Adhesion Patterns of *Neisseria Lactamica* and its Implications

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ABSTRACT

Neisseria Meningitidis is a pathogenic bacteria responsible for a wide range of serious disease, including septsis and meningitis especially in children and infants. In contrast *Neisseria Lactamica* which resides in the same ecological niche as *N.meningitidis*, the nasopharynx, does not cause disease and is commensal. It was found that the two species of *Neisseria* have converse carriage rates where *N.lactamica* has a protective role over *N.meningitidis* colonization in infants. The two species have similar structural components and genes but very different pathogenesis. It is clear that *N.meningitidis* adheres and invades host cells whereas *N.lactamica* does not invade.

Adhesion of *N.lactamica* seems to occur mostly around the sides of the cell rather than the apex or base. Adhesion numbers of *N.lactamica* increase with time of infection with a greater increase from 4 to 6 hours rather than 2 to 4 hours of infection. Adhesion rates in relation to *N.meningitidis* adhesion numbers were less which may be due to greater adhesive ability of *N.meningitidis*. Inhibitor treatment of epithelial cells and thus, a decrease in cellular inflammatory response, resulted in a greater degree of adhesion and again from 4 to 6 hours, the increase in adhesion numbers were greater than from 2 to 4 hours. Supernatant quantifications showed an increase in *N.lactamica* with time,which may reflect growth of bacteria during the course of infection.

Further experimentations with advanced techniques such as confocal microscopy and more repeats of the gentamycin assay would yield more promising results. Also, the analysis of supernatant for inflammatory mediators, bactericidal compounds, and bacterial by products is necessary in order to determine the dynamics of adhesion of *N.lactamica*.

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ABBREVIATIONS

CFU	Colony Forming Units
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
INF-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-12	Interleukin-12
LPS	Lipopolysaccharide
LOS	Lipooligosaccharide
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK-activating enzymes
MH	Mueller-Hinton
MOI	Multiplicity of Infection
N.gonorrhoeae	*Neisseria Gonorrhoeae*
N.lactamica	*Neisseria Lactamica*
N.meingitidis	*Neisseria Meningitidis*

NOD	Nucleotide Oligomerisation Domain
OD	Optical Density
OMP	Outer Membrane Proteins
OMV	Outer Membrane Vesicles
PBS	Phosphate Buffered Saline
ROS	Reactive Oxygen Species
Rho/Phal	Rhodamine/Phalloidin
RPMI	Roswell Park Memorial Institute
TLR	Toll-like Receptors
TNFα	Tumour Necrosis Factor-alpha

CHAPTER I

INTRODUCTION

The appliance of microbiology in the prevention, diagnosis, and cure of disease has revolutionised human health. The average life span of human beings has doubled and the survival rates of children are much greater. The battle against global epidemics, such as smallpox that had cost so many lives seem to be more or less conquered and at present day in the developed world more focus is on mental illnesses and degenerative disease. Mortality due to tuberculosis, which is miniscule in the Western world, reaches 3 million a year in the less-developed world (Greenwood, Slack, & Petherer 2002).

In order to free societies from fatal infections continuous and strict countermeasures must be set (Greenwood, Slack, & Petherer 2002). These are:

- continuous epidemiological surveillance
- hygiene and control of infection
- carry out immunization programmes
- accurate diagnosis and treatment

It is possible that, due to the difficulty in following the above measures in less-developed countries, there is a much larger threat towards human life. One example of an infectious bacteria that has killed so many throughout history is *Yersinia pertis* also known as plague.

It was responsible for the killing of nearly a third of Europe's population in the middle ages (www.themiddleages.net 2006). Nowadays, immunization and sanitation has eliminated plague in the Western world but in less-developed countries, such as the it still exists as endemics (WHO 2004).

These epidemiological data are evidences of the significance of bacterial infections and thus, there is still a great need for studies into pathogenic bacteria. In order to understand and develop potential treatments or vaccines against harmful pathogenic bacteria, the epidemiology, structure, genomics, and pathogenesis must be understood.

One very good example of two species of the same family that occupy a common niche within the human host are Neisseria Meningitidis (*N.meingitidis*) and Neisseria Lactamica (*N.lactamica*). These two species are closely related and they both colonise the nasopharynx of humans but with opposite consequences. *N.meningitidis* is well known to cause a range of serious disease whereas *N.lactamica* very rarely causes disease and is part of the natural bacterial flora of the nasopharynx (Collee et al. 1996;Greenwood, Slack, & Petherer 2002).

