotting *et al.*, 1992; Walochnik *et al.*, 2002).

Other infections due to *Acanthamoeba* involve nasopharyngeal and the more common cutaneous infections. The cutaneous infections are characterized by nodules and skin ulcerations and demonstrate *Acanthamoeba* trophozoites and cysts.

In healthy individuals these infections are very rare and self-limiting. However in immunocompromised patients, this provides a route of entry into the blood stream, following the spread of haematogenous to different tissues, which may be fatal (Torno *et al.*, 2000).

1.3 Shigella

Shigella species are highly adapted human pathogens that cause bacillary dysentery (shigellosis); a disease manifested ranging from mild abdominal discomfort to full-blown dysentery characterized by cramps, diarrhea, fever, vomiting, tenesmus and stools containing blood, pus, or mucus. The bacteria are primarily transmitted through the faecal-oral route. Shigellosis is one of the major global public health concerns, especially in the developing countries where sanitation is poor. A global increase in the number of food-borne *Shigella* outbreaks has been reported recently (Warren *et al.*, 2006). Following its recognition as the etiologic agent for bacillary dysentery in the 1890s, *Shigella* was adopted as a genus in the 1950s and was serologically subdivided into four species: *S. dysenteriae* (15 serotypes), *S. flexneri* (6 serotypes, with 14 subtypes), *S. boydi*i (20 serotypes), and *S. sonnei* (1 serotype). The global burden of Shigella disease(s) has been estimated by the World Health Organization to be 164.7 million cases per year, with 163.2 million and 1.5 million cases occurring in developing and industrialized countries, respectively (Kotloff *et al.*, 1999). The mortality rate in developing countries was estimated at 1.1 million deaths per year. The majority of episodes 69% and death 61% occurred in children under 5 years old. The data for shigella cases in industrialized countries were taken from surveillance data ranged from 1.8 to 6.5 cases per 100,000 population, with 31% of cases occurring in children under 5 years age. The death rate in industrialized countries was 0.2%.

The most frequently encountered species in industrialized countries are *S. sonnei* 77% and *S. flexneri* 16%, with *S. boydii* and *S. dysenteriae* comprising only 2% and 1% respectively (DuPont & Pickering, 1980; Kotloff *et al.*, 1999). The majority of infections in developing countries are caused by *S. flexneri* 60%, followed by *S. sonnei* 15%, *S. boydii* 8%, and *S. dysenteriae* 8% (Kotloff *et al.*, 1999). Among *S. flexneri* isolates, type 2a causes to 32% to 58% of infections, type 1b causes 12 % to 33%, and type 3a causes 4% to 11% of cases in developing countries; type 2a and unspecified

types 2,1, and 3 are also the most common *S. flexneri* types in the United States. *S. dysenteriae* is seen most often in the South Asia and Sub-Saharan Africa, with type 1 predominating in India, Nigeria, and Singapore and type 2 predominating in Guatemala, Hungary, and Yemen (Kotloff *et al.*, 1999). In Bangladesh, a change in the frequency of *S. dysenteriae* serotypes occurred between 1999, when *S. dysenteriae* type 1 accounted for 76% of cases, and 2002, when the number of cases decreased to 7 % (Talukder *et al.*, 2003). During this time, type 1 was supplanted first by type 4 and then by type 2. *S. dysenteriae* remerged in Indonesia in 1998 after a 15-year hiatus period when no isolates occurred; a similar situation occurred in Kolkata, India, where *S. dysenteriae* type 1 remerged to cause two outbreaks of bloody diarrhoea after only one isolate had been detected in the pervious 5 years (Dutta *et al.*, 2003; Subekti *et al.*, 2001). In the United States, types1, 2, and 3 predominate among *S. dysenteriae* isolates and are evenly distributed amongst the three types. *S. boydii* type 14 is the most common *S. boydii* type in developing countries, while type 2 accounts for the most cases in the United States (Kotloff *et al.*, 1999). Cyclic shifts in serotypes have been observed over the last century, with cycles lasting 20 to 40 years, beginning with *S. dysenteriae* type 1, which is then replaced by *S. flexneri*, which in turn is replaced by *S. sonnei* (Scerpella *et al.*, 1994). These cycles are believed to result from changes in herd immunity. A newer pattern has emerged, at least in industrialized countries, characterized by continuous low level of transmission of one serotype, which transiently peaks from time to time. The last peak occurred in 1988 with *S. sonnei*; there is no explanation for this type of cycle yet (Scerpella *et al.*, 1994).

In the United States, *Shigella* is the third most-common cause of bacterial gastroenteritis, and approximately 10% of culture cases are hospitalized (Gupta *et al.*, 2004). Between 1989 and 2002, the incidence rates for *S. dysenteriae*, *S. flexneri*, and *S. boydii* decreased by only 8%. *S. sonnei* strains account for the increasing proportion of all *Shigella*, rising from 64% in 1989 to 84% in 2002. The majority of *Shigella* isolates are recovered between July and October. Over a third of all *Shigella* isolates are recovered from children of less than 5 years; 80% of these are *S. sonnei* cases.

Pandemics caused by *S. dysenteriae* type 1 appeared in Central America and Southeast Asia in the 1970s and in sub-Saharan Africa in the 1990s. These pandemics often occur in areas disrupted by political unrest or natural disasters and are accompanied by high attack and fatality rates (Kotloff *et al.*, 1999). Approximately 20,000 Rwandan refugees fleeing into Zaire in 1994 died in one month from infections

caused by a strain of *S. dysenteriae* type1 resistant to all commonly used antibiotics (Kotloff *et al.*, 1999).

Shigellosis is primarily spread by person to person contact; in the United States, 80% of *Shigella* infections are transmitted in this manner (DuPont & Pickering, 1980; Gupta *et al.*, 2004). Close contact, poor hygienic conditions, and faecal contaminated food or water are associated with outbreaks. Secondary transmission rates are high, especially for children. Approximately 60% of those who are less than one year of age and 20% of older individuals who are exposed to a primary case become ill (DuPont & Pickering, 1980). A low infective dose (10 to 100 organisms) accounts for much of the epidemiology seen with *Shigella* (DuPont & Pickering, 1980; Rosenberg *et al.*, 1976).

Shigella outbreaks occur frequently as a result of person to person contact, especially in institutions where there is suboptimal hygiene, or from contact with food or water contaminated by human faeces. Swimming in contaminated water with oral contact resulted in an outbreak with 18% cases rate (Rosenberg *et al.*, 1976). The same *S. sonnei* strain was isolated from humans and the swimming area one month after the outbreak even after the swimming had been banned in the area. *Shigella* can survive in water at room temperature for up to 6 months (DuPont & Pickering, 1980). There is a long list of food items responsible for outbreaks, since any food may be contaminated by a food handler; examples include oyster and other shellfish (Terajima *et al.*, 2004), moose soup (Gessner & Beller, 1994), fresh pasteurized milk cheese (Garcia-Fulgueiras *et al.*, 2001), bean dip (CDC, 2000), and iceberg lettuce (Kapperud *et al.*, 1995). Removal of *Shigella* from surfaces using detergents has not proven to be any more effective than rinsing with water (Raiden *et al.*, 2003).

Shigella outbreaks in confined environments with close contact between individuals are common. Day care centres are a major setting for shigellosis, especially among those caring for toddlers and infants (Pickering, 1986). Day care centre outbreaks have been reported to have attack rates ranging from 33% to 73 %, with secondary infections identified in 26 % to 33% of other family members (Kotloff *et al.*, 1999).The transmission of *Shigella* is associated with inadequate hand washing, improper handling of diapers, and faecal contamination of water play areas such as children's pools (Gupta *et al.*, 2004). Travelling to areas where shigellosis is endemic also results in a significant number of *Shigella* infections in industrialized countries (an estimated 580,000 cases), and the illness is often more disabling than that seen with enterotoxigenic *E. coli*, the leading cause of traveller's diarrhoea (Kotloff *et al.*, 1999).

Shigella, with minor exceptions such as *S. boydii* type 13, can be thought of as virulent clones of *E. coli* that have emerged during the last 35, 000 to 270,000 years (Pupo *et al.*, 2000).

Shigella spp. are invasive enteropathogens of the large intestine (colon). The first hurdle shigellae must overcome after oral ingestion is the bactericidal effect of gastric acidity (pH 2.5). To overcome the acidic pH, *Shigella* up regulates a number of acid resistance genes (Jennison & Verma, 2004) involving *rpoS*, which encodes the alternative sigma factor RpoS. Some of these activated genes appear to include genes encoding glutamate decarboxylase and a putative glutamate-gamma-amino-butyric-acid antiporter (Waterman & Small, 2003). The result is that acid resistance is fully expressed in *Shigella* under stationary conditions, which results in that almost 50% viability is retained after 2 h of exposure to pH 2.5.

The triad of clinical symptoms associated with *Shigella* infection is the result of a series of molecular events triggering invasion of the colonic mucosa and the subsequent elicitation of an intense inflammatory response. It appears unlikely that *Shigella* can penetrate epithelial cells of the microvillus brush border directly, since the apical membrane of colonic epithelial cells is covered by mucin (Waterman & Small, 2003). Therefore, *Shigella* circumvent this second obstacle by the primary invasion of endocytic M cells located in the follicle associated epithelium (Sansonetti, 2001), other routes of invasion are also possible, including penetration of tight junctions and destruction of tissue integrity induced by polymorphonuclear leukocytes responding to cytokines (Fig 2).

Thus, the ability of *Shigella* to infect host cells, including the continuous intra and intercellular spreading is essential for leading to bacillary dysentery. The invasiveness of the *Shigella* depends on the *Shigella* type III secretion system and has been extensively characterized at the biochemical level.

1.3.1 Type III Secretion System (TTSS) of *Shigella*

The system consists of a type III secretion apparatus (TTSA) that spans the bacterial envelope, translocators that transit through the TTSA and insert into the host cell membrane to form pores (translocon), effectors that transit through the TTSA and the translocon and are injected within the eukaryotic cell, chaperones that bind to translocators and some effectors prior to their transit through the TTSA, and transcription activators.