Now, for the purpose of this project, the epidemiology, structure, genomics, pathogenesis, and key similarities and differences will be discussed regarding these two species of the Neisseria family, *N.meningitidis* and *N.lactamica*.

I.1. *Neisseria Meningitidis*

N.meningitidis belong to the genus Neisseria, which include other members such as the pathogen, *N.gonorrhoeae* and the non-pathogenic commensal *N.lactamica*. They are gram-negative diplococci and humans are the only natural carriers, found predominantly in the nasopharynx (Collee et al. 1996;Murray, Rosenthal, & Pfaller 2005). Their capsular polysaccharide antigens define the serogroups to which each member belongs to. The serogroups are A, B, C, X, Y, Z, 29-E, and W135 of which groups, A, B, and C are associated with epidemics (Dupuy et al. 2006). It causes a wide range of disease depending on the site of infection but almost a third of cases present as fatal septicaemia with the remaining two thirds as meningitis (Collee et al. 1996;Greenwood, Slack, & Petherer 2002)

I.1.1. Epidemiology

N.meningitidis is a pathogen of global significance and remains as one of the most feared infections. Its rapid progression and tendency to cause widespread epidemics are the cause of a high mortality rate of 20-50% (Morley & Pollard 2002;Rosenstein et al. 1999). Although other serious infectious disease are prevented with successful vaccination programs in temperate countries, meningococcal disease remains as the most frequent cause of death in childhood due to infections (Morley & Pollard 2002).

Clinical meningococcal disease was first described in 1805 during an outbreak in the vicinity of Geneva, Switzerland by Vieusseux (Evens & Brachman 1998). The major outbreaks during the 20th century were during the two World Wars possible due to crowded and enclosed conditions during large scale mobilization. But meningococcal meningitis has occurred throughout the world sporadically in small clusters (Fig.1.1) (Dupuy et al. 2006;WHO 2006). Regardless of the climate of the country, meningococcal epidemics can occur anywhere especially dry seasons.

Fig.1.1. Major epidemics of meningococcal meningitis from 1971 to 1997 (Dupuy et al. 2006)

But by far the highest burden of meningococcal disease occurs in sub-Saharan Africa, distinctively in the region called the 'Meningitis belt' (Fig.1.2.). This hyperendemic area is home to attack rates of 100-800 per 100,000 population during major African epidemics in which serogroups, A, C, and W135 are the main players of meningococcal disease. High susceptibility in this area is due to the climate during the dry season where respiratory tract infections are common due to dust winds and cold nights. Also the transmission of *N.meningitidis* is escalated by overcrowded housing and large scale mobilizations during pilgrimages (Dupuy et al. 2006;WHO 2006).

Fig.1.2. The African meningitis belt. Extending from Ethiopia in the East, to Senegal in the West. (Dupuy et al. 2006)

Epidemiological data reveals certain behavior or geographical location that increases susceptibility to infection. It is indeed true that 'overcrowding' sharply increased carrier rate of *N.meningitidis*. Especially during World War I and the massive epidemic in Chille during 1941-1942, population density was very high leading to amplified carriage rates (Evens & Brachman 1998). Temporal factors also play a role in sporadic endemic cases where most cases are seen during dry seasons in Africa and in the USA, incidence peaks in late winter and early spring. This may be due to a change in sleep patterns in more crowded rooms (Evens & Brachman 1998;Rosenstein et al. 1999). Another key factor in meningococcal disease is age. Meningitis is a childhood disease and most often seen in children below the age of 15. Age specific incidence is highest among infants between 3 and 12 months in temperate countries and mortality reached 80-90% in children in some epidemics before effective therapeutics (Evens & Brachman 1998). Incidence of meningococcal infections during 1984 and 1991 in England and Wales was maximum at 6 months of age and declined towards the age of 4. Although certain strains were found to be associated with disease in older children and young adults (Jones & Mallard 1993).

I.1.2. Clinical manifestation

Spread of *N.meningitidis* usually occurs by droplet exposure which results in asymptomatic carriage most of the time. The progression from carriage to invasive disease results in septicaemia and/or meningitis with fever and a characteristic feature is a non-blanching purpuric rash. The severity varies greatly amongst individuals from bacteraemia to severe septicaemia and invasive disease rarely can take the form of arthritis or pneumonia (Morley & Pollard 2002).