All of the TTSS genes necessary for entry of the bacterium into epithelial cell host have been identified and sequenced (Maurelli *et al.*, 1985; Parsot, 1994; Sansonetti *et*

al., 1982). They are clustered in a 30 kb region of a 220 kb virulence plasmid (Cossart & Sansonetti, 2004; Sasakawa *et al.*, 1988), plasmid cured *Shigella* strains are non virulent and cannot penetrate cultured epithelial cells (Sansonetti *et al.*, 1982). This region contains two sets of genes. The first one corresponds to the 13 ipa and ipg (Invasion plasmid antigen and gene) genes that encodes the IpaA-D, IpgB and IpgD effector proteins, and their dedicated cytoplasmic chaperones (Buchrieser *et al.*, 2000). Effectors are defined as proteins that through the TTSA and the translocon, are injected into cell, where they affect cellular functions. The chaperones are required to stabilize and partition the effector(s) before secretion (Parsot *et al.*, 2003). The repertoire of effectors secreted by a TTSA consists from 25 proteins. The second region of the 30 kb gene cluster corresponds to the 20 mxi and spa (membrane expression of Ipa and surface presentation of Ipa antigens) genes that encode mostly components of the TTSA.

The TTSA assembled by bacteria growing in culture at 37°C is only passively active and is activated upon contact of bacteria with epithelial cells. TTSA activation leads to an increase in the transcription by MxiE of genes encoding TTSA substrates (Demers *et al.*, 1998; Kueltzo *et al.*, 2003; Mavris *et al.*, 2002). The activation of MxiE requires IpgC, the chaperone of IpaB and IpaC, to act as a co-activator (Mavris *et al.*, 2002). In conditions of non secretion, IpaB and IpaC are transported through the TTSA (Menard *et al.*, 1993). Both proteins are inserted into the host cell membrane to form the translocon pores through which other effectors proteins subsequently pass through (Blocker *et al.*, 1999). IpaB also interacts with CDC44 on the cell surface. Additionally, IpaB and IpaC posses direct effectors function that alters the host cell physiology, including the induction of apotosis in macrophages (Zychlinsky *et al.*, 1994) and cytoskeletal rearrangements in epithelial cells (Kueltzo *et al.*, 2003; Tran *et al.*, 2000; Tran Van Nhieu *et al.*, 1999) respectively. Transit of IpaB and IpaC via the TTSA liberates IpgC, but this is not sufficient to activate MxiE as long as OspD1 is present in the cytoplasm. Following transit of OspD1 through the TTSA, MxiE is liberated and an interaction between MxiE and IpgC would allow MxiE to bind and activate the transcription of other substrates.

Other proteins which are secreted via the type III pathway, and facilitate entry of bacteria into host are IpaD, IpgD, IpaA, IpgB1, and VirA (High *et al.*, 1992; Menard *et al.*, 1993; Tran Van Nhieu *et al.*, 1997). Although not part of the translocon, IpaD is probably required for insertion of IpaB and IpaC in the cell membranes (Menard *et al.*, 1993; Picking *et al.*, 2005).

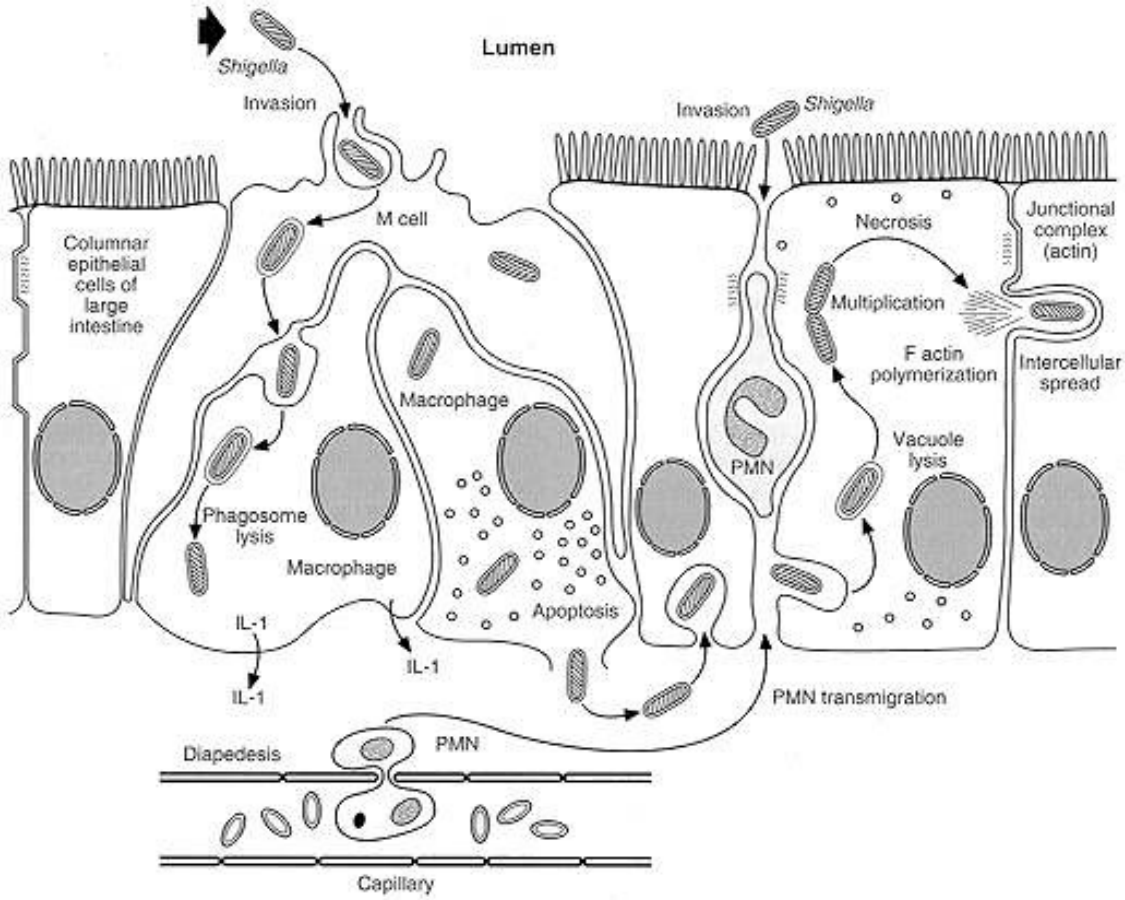

Figure 2 Life cycle of *Shigella* bacteria; omitted from courtesy of Samuel Baron.

1.4 Vibrio cholerae

In 1854 an Italian scientist named Pacini, was the first person to describe the agent responsible for cholera, he found a large number of curved bacteria in the intestinal contents of cholera victims which he called *Vibrio cholerae* (Pollitzer, 1959). This initial discovery was overshadowed by the work of Robert Koch, who studied cholera in Egypt and demonstrated in 1883 that cholera was caused by this comma shaped organisms (Pollitzer, 1959).

However, the classic epidemiological study of John Snow in 1854 in London showed the associations of the disease with contaminated drinking water even before any bacteria were known to exist (Fabiano L. Thompson *et al.*, 2006).

Vibrio species are now known to be ubiquitous in marine, estuarine, and freshwater environments and to encompass a diverse group of bacteria. Currently, the genus *Vibrio* consists of 65 species, of which more than 12 species are known to be associated with human disease (Abbott & Janda, 1994; Blake *et al.*, 1979; Mitra *et al.*, 1996; Perez-Tirse *et al.*, 1993; Tacket *et al.*, 1984).

V. cholerae strains belonging to the O1 and O139 serogroups that cause the severest form of the clinical symptoms of the disease named cholera, which usually occurs as explosive outbreaks. Cholera characterized by the passage of large volume of watery stools that rapidly causes dehydration, hypovolemic shock, and acidosis and can lead to death if the appropriate treatment is not initiated.

Pathogenic *V. cholerae* enters its host through an oral route of infection, colonizes the small intestine, and produce a potent enterotoxin known as cholera toxin (CT), which is mainly responsible for the manifestation of the disease (Faruque *et al.*, 1995; Kaper *et al.*, 1995). In toxigenic *V. cholera*, CT is encoded by a lysogenic phage referred to as CTXϕ (Waldor & Mekalanos, 1996). In addition to CT, the ability of pathogenic *V. cholerae* to cause disease depends primarily on the expression of a pilus colonization factor known as toxin coregulated pilus (TCP), so named because expression of TCP is under the same genetic control as that of CT (Faruque *et al.*, 1998; Faruque *et al.*, 2004). Both these pathogenic factors and their genetic determinations have been characterized to a considerable extent.

Cholera is a primary waterborne disease, and populations interacting with contaminated surface water may be affected at a high rate. The disease is endemic in Southern Asia and parts of Africa and Latin America, where outbreaks occur widely and it is associated with poverty and unstable countries (Faruque *et al.*, 1998; Kaper *et al.*, 1995). The fecal oral route of cholera usually occurs due to uptake of contaminated water as well as food been recognized to be an important source of transmission of cholera. After the uptake of contaminated water or food with the organisms, the bacteria passes through the acid barrier of the stomach, colonizes the small intestine, and produces CT, which is mainly responsible for the manifestation of the disease (Kaper *et al.*, 1995). CT acts as a classical A-B type toxin, leading to ADP- ribosylation of a small G protein and constitutive activation of adenylate cyclase, thus giving rise to an increase in the level of cyclic AMP within the host cell. This results in the rapid efflux of chloride ions and water from host intestinal cells. The subsequent loss of water and electrolytes leads to the severe diarrhea and vomiting characteristic of cholera. Massive outpouring of fluid and electrolytes leads to severe dehydration, electrolyte abnormalities, and metabolic acidosis (Kaper *et al.*, 1995). In severe disease, death may occur in as high as 50% to 70% of cases if they are not hydrated.

V. cholerae O1 and O 139 strains are human pathogens, these bacteria belong to a group of organisms that their normal inhabitants of the aquatic environment. The physicochemical conditions for the survival of the *V. cholerae* O1 have been

investigated, and the possibility of survival of the organism in an estuarine environments and other brackish waters is widely accepted. The survival might depend on several factors, such as the presence of particular physicochemical conditions, interaction of bacteria with aquatic plants or animals, and/or the existence of specific ecological association involving several components of the aquatic environment. It has been postulated that under unsuitable environmental conditions the bacteria are converted to a viable but non-cultivable (VBNC) form that cannot be recovered by standard culture techniques, and that such forms are able to produce infection and can revert to the cultivable form (Colwell & Huq, 1994; Kaper *et al.*, 1995). Contrary to this proposition, laboratory based studies on a marine *Vibrio* strain (Novitsky & Morita, 1976; Novitsky & Morita, 1977) showed that the organism responds to starvation by reducing metabolic activities and inducing morphological changes (Colwell & Huq, 1994). The public health and ecological importance of the possible survival forms, such as VBNC, depends on whether these forms are re-convertible to live infectious bacteria.