Children are at greatest risk from disease although many studies have indicated protection against *N.meningitis* in infants under the age of 6 months. Risk factors include, active/passive smoking (Ahmer et al. 1999), overcrowding, poverty, young age, exposure to respiratory infections, and complement deficiency.

I.1.3. Structure and Classification

N.meningitidis are bean-shaped gram-negative diplococci with either flat or concave opposing edges (Fig.1.3.). They are 0.8μm in diameter and have an outer membrane, a layer of peptidoglycan in the periplasmic space, and an inner membrane that contains cytoplasm and DNA (Fig.1.4.) (Brooks, Butel, & Morse 2004;Collee et al. 1996).

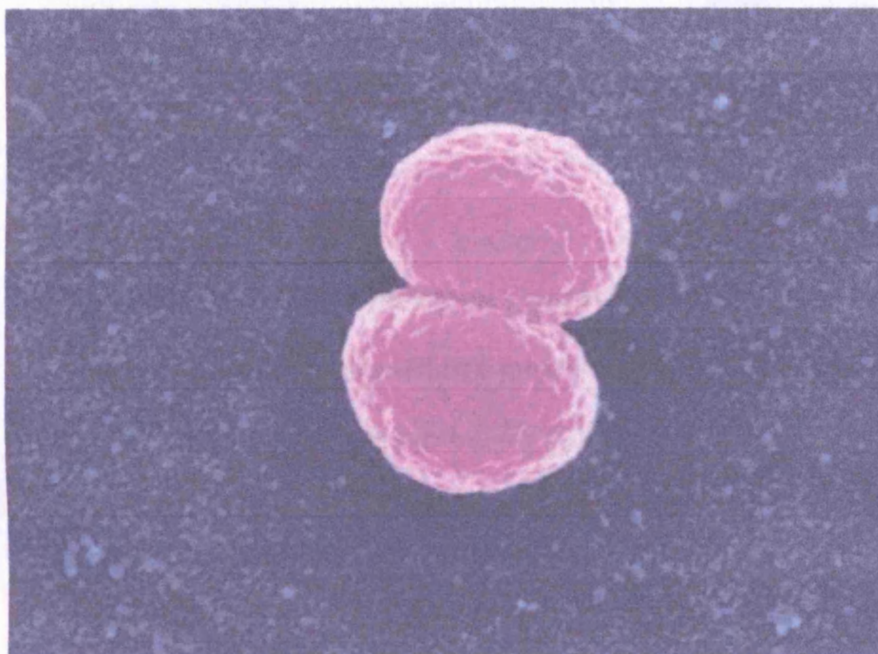

Fig.1.3. Scanning Electron Microscope image of *N.meningitidis*. (taken from www.waterscan.co.yu)