Water is clearly a way for transmission of *V. cholerae*, the physical, chemical, and biological parameters that support the seasonal pattern of epidemics are not clear. Several models have been proposed to explain this epidemiological observation. Thus, during interepidemic periods, toxigenic *V. cholerae* may exist in an unexplained ecological association with aquatic organisms such as *A. castellanii* in a possible non-cultivable form until the next epidemic season, when environmental factor trigger the dormant bacteria to multiply and lead to cholera outbreaks (Colwell & Huq, 1994).

Studies so far suggest that the cause of cholera in humans is also linked with a natural process of enrichment of toxigenic *V. cholerae*, and partly explain the benefit imparted to the pathogen during the disease in humans. However, to further understand the general epidemiological behavior of *V. cholerae*, which includes mechanisms leading to seasonal pattern of epidemics, transient appearance of new epidemic colonies; it is important to study the interaction among the bacteria, genetic elements mediating the transfer of virulence genes, the human host, and possible environmental factors.

The O-Ag polysaccharide (O-PS) has known as an important compound of the *V. cholerae* LPS. Analyses have shown the important role of *V. cholerae* LPS in the virulence and spread of the cholera. The O-PS of *V. cholerae* LPS is mainly responsible for its immunogenicity and production of vibriocidal antibodies in the host cell.

Gram-negative bacteria were known to manifest a wide spectrum of endotoxic activities (Raziuddin, 1978). In conformity with this, the LPS of *V. cholerae* was shown

to exhibit several endo-toxic activities (Kabir & Mann, 1980; Raziuddin, 1978). *V. cholerae* LPS was also shown to exhibit mitogenic effects and possess adjuvant properties (Kabir & Mann, 1980).

In Gram negative bacteria lipid A among all the constituents of LPS is responsible for endo-toxic activities (Galanos *et al.*, 1972). Lipid A is the active domain which is responsible for the induction of all known pathophysiological LPS effects (Broady *et al.*, 1981; Galanos *et al.*, 1972). Lipid A of *V. cholerae* LPS is similar in structural with the lipid A of many of other Gram negative bacteria (Broady *et al.*, 1981).

It was been showed that the O Ag is a protective Ag and was involved in the adherence and colonization of *V. cholerae* (Gupta *et al.*, 1992; Manning *et al.*, 1986; Neoh & Rowley, 1970). In an experiment by Fuerst and Perry (Fuerst & Perry, 1988) showed that LPS on sheathed flagella of *V. cholerae* O1 by protein A gold immunoelectron microscopy. The flagellum was found very important for in vitro attachment and enhanced initial colonization of the host intestinal surface in the infant mouse cholera model (Attridge & Rowley, 1983). Therefore the LPS on the flagellum was found to act as a carrier of adhesions (Chitnis *et al.*, 1982). It was been showed (Mukhopadhyay *et al.*, 2000) in the mouse model, the anti LPS antibodies induced passive protection through microagglutination and/or immobilization of vibrios, which did not allow the vibrios to adhere and colonize the intestine of the host.

1.5 Interaction with bacteria

Acanthamoebae feed on other microorganisms by phagocytosis, many bacteria are able to survive after the uptake with the amoebae cell and multiply within phagosomes, or in the cytoplasm or lyses the amoebae host cell. In 1956 Drozanski found a bacterium similar to *Pseudomonas* as agent of a fatal infection of *Acanthamoebae* (Drozanski, 1956).

In the mean time a wide range of bacteria, including several human pathogens, have been identified as being able to survive within *Acanthamoeba* cells. The intracellular growth has been associated with enhanced environmental survival of the bacteria, increased virulence and increased resistant against antibiotic substances (Barker *et al.*, 1999; Cirillo *et al.*, 1994).

Many Gram-negative or Gram-positive bacteria, including *Mycobacteruim*, and *Legionella* survive intracellular with the free-living amoebae establishing an endosymbioiontic relationship (Greub & Raoult, 2004; Hilbi *et al.*, 2007). The term endosymbionts defined by Buchner as ‘a regulated, harmonious cohabitation of two

nonrelated partners, in which one of them lives in the body of the other (Greub & Raoult, 2004).

Mycobacteruim spp. are ubiquitously present in natural and municipal water sources and most of the species can cause infection to human and fish. They have been shown that *M. marinum*, *M. fortutum* and *M. avium* replicate with *A. castellanii*, while non pathogenic species *Mycobacteruim smegmatis* is eatable by *A. castellanii* (Cirillo *et al.*, 1997). Moreover, *M. marinum* survives within cysts of *A. polyphaga* (Steinert *et al.*, 1998). Amoebae affect the virulence and antibiotic susceptibility of *M. avium* compared by bacteria culture alone. They found that *M. avium* grown in *A. castellanii* invade better the epithelial cells and macrophage, and multiply better in moncytes and macrophages and are more virulent to mice (Cirillo *et al.*, 1997). The anaerobe bacterium *Mobiluncus curtisii* is able to survive in an otherwise aerobic environment by multiplication and persistence within *Acanthamoeba*.

Acanthamoebae were suggested as the possible new sources, reservoirs and transfer mechanisms of infections caused by bacteria (Berk *et al.*, 1998; Tomov *et al.*, 1999). The intereaction between free-living amoebae and *L. pneumophila* was subject to many studies, showing that *L. pneumophila* depends on intra-amoeba multiplication prior to the infection of human (Rowbotham, 1986). Moreover, multiplication of *Legionella* seems to be restricted to protozoa cells, since they are unable to multiply extracellular in environment water (Abu Kwaik *et al.*, 1998). Thus free-living amoebae are considered to be a natural reservoir for *Legionella.*. After the attachment of the *L. pneumophila* to the amoeba cell, the bacteria are phagocytosed, actively to the lysosomal fusion and rapidly multiply within the amoeba phagosome. In their stationary growth phase the *Legionella* trigger a cytolytic mechanism which eventually leads to lysis of the amoeba cell and the release of vesicles filled with bacteria, which represent the infective particles for human (Berk *et al.*, 1998; Gao & Kwaik, 2000). Interestingly, *L. pneumophila* is also able to survive in the amoeba cyst, within the *Acanthamoeba* cyst bacteria are protected against chlorine at concentration commonly used for disinfection (Kilvington & Price, 1990; King *et al.*, 1988).

Taking the advantage of the ability of *Legionellae* to survive within amoebae, the *Acanthamoebae* have been used successfully for the recovery of viable *L. pneumophila* from clinical and environmental samples (Rowbotham, 1986) and for resuscitation of viable but non-culturable *Legionellae* (Steinert *et al.*, 1997).

In addition to *L. pneumophila*, a number of studies reported on the occurrence of intracellular bacteria in free-living amoebae, which also lyses their amoebae hosts and

thus were named *Legionella*-like amoeba pathogens (LLAP). Most of these LLAPs were originally found in amoeba isolates from sources associated with confirmed cases or outbreaks of Legionnair's diseases (Adeleke *et al.*, 1996).

Taking advantages of these free-living amoebae (non mammalian cells) allowed researchers to use the non mammalian cells as a model to study and analysis of bacteria virulence and test many hypotheses in pathogencity. The non mammalian cells are easy to handle experimentally and have longer age process compared to mammalian cells and allow researchers to have in depth analysis of host factor for host pathogens interaction.

Bacteria are manipulated genetically, which allow researchers to have several mutants which can be used to study the bacteria host interaction. In addition of that the interaction between free-living amoeba and bacteria will give researchers a lot of useful information which allow researchers to understand the host pathogen relationships. A better understanding of these interactions will lead to the development of the therapeutic agents to recognized and stop infectious agents.

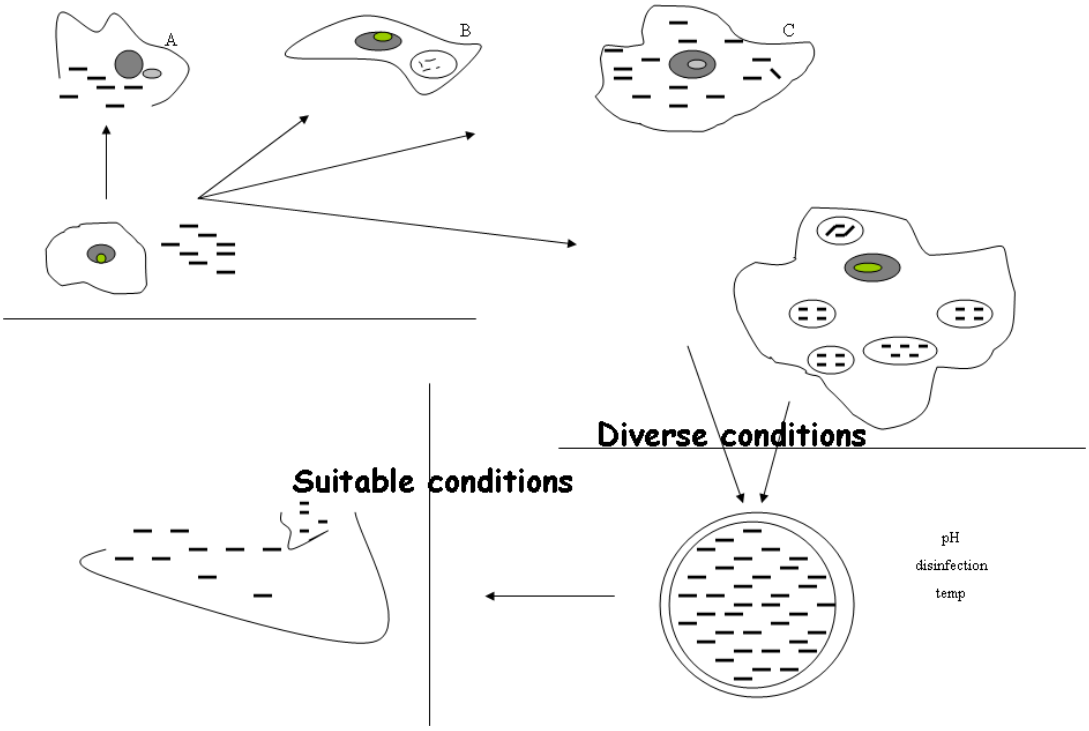

Figure 3 Interaction between *Acanthamoebae* and bacteria.

2 Aims of the thesis

The main aim of the thesis was to study the interaction between free-living amoebae *A. castellanii* and waterborne bacteria**.**

The specific aims of the thesis were

1. Does *A. castellanii* enhance growth and the survival of the *V. cholerae?*
2. Is *A. castellanii* an environmental host for *Shigella* species ?
3. To determine the role of *V. cholera* O139 and MO10 capsule and LPS on the intracellular growth of *V. cholerae.*
4. To determine the role of IpaB of *S. flexneri* in the interaction with *A. castellanii*.