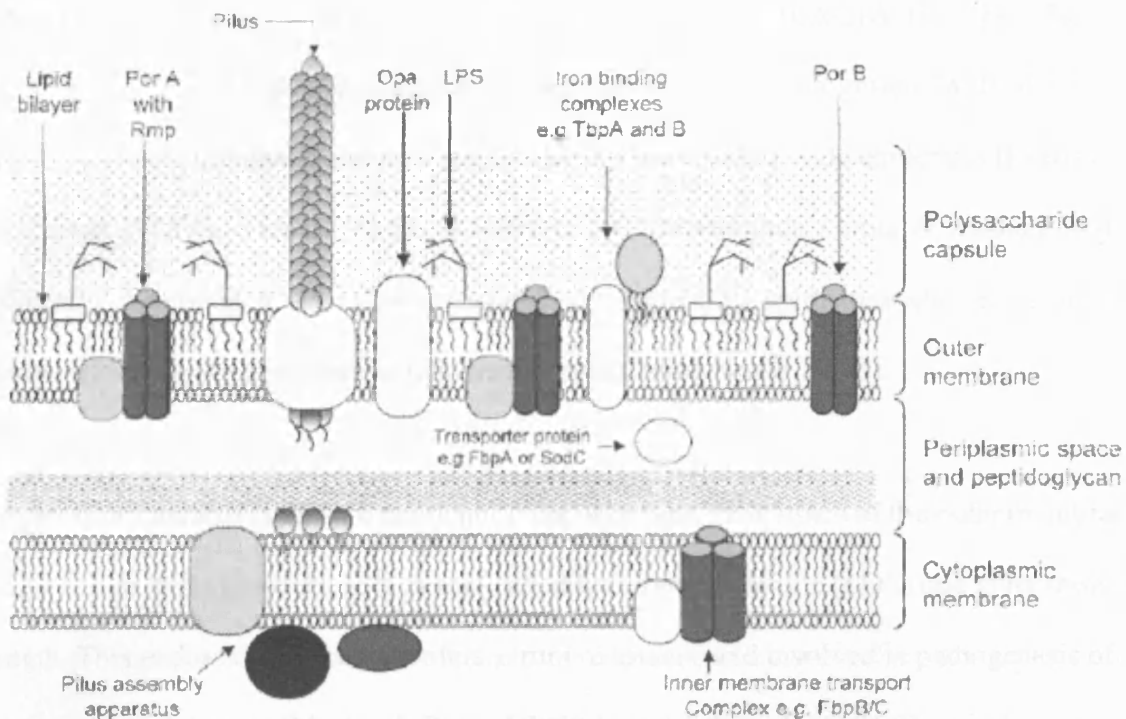

Fig.1.4. ***N.meningitidis*** **membrane structures. Outer and inner membrane structures and peptidoglycan structures shown together with various outer membrane proteins, (PorA, PorB, Opa), Lipopolysaccharide (LPS), and Pilus. (Morley & Pollard 2002)**

As shown by Fig.1.4., the outer membrane is surrounded by a polysaccharide capsule which protects *N.meningitidis* against phagocytosis, complement-mediated lysis and other environmental abuse but do not induce an inflammatory response in the host (Brandtzaeg & van Deuren 2005;Morley & Pollard 2002). Due to these protective properties, it is clear that only pathogenic meningococci contain a polysaccharide capsule (Manchanda, Gupta, & Bhalla 2006). According to the antigenic and chemical properties of specific polysaccharide

capsules, *N.meningitidis* can be classified into 12 distinct serogroups (A, B, C, H, I, K, L, M, X, Y, Z, 29E, and W135), of which *meningitidis* from the 5 serogroups (A, B, C, Y and W135) are most infectious and thus responsible for most world-wide epidemics (Evens & Brachman 1998;Greenwood, Slack, & Petherer 2002;Manchanda, Gupta, & Bhalla 2006). Typically, serogroup A strains are associated with large scale epidemics whereas serogroup B strains occur in hyperendemic or sporadic cases (Tettelin et al. 2000).

Lipopolysaccharides (LPS) are the major components on the surface of the outer membrane (Fig.1.4.). In meningococci, LPS is also called lipooligosaccharide (LOS) due to its shorter length. This endotoxin structure confers serum resistance and involved in pathogenesis of meningococcal disease (Morley & Pollard 2002;Vogel & Frosch 1999). They induce strong proinflammatory immune responses in the host and are thought to hide away from the immune system by undergoing phase variation in order to pass through the mucosal barrier (Brandtzaeg & van Deuren 2005;Plant et al. 2006).

Another group of structures found on the outer membrane are outer membrane proteins (OMP) embedded within the lipid bilayer. As shown by Fig.1.4., PorA and PorB proteins are porins that allow ion transportation across the impermeable lipid bilayer. These proteins are major epitopes recognized by the immune system and are also highly variable (Brandtzaeg & van Deuren 2005). Depending on the antigenic differences of these OMPs, *N.meningitidis* can be further classified into serotypes and subtypes based on the PorB and

PorA proteins respectively (Morley & Pollard 2002). Other protein structures include Opa and Opc opacity proteins which contributes to cell adhesion and invasion.
Thus, *N.meningitidis* are currently classified as [serogroup]:[serotype]:[subtype]:[LOS immunotype] according to the characteristics of the surface antigens. Classification is useful in epidemiologic studies as some serotypes are associated with epidemics (Greenwood, Slack, & Petherer 2002) and it is also important for vaccine development (Manchanda, Gupta, & Bhalla 2006).

Finally, a long filamentous structure consisting of several subunits are found on the outer surface. This structure is called pili and *N.meningitidis* produce a type IV pili which attaches to CD46, expressed on most human cells. Pili are important for pathogenic Neisseria species to exert virulence and are encoded by expression of the *pilE* locus (Aho, Keating, & McGillivray 2000). They are also required for obtaining bacterial DNA required for displaying genetic diversity (Brandtzaeg & van Deuren 2005).

I.1.4. Genomics

Elucidation of the genomics of *N.meningitidis* is important in order to reveal mechanisms of interaction with the host. Still our understanding of meningococcal invasion and infection is incomplete but with new genomic technologies, soon complete genome

sequences for all pathogenic *N.meningitidis* strains will help to uncover disease-causing mechanisms (Feavers 2000;Grifantini et al. 2002).

Currently, the genomes of two strains of *N.meningitidis* have been sequenced. Complete genome sequences of *N.meningitidis* serogroup A strain Z2491 (Parkhill et al. 2000) and serogroup B strain MC58 are now available (Tettelin et al. 2000). Both these sequences are around 2.2MB in length and have a GC content of about 52%.

The prominent feature of these genome sequences was the presence of highly repetitive elements from short (10-200 bp) to large sequences (Nassif 2002). These elements are involved in antigenic variation and especially important for the natural transformability and fluidity of the genome of *N.meningitidis* (Nassif 2002;Parkhill et al. 2000). A large proportion of these sequences were associated with genes encoding outer membrane proteins involved in interaction with host cells (Feavers 2000). These include genes for the type IV pilus, Opa proteins and Opc proteins. Especially, antigenic variation of the pilus proteins is important in evasion of host clearance mechanisms (Tettelin et al. 2000). The mechanism is shown in Fig.1.5. where the variable region of the *pilE* expression locus receives a transfer of DNA from numerous silent *pilS* loci via RecA-mediated recombination (Davidsen & Tonjum 2006). Additionally, previous identified genes encoding the capsular polysaccharide and LPS were also present(Klein et al. 1996).

Phase variation controls gene expression and most genes responsible for host interactions are found to be phase-variable. These genes were found at a much greater number than in other organisms (Tettelin et al. 2000).

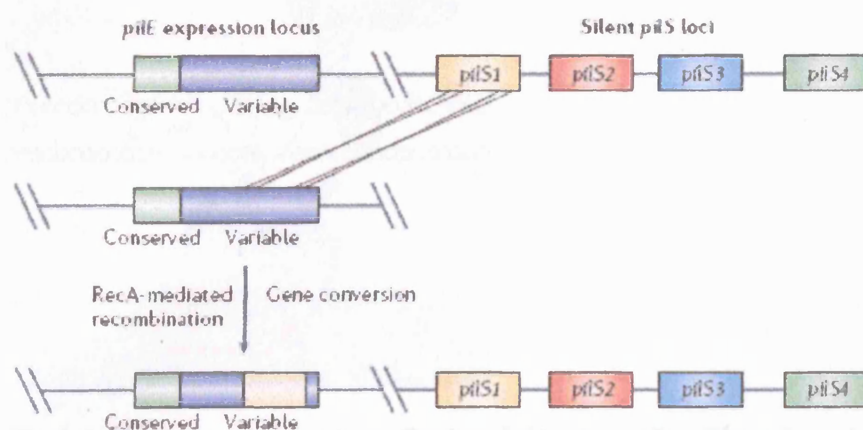

Fig.1.5. Antigenic variation of *pilE* expression locus.

Gene conversion occurs by DNA transfer from pilS loci to the variable pilE locus via RecA. (Davidsen & Tonjum 2006)

Additionally, due to absent DNA-repair genes such as those found in *Esherichia coli*, *N.meningitidis* produces an excess of spontaneous genetic variants. This again is advantageous in its interaction with the host bring about antigenic changes relevant to adherence and invasion (Davidsen & Tonjum 2006).

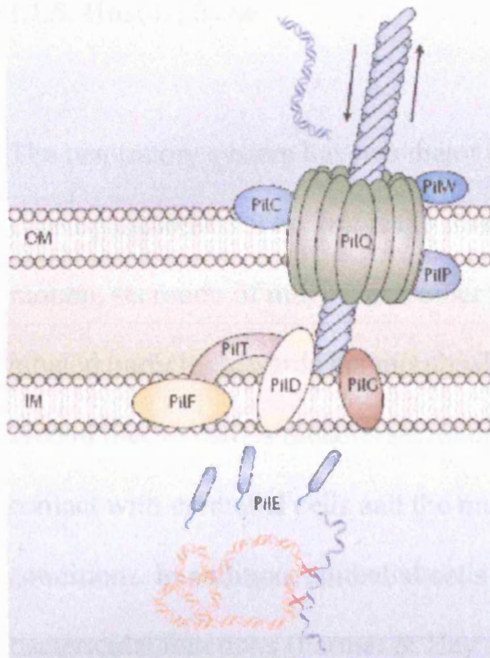

Fig.1.6. Horizontal gene transfer involving type IV pili, and various *Pil* proteins.