3 Materials and Methods

3.1 Microorganisms

Acanthamoeba castellanii (ATCC 30010 and 30234) were obtained from the American Type Culture Collection, 10801 University Blvd. Manassas, VA 20110-2209, USA.

12 strains of *V. cholerae* O1 classical and El Tor listed in table 1 were from the culture collection of Laboratory Science division, International Centre for Diarrheal Disease Research, Bangladesh. The plasmid (pGFPuv) carrying GFPuv gene and confers resistance to ampicillin (100 mg/ml), was obtained from BD Biosciences Clontech, USA and introduced by electroporation into *V. cholerae* O1 classical strain C-19385 and *V. cholerae* O1 El Tor strain AK-38670. All mentioned *V. cholerae* strains used in paper I.

Table 1

Strain no	Biotype	Laboratory ID	Year of isolation
1	Classical	C-19385	1965
2	El Tor	Q-5970	1977
3	Classical	F-2427	1968
4	El Tor	AE-8182	1989
5	El Tor	AK-38670	1995
6	Classical	H-18	1970
7	El Tor	AR-32732	2002
8	Classical	X-19850	1982
9	Classical	Y-8661	1983
10	El Tor	AS-6522	2003
11	El Tor	MQ-1194	2001
12	Classical	AA-5117	1985

V. cholerae O1 classical- Ogawa strain 395, *V. cholerae* O1 El Tor-Inaba strain N16961 and *V. cholerae* O139 were obtained from the collection of Department of Laboratory Medicine, Division of Clinical Microbiology F 82, Karolinska University Hospital, Huddinge, SE-141 86, Stockholm, Sweden were used in paper II.

Vibrio cholerae O139 and MO10 is a wild type producing a capsule and a short LPS O side chain. A clinical isolate, from an emerged in 1992 and caused epidemic cholera in India. MO10-T4 strain is a spontaneous capsule mutant of MO10 (Waldor *et al.*, 1994). Bengal-2R strain (capsule + LPS double mutant) is a negative transposon Tn5lac insertion mutant of Bengal-2 (Knirel *et al.*, 1997), which is a vaccine derivative of MO10 (Waldor *et al.*, 1994). The strains were obtained from the culture collection of Laboratory Science Division, International Centre for Diarrhoeal Disease Research, Bangladesh were used in paper III.

The *Shigella* strains are *S. sonnei* (CCUG 37894) and *S. dysenteriae* (CCUG 37889) obtained from Culture Collection Göteborg University (University of Gothenburg), Göteborg, Sweden and were used in paper IV.

Shigella flexneri serogroup 5a strains M90T (Sansonetti *et al.*, 1982), BS176 (virulence-plasmid-cured derivative of M90T), and SF620 (non-polar *IpaB* deletion mutant) (Menard et al., 1993) were used in manuscript V.

3.2 Culture media and growth conditions

3.2.1 Growth of amoebae

Acanthamoeba castellanii was grown stationary at 30 ºC to a final concentration of 10^6cells /ml in ATCC medium no. 712 (ATCC, Manassas, VA).

3.2.2 Growth of bacteria

V. cholerae were grown on Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar plates (Oxoid, England) for 24 h at 37 ºC (Paper I, II and III). *Shigella* were grown on Blood agar plates for 24 h at 37 ºC (Paper IV and V). Then all bacteria were cultured in Luria-Bertani (LB) medium at 37 ºC to an absorbance at 600 nm of 0.4 to 0.6 OD units.

3.3 Co-cultivation of bacteria and amoebae

The co-cultivation assay was based on a method presented previously (Abd *et al.*, 2005). Axenically maintained amoebae were grown at 30 ºC to a final concentration of 2 x 10^5 cells /ml in ATCC medium as described above. Co-cultivations of each bacterium with *A. castellanii* were incubated in NUNC tissue culture flasks (75 cm) purchased from VWR International (Stockholm, Sweden) filled with 50 ml ATCC medium 712 containing *A. castellanii* at a concentration of 2 x 10^5 cells /ml and the particular bacteria at a concentration of 2 x 10^6 cells /ml.

3.4 Cultivation of control microorganisms

Control flask for each microorganism were cultivated separately and prepared in the same way and with the same initial concentration as for co-cultivated microorganisms. The flasks were incubated at the proper temperature without shaking.

3.5 Microscopic analysis

Alone amoebae and co-cultivated amoebae were counted in a Bürker chamber (Eurolab, Merck) under a light microscope (Carl Zeiss, Zeiss) using erythrosine B stain (ATCC), which stains dead amoebae only. The locations of bacteria in amoebae were disclosed by electron microscopy. Samples (5 ml) of alone and co-cultivated amoebae were centrifuged for 10 min at 300g in a Labofuge GL centrifuge (VWR International), and then washed in PBS. The pellets thus obtained were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 with 0.1 M sucrose and 3mM $Cacl_2$ for 30 min at room temperature. Samples were washed in sodium cacodylate buffer and post-fixed in 2% osmium tetroxide in the same buffer for 1 h. The samples were centrifuged, dehydrated and embedded in Epoxy resin, LX-112. The embedded samples were cut into ultra-thin sections, placed on grids, stained with uranyl acetate and lead citrate. The stained sections were examined in a Philips transmission electron microscope.

3.6 Bacterial uptake, intracellular growth and survival

Samples (2 ml) of co-cultivated amoebae were diluted with 8 ml PBS, centrifuged as described above and washed six times with PBS to minimize extracellular bacteria. The pellets were re-suspended in 1 ml PBS and incubated with 300µg /ml of gentamicin for 1 h at room temperature. The samples were then diluted with 9 ml PBS and centrifuged as described above. The supernatant was withdrawn and 100 µl was spread on blood agar plates as control for gentamicin assay. The pellet was diluted two-fold with 0.1% sodium deoxycholate. Series of tenfold dilution from 10^{-1} to 10^{-4} were prepared and spread on blood agar plates. All plates were incubated at 37 ºC for 24 h, and viable counts of bacteria colonies were counted.

3.7 Flow cytometry analysis

To detect necrosis or apoptosis by flow cytometery, V: FITC assay kit (ANNEX100F) from MorphoSys UK Ltd (Oxford, UK) was used. Samples of amoebae were prepared and examined in the absence and presence of bacteria. Cells were labeled with Annexin-V: FITC, according to the manufacturer's protocol. In brief, 2 x 10^5 amoeba cells /ml washed with binding buffer, addition of 5 µl Annexin-V: FITC, incubated in dark for 10 min at room temperature, resuspended in 190 µl binding

buffer, and addition of 10 µl of the propidium idodide, (PI) solution. Cells were immediately analysed on FACSAria (BD Biosciences, NJ, USA).

3.8 Statistical analysis

Student's t-test and Chi-square test were used to examine for significant differences in growth between alone and co-cultivated amoebae as well as bacteria.

3.9 Methodological considerations

In microbiological work, technical problems vary between microorganisms genera or species, and even between strains within a species. *A. castellanii* is easily cultivated in the ATCC media, easily to count and stable in the media used.

The technical problem was faced in preparatory work. Two problems required methodological development, one related to the concentration of gentamicin to kill all the extracellular bacteria, and the other was difficulty to adjust the flow cytometry analysis. Although similar methods were used in the publications, the reproducibility of the methods allowed improvement of the methods used.

4 Result and Discussion

In this doctoral thesis the interaction of *Acanthamoeba castellanii* with different serogroups of *Vibrio cholerae* the causative agents of cholera and *Shigella* species, the causative agents of shigellosis were been studied. Cholera and shigellosis affect millions of people round the world and it is very important to study ecology of the bacteria and how they interact with other microorganisms in the aquatic environments.

Here the presentation and discussion of the results in two parts; interaction with *V. cholerae* and with *Shigella* species, followed by concluding remarks.

4.1 Paper I, II and III

Microorganisms are found in aquatic environments and in biofilms where a complex interaction between bacteria and eukaryotic cells occur. *V. cholerae* interacts symbiotically with zooplankton (Abd *et al.*, 2005; Reidl & Klose, 2002) and associates with algae (Islam *et al.*, 1989).

In this thesis, we examined the ability of unencapsulated *V. cholerae* O1 classical and El Tor strains to grow and survive inside *A. castellanii*. The results showed that co-cultivated bacteria grew inside the *A. castellanii* during the experiment time (Fig 4), and the presence of the *A. castellanii* enhanced the survival of *V. cholerae* O1 strains for more than 2 weeks, while the number of cultivated *V. cholerae* O1 in the absence of *A. castellanii* decreased to non-detectable levels in a few days (Fig 5).

To study whether the alone-cultivated bacteria died or entered the VBNC state, the cultivability of *V. cholerae* O1 GFP colonies were estimated by cultivation on blood agar plates, and viability was detected by production of fluorescence light. The cultivated bacteria were green fluorescent on day 0,1 and 2 and the fluorescence became weak and weaker on day 3 and 4 respectively and the fluorescence light disappeared on day 5 which confirmed that the bacteria died and they do not enter the VBNC state, because the half-time of GFP was 24 h.