The exogenous DNA is intergrated into the meningitidis genome by homologus recombination. (Davidsen & Tonjum 2006)

Also, transformations that contribute to horizontal gene transfer are another way to increase antigenic variance. As shown by Fig.1.6., exogenous DNA is caught by the retracting type IV pili and taken up and intergrated into the *N.meningitidis* genome by various *Pil* proteins. Reassuringly, 3 major regions with unusual nucleotide composition obtained by horizontal transfer were also present in the full genome sequence of strain, MC58 (Tettelin et al. 2000). The wide availability of the genome sequences of *N.meningitidis* strains will definitely accelerate development of vaccines providing protection against pathogenic meningococci.

I.1.5. Host Defense

The respiratory system has two major mechanisms to protect against potential disease-causing pathogens. The first is the mucociliary clearance, where a combination of ciliary motion, secretion of mucins and other macromolecules and fluid facilitates the clearance of inhaled particles towards the mouth where it is swallowed (Farmer & Hay 1995). The second mechanism is mucous secretion. Mucus entraps inhaled particles and prevents direct contact with epithelial cells and the mucous gel becomes thicker in inflammatory conditions. In addition, epithelial cells secrete a large variety of macromolecules with bactericidal functions (Farmer & Hay 1995).

The epithelium plays a key role in host defense against microbes. Recognition systems such as the Toll-like receptors (TLRs), Nucleotide oligomerisation domain (NOD) receptors are present to activate antibacterial effector mechanisms on demand (Bajaj-Elliott 2005). The overall response is that the epithelium increases production of cytokines, chemokines and antimicrobial peptides which mediate effector molecules for killing microbes but also as regulators of cellular inflammation and immunity (Bals & Hiemstra 2004).

I.1.6. Pathogenesis

N.meningitidis is a known pathogen of significance but in actual fact, many *N.meningitidis* do not produce disease and reside in the nasopharynx and the host presents as a carrier. The reason behind the small percentage of invasive meningococcal disease is unknown but predisposing factors such as inhalation of dry dusty air or passive smoking increase the risk of *N.meningitidis* invasion (Pathan, Faust, & Levin 2003).

In order for *N.meningitidis* to produce the systemic infections, it must rise above specific and non-specific mucosal defences, it must be able to cross the upper respiratory mucociliary sheath, and also needs to attach to epithelial cells. All these features must be overcome in order to successfully colonize the nasopharnynx (Tzeng & Stephans 2000). Thus, *N.meningitidis* has evolved genetic mechanisms to survive as a commensal and at times an invader into the bloodstream. Two prominent mechanisms are 'antigenic variation' of components of the outer membrane and 'horizontal DNA exchange' through transformations as explained above (Tzeng & Stephans 2000).
Additionally, structures on its outer membrane are crucial for its survival in the host. Its polysaccharide capsule promotes adherence and inhibits opsonophagocytosis and the production of various factors inhibit ciliary clearance activity (Pathan, Faust, & Levin 2003)

Major structures that give *N.meningitidis* its virulence are the structures found on the outer surface of the bacteria. These are the pili, lipooligosaccharides (LOS), capsular polysaccharide, and outer membrane proteins (OMPs) (Tzeng & Stephans 2000).

Meningitis and its clinical outcome are due to activation of inflammatory cytokines, leukocyte infiltration across the blood brain barrier, breakdown of blood brain barrier due to oedema, induction of cellular apoptosis, coagulation of vessels and ischaemia.

In reality, more damage is done to host tissues by the immune system. As shown in Fig.1.7., the meningococcal endotoxin is the prime factor that triggers the host immune system. Endotoxin is released via 'blebbing' of the outer membrane and large amounts are delivered in pockets of vesicles into the bloodstream (Fig.1.8.). CD14 is the prime receptor that binds endotoxin which in turn activates macrophages to produce proinflammatory cytokines (TNFα and IL-1β). It can also activate the 3 different types of complement pathway (classical, alternative, or mannose-binding lectin pathway), which in turn can activate neutrophils to excessively release reactive oxygen species (ROS) resulting in endothelial cell damage (Hackett, Thomsom, & Hart 2001;Pathan, Faust, & Levin 2003).

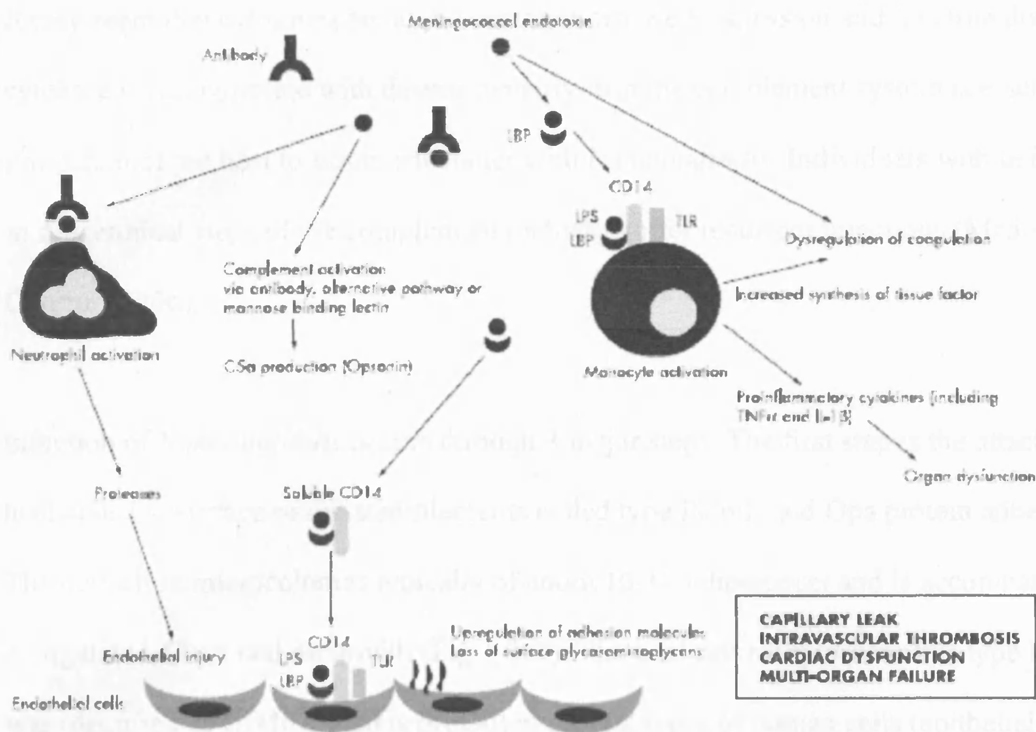

Fig. 1.7. Inflammatory cascade in meningococcal septicaemia.

(Pathan, Faust, & Levin 2003)

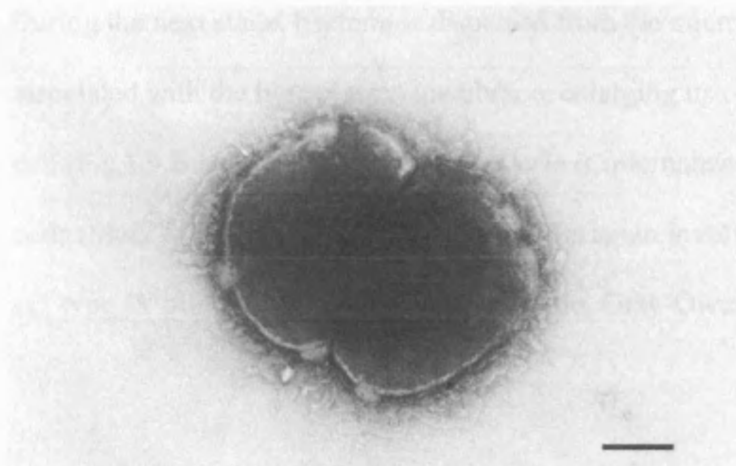

Fig. 1.8. Membrane blebbing in Meningococci. (Hackett, Thomsom, & Hart 2001)

It may seem that cytokines are at the centre of disease progression and it is true that cytokine levels correlate with disease severity. But the complement system is essential for protection of the host to future encounter with *N.meningitidis*. Individuals with deficiencies to the terminal steps of the complement pathway suffer recurrent infections (Mathew & Overturf 2006).