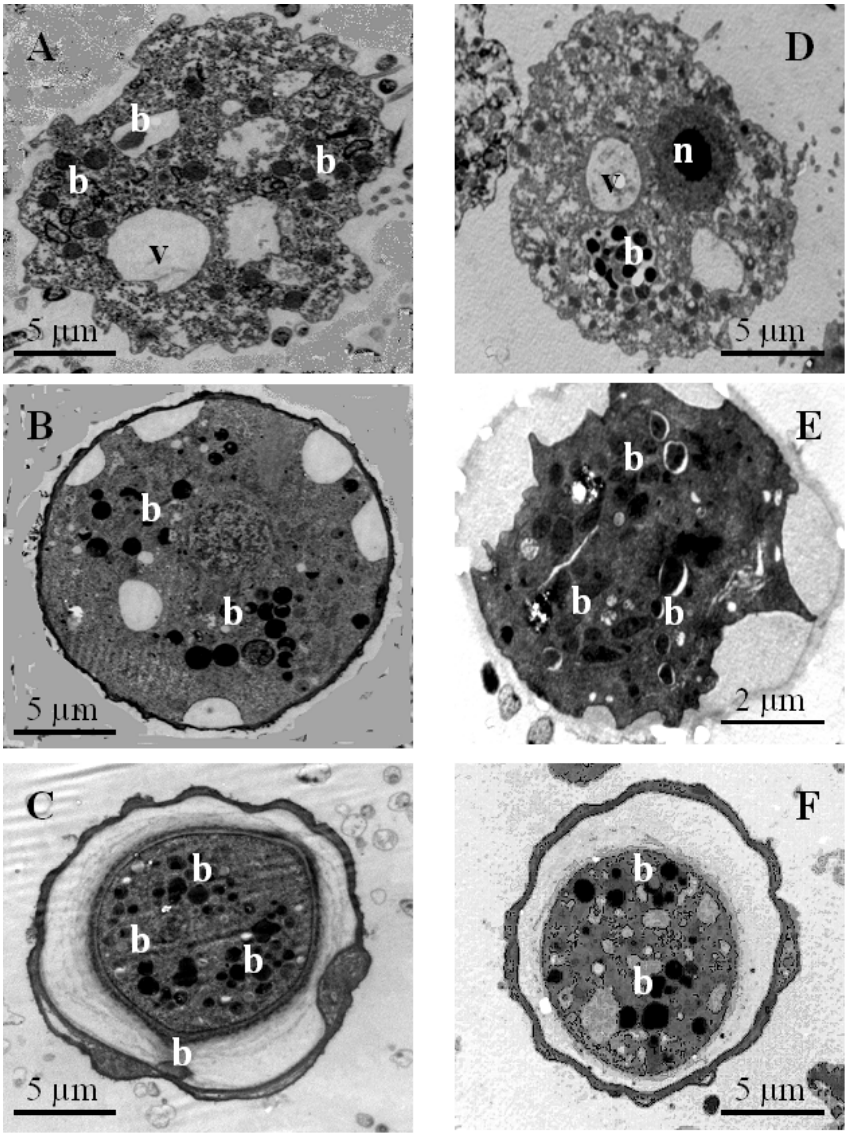

Figure 4 Electron microscopy photomicrographs of the intracellular localization of *V. cholerae* in *A. castellanii*. n, nucleus; v, vacuole; b, bacteria. (A) *A. castellanii* contains *V. cholerae*, 1 day after co-cultivation. (B) *A. castellanii* cyst contains *V. cholerae*, 1 day after co-cultivation (C) *A. castellanii* cyst contains *V. cholerae*, 7 days after co-cultivation (D) *A. castellanii* contains *V. cholerae*, 3 days after co-cultivation (E) and (F) *A. castellanii* cyst contains *V. cholerae*, 3 days after co-cultivation.

V. cholerae O1 strains grew and survived intracellularly in *A. castellanii* for the duration of the experiment. From these result *A. castellanii* supported the survival of *V. cholerae* O1 strains which maintained by the continuous growth of intracellular *V. cholerae* which increased from 0 at the start of the experiment to 10^3 on day 1, to 10^4 on 4 day, and to 10^5 on day 14 (Fig 5). The number of *A. castellanii* and the intracellular *V. cholerae* O1 increased over the time, and the statistical analysis showed that the growth of intracellular *V. cholerae* were dependent on the growth of *A. castellanii*.

Here we presented that the *A. castellanii* may worked as biological host for *V. cholerae* O1 which support the intracellular growth and survival of the bacteria. The behavior of *V. cholerae* O1 similar to the behavior of the facultative intracellular bacterium *F. tularensis* (Abd et al 2003) and differ from the behavior of extracellular bacteria *Pseudomonas aeruginosa* (Abd et al 2008).

Interestingly, *V. choerae* O1, which is well known as an extracellular bacterium, behaved as a facultative intracellular bacterium in this thesis. The facultative intracellular behavior of *V. cholerae* shown in this study explains the ability of *V. cholerae* to survive and to grow in the cytoplasm of the amoebae.

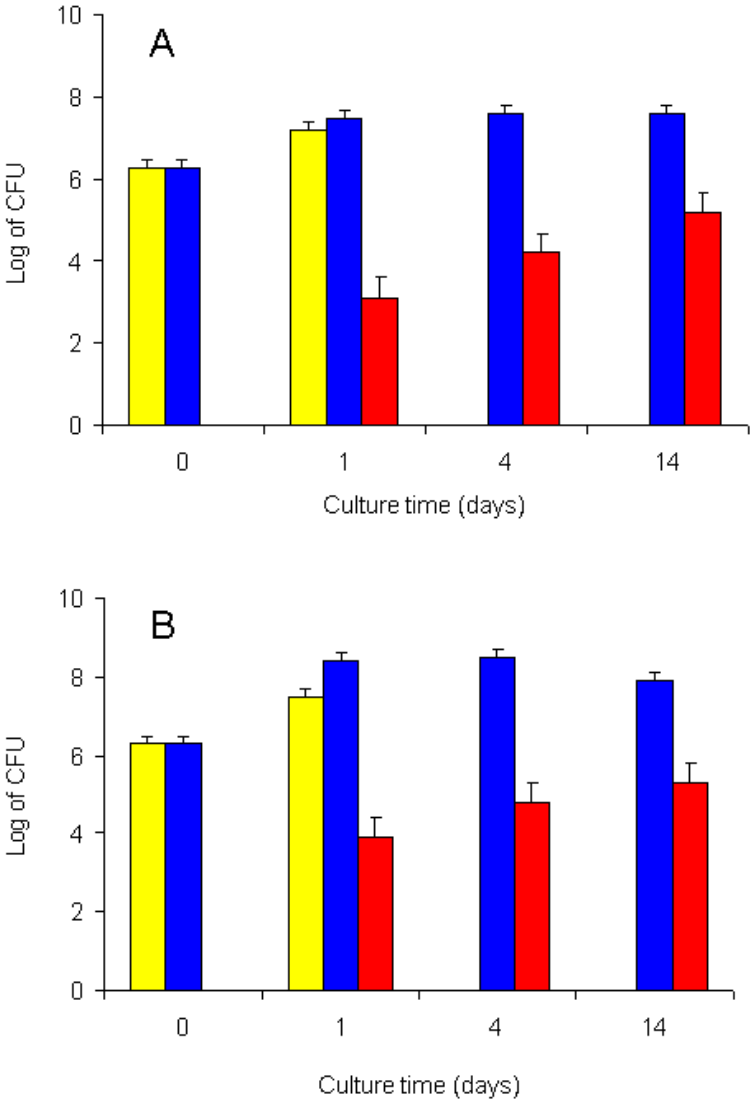

Figure 5 Viable counts of *V. cholerae* O1 strains: (a) counts of classical strains, and (b) counts of El Tor strains. Yellow staples indicate *V. cholerae* in the absence of *A. castellanii*; blue staples indicate *V. cholerae* in the presence of *A. castellanii*; and red staples indicate intracellular *V. cholerae*. Data indicate means value of six independent experiments.

The ability of *V. cholerae* to grow and survive intracellularly in *A. castellanii* presented here may have revealed one of the biological factors that could enhance the survival of *V. cholerae* in environments, because the amoeba is a possible biological factor that enhances growth of the bacteria in water and may increase the possibility that humans become infected with cholera.

The intracellular survival of the bacteria may explain why the *V. cholerae* experience losses to predators in the environment. Moreover, *V. cholerae* may utilize the amoeba as an environmental host which may enhance the growth of the bacteria, and protect the *V. cholerae* form the unsuitable conditions in the environment.

In this thesis we found that *V. cholerae* O1 has a facultative intracellular behavior as a new common property, which extend our knowledge in studying the ecology, immunity, treatment, and support the role of free living amoebae as environmental hosts of the epidemic *V. cholerae*.

The infective dose of *V. cholerae* is very high, which reflect the needs of the bacterium to environmental host to enhance the multiplication of the bacteria. Therefore the role of *A. castellanii* which may enhance growth and multiplication of the *V. cholerae* O 139 and O1 and the symbiotic relationship between these microorganisms were been studied. The analysis showed that co-cultivated *V. cholerae* strains grew hundred folds after one day and survived two weeks in comparison to singly cultivated bacteria. The symbiotic relationship between intracellularly growing *V. cholerae* and the host cell *A. castellanii* was examined by recultivation of $4x10^5$ cell/ mL *A. castellanii* hosting $2x10^5$ cell/ mL *V. cholerae* in 50 mL ATCC medium 712 for 2 weeks. The result showed an enhanced growth of both microorganisms. Viable *A. castellanii* number increased from $4x10^5$ cell/ mL on day 0 to $2.3x10^6$ cell/ mL on day 14 and the viable count of *V. cholerae* increased from $2x10^5$ cell/ mL on day 0 to $2.5x10^9$ and to $3x10^{10}$ cell/ mL on days 1 and 4 and bacteria survived during 14 days at a concentration of $3x10^8$ cell/ mL (Fig. 6). Recultivation of *A. castellanii* hosting intracellular *V. cholerae* as endosymbionts enhanced growth and multiplication of the amoeba as well as the bacteria. The present study examined if the interaction between *V. cholerae* and *A. castellanii* showed endosymbiont-host relation. The results showed enhanced growth and survival of co-cultivated *V. cholerae* species, which did not inhibit growth of the amoebae in co-cultivation. Growth of *V. cholerae* inside *A. castellanii* trophozoites did not affect viability of the trophozoites to develop to cysts hosting viable *V. cholerae*. Thus, the *V. cholerae* interact with *A. castellanii* and used the amoebae as production unit to reach the high number of bacteria needed to cause infection in humans.

To study the effect of clinical isolate of *V. cholerae* MO10 on the amoebae, growth of *A. castellanii* in absence or in presence of *V. cholerae* MO10 strains was studied. The concentration of the amoebae (trophozoites and cysts) in absence or presence of *V. cholerae* MO10 strains was $2x10^5$ cells/mL, which increased ten-fold in absence of the bacteria after 14 days. The number of *A. castellanii* increased ten-fold in the presence of each of wild type *V. cholerae* MO10, capsule mutant strain, and capsule +LPS double mutant of MO10 strain. Despite count of amoeba in presence of the capsule mutant strain, the number of amoebae is not as many as shown by the counts in

presence of other bacterial strains. Growth of *A. castellanii* in absence or presence of MO10 strains did not show any statistically significant (t-test, $p > 0.05$).

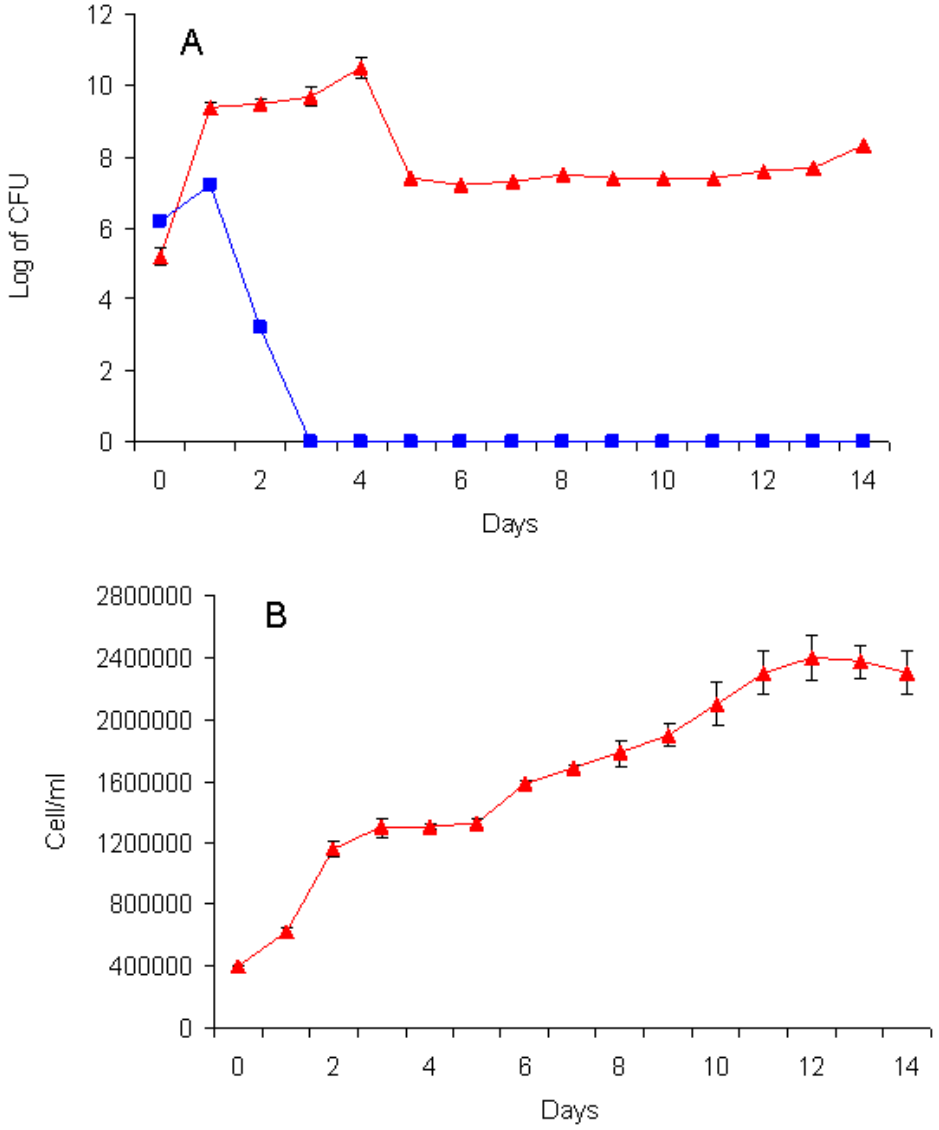

Figure 6 Symbiotical growths of amoeba and bacteria. A. Growth of *V. cholerae* from recultivation of amoeba hosting intracellular bacteria (red triangles) and growth of singly cultivated bacteria (Blue squares). Data indicate mean values ± SD of double measurements. B. Growth of recultivated *A. castellanii* hosting intracellular *V. cholerae* O1. Data indicate mean values ± SD of double measurements.

The effect of *A. castellanii* on growth and viability of *V. cholerae* strains was studied, *V. cholerae* strains cultivated in ATCC medium are compared to growth and viability of the same strains co-cultivated with amoebae in the same medium.

The viable counts of wild type MO10, capsule mutant strain and capsule + LPS double mutant strain in the presence of *A. castellanii* showed 1000-, 1000- and 10-fold increase after one day, respectively, and all bacterial strains survived for more than 2

weeks. Viable counts of all *V. cholerae* MO10 strains in the absence of amoebae increased 100-, 100- and 10-fold during the first day, respectively, followed by a decrease to non detectable levels on day 4 and 5. Student's t-test showed a significant statistical difference in the growth of the bacteria in the absence or presence of *A. castellanii* ($p < 0.0001$).

The effect of the capsule or LPS on the uptake of the bacteria, the number of engulfed wild type MO10, capsule mutant strain and capsule + LPS double mutant strain estimated by viable count and gentamicin assay after 2h of co-cultivation was $2.6 \times 10^2 \pm 20$ CFU/mL, $2.4 \times 10^2 \pm 10$ and $2.2 \times 10^2 \pm 20$ CFU/mL, respectively. The difference in the uptake of *V. cholerae* strains was not significant; p value of χ^2 test was 0.99 (Fig 7).

The growth and survival of the engulfed bacteria following gentamicin treatment and re-cultivation, the number of intracellularly growing bacteria was estimated by viable count. Viable counts of engulfed wild type MO10, capsule mutant strain and capsule + LPS double mutant strain increased intracellularly to 10^3 CFU/mL after 24h and to 10^5 CFU/mL after 48h, respectively and they survived at 10^5 CFU/mL for more than two weeks. χ^2 test did not show any statistical difference in the intracellular growth of *V. cholerae* strains ($p = 0.999$). Growth of the wild type, capsule mutant strain, and capsule + LPS double mutant strains were enhanced in the presence of *A. castellanii* and the bacteria could grow inside the amoebae. However, the intracellular growth of the mutant strains was not significantly different from their wild type strain.

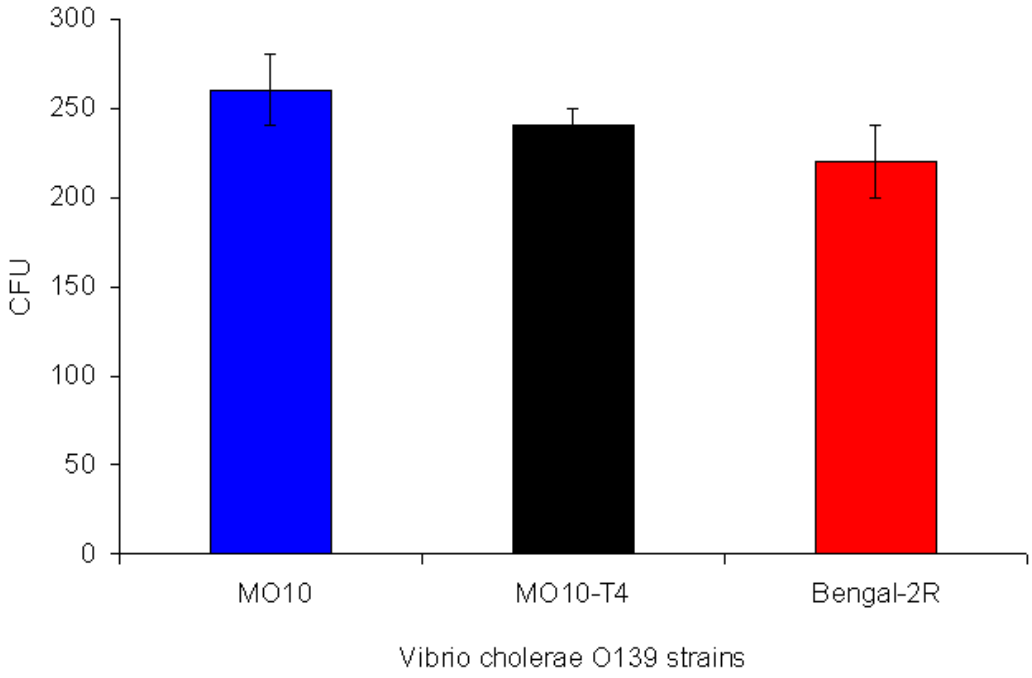

Figure 7 Uptake of *V. cholerae* by *A. castellanii*. The number of engulfed *V. cholerae* MO10, MO10-T4 and Bengal-2R estimated by viable count and gentamicin assay after 2h of co-cultivation. Data indicate mean ± SD of three repeated times at each time point.

Electron microscopy was used to visualise the intracellular localization of wild type MO10, capsule mutant strain and capsule + LPS double mutant strain in *A. castellanii*. Samples from co-cultures containing *A. castellanii* and each *V. cholerae* strain were prepared separately for electron microscopy. The intracellular localization of the bacteria was seen in the cytoplasm of trophozoites a few hours after co-cultivation. Multiplication of bacterial cells occurred in the cytoplasm of trophozoites one day after co-cultivation. Moreover, bacteria were found in the cysts of *A. castellanii* 6 and 7 days after co-cultivation (paper III).

The result showed that the clinical isolate *V. cholerae* MO10 grew and survived symbiotically in *A. castellanii* during the experiment time. The intracellular growth of the wild type, capsule mutant strain, and capsule + LPS double mutant strains, was not significant despite the strains differences in cell composition of each strain.

4.2 Paper IV and V

Free-living amoebae are always found in water environments in which *Shigella* also occur. Thus, *Shigella* and amoebae share the same water environment, and accordingly the two microorganisms could interact. Many studies have demonstrated that *Acanthamoeba* interacts with various water-borne pathogens, and these studies

establish the role of *Acanthamoeba* as a reservoir for such pathogens (Abd *et al.*, 2005; Jeong *et al.*, 2007). *Shigella* is a facultative intracellular bacterium that can multiply and survive either inside or outside of host cells (Arias Fernandez *et al.*, 1989), which is important to remember when considering *Shigella's* interaction with *Acanthamoeba*. Many enteropathogens, including *Shigella* species, survive chlorine treatment more readily if they are incubated with protozoa. King *et al.* showed that *S. sonnei* is capable of surviving within protozoa, which increases its resistance to killing by chlorine by a factor greater than 50 (King *et al.*, 1988).

The interaction between *A. castellanii* and *Shigella* bacteria were studied. The growths of *A. castellanii*, in absence or present of *Shigella* species, were determined by amoebae cell count during the experiments time. The number of the cells of *A. castellanii* alone and in presence of *S. sonnei* and *S. dysenteriae* increased ten folds. The growth and survival of *S. sonnei* and *S. dysenteriae* in the cytoplasm of *A. castellanii* resemble their pattern of growth and survival in macrophages (Ismail *et al.*, 2002), and it confirms that these bacteria are facultative intracellular. Moreover it resembles the symbiotic growth and survival of *V. cholerae* bacteria inside *A. castellanii* (Abd *et al.*, 2005).

S. sonnei and *S. dysenteriae* co-cultivated with amoebae increased 10, 100 folds respectively in 18 days (Fig 8), while the concentrations of *S. sonnei* and S. *dysenteriae* in the absence of amoebae decreased from 2.0×10^6 CFU/ml on day 0 to non-detectable levels at day 15 and 18, respectively (Fig 8). These results showed that the presence of amoebae enhanced growth of *S. sonnei* and of *S. dysenteriae* in comparison to alone cultured *Shigella*. *S. sonnei* increased ten fold, and *S. dysenteriae* 100 hundred fold.

Samples containing amoebae co-cultivated with *S. sonnei* and *S. dysenteriae* were washed and treated with gentamicin to kill extracellular *Shigella* following treatment with deoxycholate solution to release intracellular bacteria. The intracellular bacteria could be detected by viable count assay after 2h and up to the 3 weeks (Fig 8). The intracellular bacteria grew and survived in the cytoplasm of the amoebae for three weeks.

Erythrosine B staining showed that the co-existence of the microorganisms did not affect the viability of the amoebae. *A. castellanii* trophozoites and cysts in which *Shigella* was present were viable, and the intracellular bacteria did not affect the viability of the amoeba. There were no differences in the number of cysts of *A. castellanii* in the absence or presence of *A. castellanii.* Thus, the relationship between *S. dysenteriae*, *S. sonnei* and *A. castellanii,* can be considered as symbiosis.

Accordingly, *A. castellanii* might act as an environmental reservoir for both *S. sonnei* and *S. dysenteriae*, which may contribute to the explanation of why *S. sonnei* causes 78% of the outbreaks of shigellosis in the industrialized countries where hygiene is high (Pal, 1984; Sultana *et al.*, 2002).

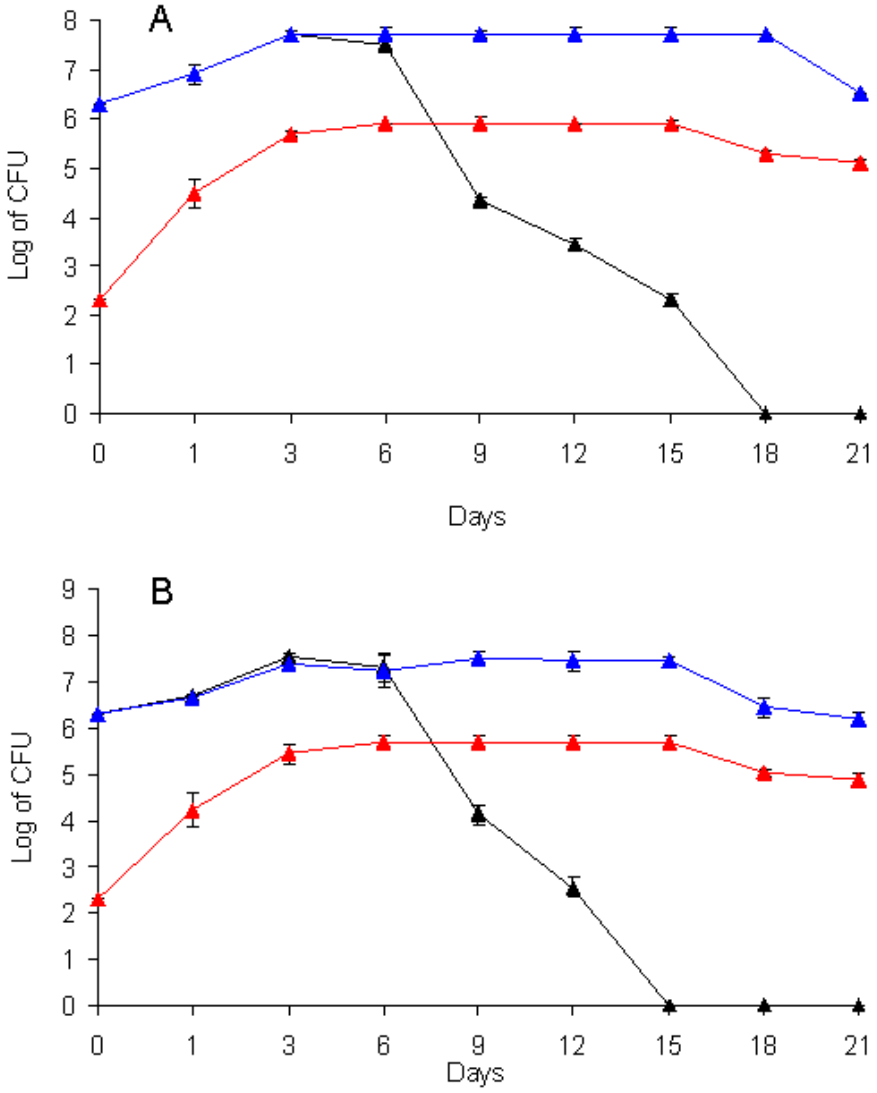

Figure 8 A Viable count of *S. dysenteriae*. The numbers of *S. dysenteriae* cultivated alone (black), *S. dysenteriae* co-cultivated with amoebae (blue), and intracellular *S. dysenteriae* (red) B. Viable count of *S. sonnei*. The numbers of *S. sonnei* cultivated alone (black), *S. sonnei* co-cultivated with amoebae (blue), and intracellular *S. sonnei* (red). The points are mean values of two independent experiments, and bars indicate their standard deviations.

Electron microscopy of *A. castellanii* cells from culture in the absence of *Shigella* compared to the amoebae in presence of *Shigella* showed that *S. sonnei* or *S.*

dysenteriae were located in vacuoles or in the cytoplasm of amoebae (Figures 9C and 9D). The bacteria were present in trophozoites and in cysts on days 1 and 6 (Figure 9).

Intracellular bacteria utilize different mechanisms to survive and multiply inside the host cells such as amoebae and macrophages. *Francisella tularensis* survives within membrane-bounded vacuoles in macrophages (Greco *et al.*, 1987) as well as in *A. castellanii* (Abd *et al.*, 2003). In contrast, *Shigella* lyses the surrounding membranes of the vacuoles and escapes into the cytoplasm of macrophage in a short time (Clerc *et al.*, 1987; Shere *et al.*, 1997). We found that *Shigella* cells were located in vacuoles or in the cytoplasm of *A. castellanii* trophozoites at day 1 and in the cysts at day 7 (Fig 8 C, D, E and F). Moreover, the intracellular *S. dysenteriae* or *S. sonnei* survived for more than 3 weeks at the level of 10^5 or 10^6 CFU/ml, respectively. This thesis presents more details about the interaction between *S. dysenteriae*, *S. sonnei* and *A. castellanii* to provide more evidences about ability of *A. castellanii* to act as an environmental host for *S. dysenteriae* as well as *S. sonnei*.

The growth and survival of *S. dysenteriae* and *S. sonnei* in the cytoplasm of *A. castellanii* resemble their pattern of growth and survival in macrophages (Ismail *et al.*, 2002), and it confirms that these bacteria are facultative intracellular. Moreover it resembles the symbiotic growth and survival of *V. cholerae* inside *A. castellanii* (Abd *et al.*, 2005).

S. dysenteriae and *S. sonnei* cultivated with *A. castellanii* grew at the same rate at 20 ºC as it did at 30 ºC. The *Shigella* species kill the amoebae at 37 °C because *Shigella* expresses virulence genes at 37 ºC. A regulatory factor VirF regulates all of the genes that are needed to activate invasion genes. VirF is activated when the temperature in the environment of the *Shigella* reaches 37 ºC (Falconi *et al.*, 1998).

Although free-living amoebae represent important predators of bacteria and fungi, some microorganisms have the ability to resist destruction by free-living amoebae. The interaction between *A. castellanii* and *S. dysenteriae* or *S. sonnei* was investigated in this thesis and it was found that the presence of the free-living amoeba could enhance the growth and survival of the *S. dysenteriae* and *S. sonnei*. This result suggests that amoeba acts as a reservoir for *S. dysenteriae* and *S. sonnei.* Free-living amoebae are ubiquitous in nature found in many aquatic environments. *S. dysenteriae* and *S. sonnei* are known waterborne pathogens; therefore it is likely that amoebae play a role in the transmission of the microorganism in the environment.

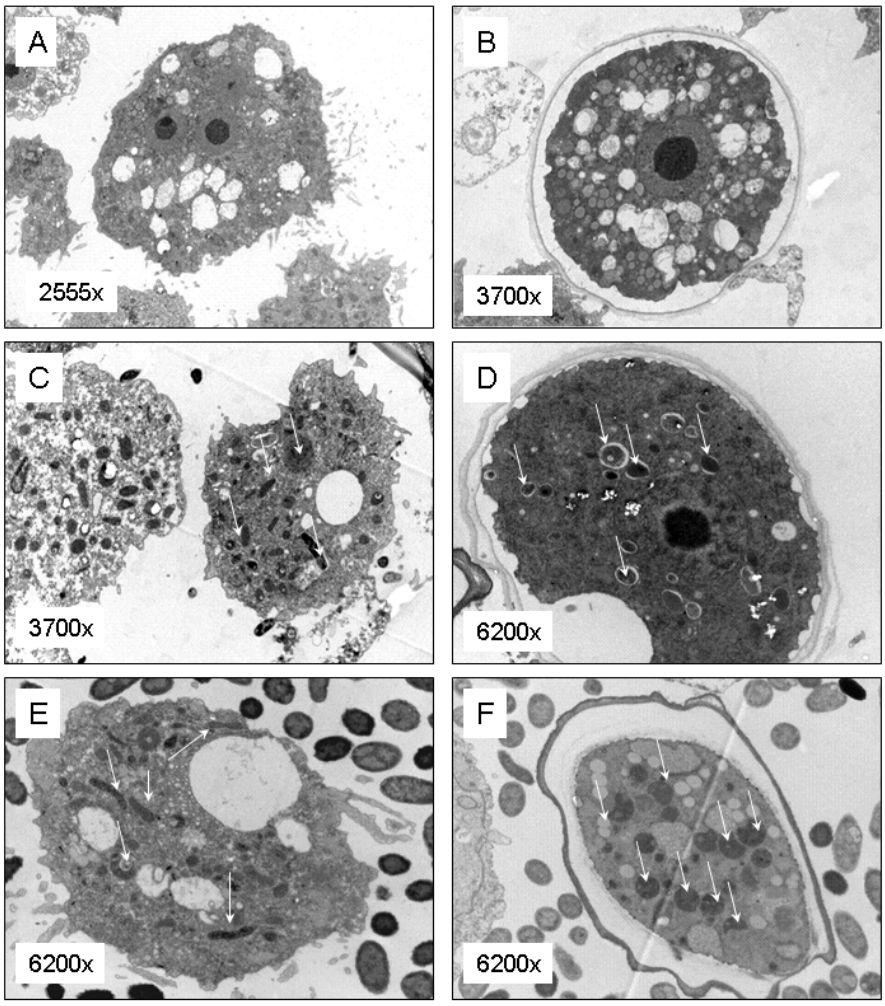

Figure 9 Electron micrographs showing the intracellular localization of *Shigella* in *A. castellanii.* The arrows head indicate pointed to the *Shigella*. A- *A. castellanii* trophozoite alone. B- *A. castellanii* cyst alone c - *A. castellanii* trophozoite co-cultured with *S. sonnei,* Day 1. D- *A. castellanii* cyst co-cultured with *S. sonnei,* Day 6. E - *A. castellanii* trophozoite co-cultured with *S. dysenteriae,* Day 1. F- *A. castellanii* cyst co-cultured with *S. dysenteriae,* Day 6.

S. flexneri is a waterborne pathogen, it may interact with *A. castellanii* present in water and this may prime the *S. flexneri* for infection of the host cell. The interaction between *A. castellanii* and *S. flexneri* was examined as well as the role of the IpaB protein on the *A. castellanii*.

The growth of *A. castellanii,* in absence of *S. flexneri* increased 10 fold from day 5 to day 10. The number of *A. castellanii* co-cultivated with wild type, IpaB mutant, or plasmid cured strain *S. flexneri* decreased 100 fold on day 6, but started to grow again on day 9 ending in the initial number on day10.

Wild type *S. flexneri* at 30 ºC inhibits the growth of *A. castellanii* to a rate that is lower than alone grown amoebae ($p \leq 0.001$) but the affect did not result in killing of the

amoebae. However at 37 °C it was found that the wild type killed *A. castellanii* in 1 day. In this context, it is well known that the invasive property of *S. flexneri* is depended on the 220-kb plasmid, which is strongly temperature regulated. The organism is noninvasive at 30 °C but fully invasive at 37 °C (Maurelli *et al.*, 1984).

To examine affect of the amoebae on the bacterial growth viable count of the bacteria was performed. It was found that both alone and co-cultivated bacteria increased 100 fold on day 1. The decline phase started on day 9. However, the alone and co-cultivated *S. flexneri* died on days 15 and 18, respectively.

To study the role of wild type *S. flexneri* and with the mutant, *A. castellanii* co-cultivated with wild type *S. flexneri* encoding TTSS system or with its mutant (virulence-plasmid-cured strain) showed that the wild type *S. flexneri* killed amoeba population in 1 day and TTSS mutant killed the amoebae after 6 days. However, *S. flexneri* IpaB mutant, killed the amoeba population in 3 days. The number of *A. castellanii* in absence of the wild type *S. flexneri* increased two times on day 3 and survived through the experiment time.

To study the affect of TTSS I focused on IpaB, which, was found to be important in the entry into host cells and in the lysis of phagocytic vacuoles (Menard *et al.*, 1993).

IpaB was found to play an important role in killing the amoebae in 3 days. When the TTSS is activated and the virulence plasmid is absent, *A. castellanii* cells are still destroyed.

These results showed that IpaB plays an important role in killing *A. castellanii* when compared to the IpaB mutant and virulence plasmid-cured strains. Thus means, that there are other components of TTSS play part in the destruction of *A. castellanii* in the absence of IpaB or that these other components could work together with IpaB to destroy *A. castellanii.* Furthermore, these results confirm that there are other mechanisms not existing in the plasmid involves in the killing of *A. castellanii.*

Flow cytometry showed that percentages of necrosis in amoeba cells caused by *S. flexneri* wild type, IpaB mutant and TTSS mutant strains were 90.9%, 81.9% and 66.9%, respectively (Fig. 10). Analysis of these data showed that TTSS virulence factors caused 23.6% of the necrosis and IpaB protein caused 38% of the necrosis caused by TTSS indicating that IpaB protein and other TTSS virulence factors participate strongly in amoeba killing as well. However, percentage of necrosis caused by TTSS in plasmid compared to those not present in plasmid was 35%. High percentage of necrosis caused by the plasmid-cured strain (TTSS mutant strain)

showing that other components rather than plasmid located TTSS participated in the killing of the amoeba populations.

Despite that most of the amoebae killing was caused by necrosis, the flow cytometry detected low percentages of apoptosis less or not much more than alone cultured amoebae (Fig. 10). Killing of *A. castellanii* by wild type *S. flexneri* possessing TTSS or mutant in TTSS was statistically significant (p of $\chi 2 = 0.007$).

Moreover, difference in viability of *A. castellanii* alone or co-cultivated with wild type *S. flexneri* was also found to be significant (p of $\chi 2 = 1.4 \times 10^{-11}$). The flow cytometry results demonstrated that wild type, IpaB mutant and a plasmid-cured strain of *S. flexneri* induce necrosis in *A. castellanii*.

Taking together these findings conclude that *Shigella* has the ability to control the way it kills the host cells, as it clearly commands mechanisms that lead to either apoptosis or necrosis. Thus, such ability must play a crucial role in the interaction of *Shigella* with different host cells. *S. flexneri* utilizes its TTSS to induce necrosis in *A. castellanii* cells. However, there are other mechanisms, which assist the virulence plasmid or takes over when it is not operating efficiently.

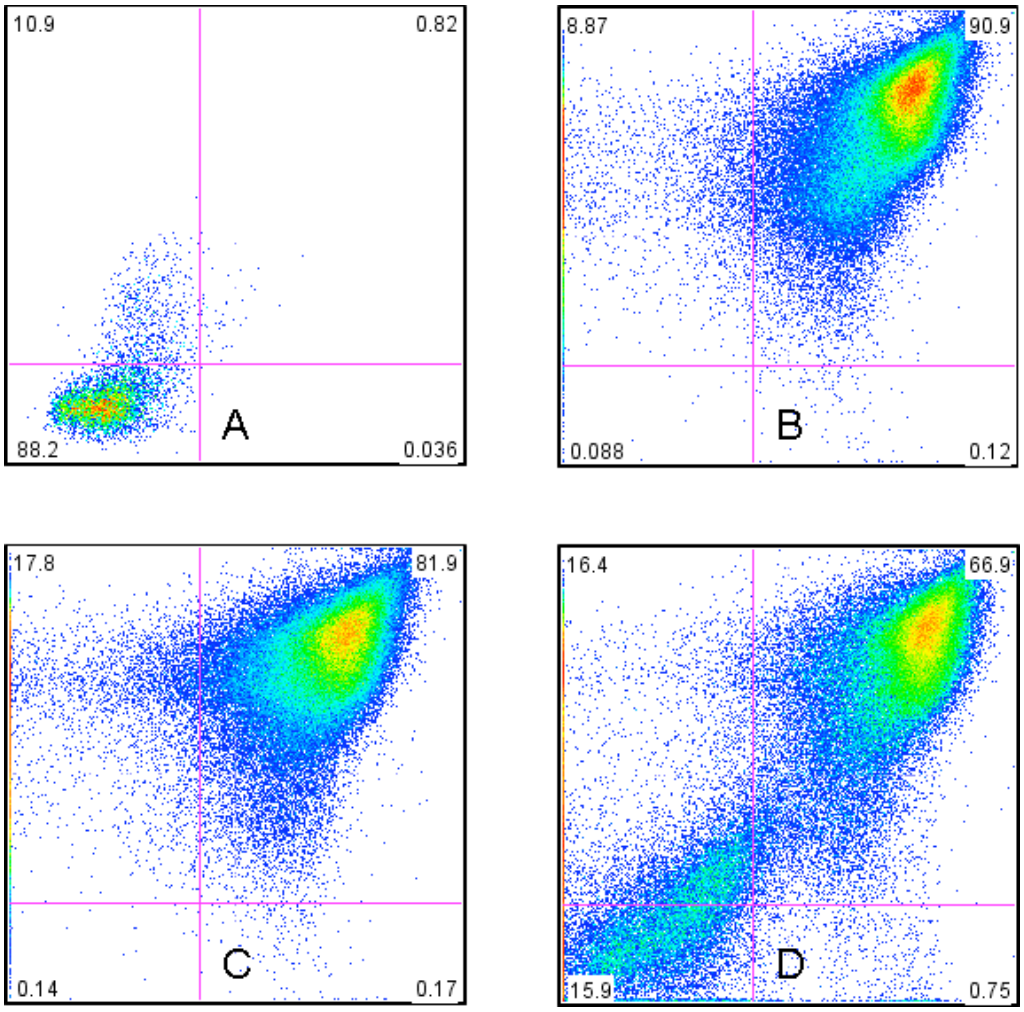

Figure 10 Flow cytometric analyses. Right upper panel shows necrosis % and right lower apoptosis %. A. ***A. castellanii*** **alone. B.** ***A. castellanii*** **co-cultivated with wild type** ***S. flexneri.*** **C.** ***A. castellanii*** **co-cultivated with IpaB mutant** ***S. flexneri.*** **D.** ***A. castellanii*** **co-cultivated with plasmid-cured** ***S. flexneri.***

5 Concluding Remarks

In summary, the work presented in this thesis has demonstrated that:

1. *V. cholerae* are facultative intracellular bacteria.
2. *A. castellanii* enhances the growth and survival of the *V. cholerae. V. cholerae* does not inhibit growth of the amoebae indicating endosymbionts-host relation between *V. cholerae* and *A. castellanii*.
3. The capsule and a short LPS O side chain of *V. cholerae* MO10 do not had any role on the intracellular growth of the bacteria inside *A. castellanii.*
4. *A. castellanii* may play a role in the transmission of *S. dysenteriae* and *S. sonnei* in the environment.
5. IpaB of *S. flexneri* plays an important role in killing *A. castellanii and* there is another mechanism exists in killing the *A. castellanii* not present on the plasmid of *S. flexneri.*

Acanthamoebae support and enhanced bacterial growth, survival and provide the suitable environmental condition to the bacteria which will increase the risk of human illness caused by water-borne bacteria. *Acanthamoeba* is an ideal non mammalian model to study bacterial virulence. The advantages of *Acanthamoeba* as a model to study bacterial virulence are that it is an inexpensive cell line, it is easy to handle experimentally and it allows analyses of large pools of mutants.

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