Infection of *N.meningitidis* occurs through 3 major steps. The first step is the attachment to host cells via surface-associated filaments celled type IV pili and Opa protein adhesins. They attach in microcolonies typically of about 10-100 diplococci and is accompanied by elongation of host cell microvilli (Fig.1.9.A). The host cell receptors for this type IV pili was identified as CD46 which is present in several types of human cells (epithelial, endotheial and sperm cells) (Kallstrom et al. 1997;Merz & So 2000). The binding of CD46 was shown to result in decreased interleukin-12 (IL-12) expression leading to decreased immunity (Karp et al. 1996), which *N.meningitidis* takes advantage of during attachment. During the next stage, bacteria is dispersed from the microcolonies and become closely associated with the host plasma membrane, enlarging its contact surface area with the host cell (Fig.1.9.B and C). And finally *Neisseria* is internalized by engulfment by the epithelial cells (Merz & So 2000). The invasion process again involves various surface proteins such as, type IV pili, Opa and Opc proteins (Dehio, Gray-Owen, & Meyer 2000).

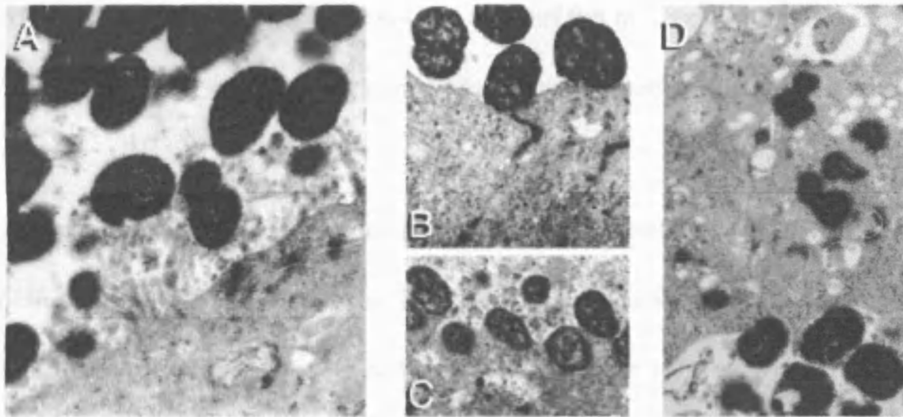

Fig.1.9. Electron micrograph of *Neisserial* attachment and invasion of T84 human epithelial cells. (Merz & So 2000)

A: Adherence of *Neisseria* to the apical plasma membrane in microcolonies. B: *Neisseria* is dispersed and attach as single layer. C: Contact area between *Neisseria* and host cell enlarges. D: *Neisseria* is internalized.

I.2. *Neisseria Lactamica*

Much research was focused on the pathogenic species of Neissera due to its disease-causing properties and thus naturally less attention has been on the non-pathogenic *N.lactamica* species. But more interest is directed towards these commensal bacteria owing to its similarity with *N.meningitidis* in terms of genomic sequence and key membrane structures.

At present time, much is still unknown about the host cell interactions with structures on *N.lactamica* but some progress has been made, especially in vaccine development.

Lactamica belongs to the genus *Neisseria*, which includes *meningitidis* and *gonorrhoeae*. *N.lactamica* was first described by Hollis et al in 1969 (Hollis, Wiggins, & Weaver 1969). Studies before this date were not able to distinguish it from *N.meningitidis* because acid production from lactose was not used as a differential test.

I.2.1. Epidemiology

N.lactamica does not cause disease but is frequently found in the nasopharynx of humans. Carriage is highest in infants and young children but declines with age, which is opposite to *N.meningitidis* where carriage is low in infancy but rises to peak in adolescents and young adults (Bennett et al. 2005;Blakebrough et al. 1982).

This converse carriage rate between *N.lactamica* and *N.meningitidis* and the fact that antimeningococcal bactericidal levels increase in infants with low meningococcal carriage is an indirect evidence that *N.lactamica* may provide protection against *N.meningitidis* by induction of the immune system (Alber et al. 2001;Bennett et al. 2005;Blakebrough et al. 1982).

Evidence comes from a study with children in Turkey where the key findings were, 1) children under the age of 2, meningococcal carriage was 5-fold less and *N.lactamica* carriage was higher than that of school children. 2) *N.lactamica* carriage was significantly higher in areas with low incidence of meningococcal disease (Bakir et al. 2001) and also from a mathematical model predicting prevalence and incidence, *lactamica* was found to inhibit *meningitidis* (Coen, Cartwright, & Stuart 2000).

Additionally in a study with experimental mouse models, immunization with *N.lactamica* killed whole cells, outer membrane vesicles and OMPs and protected against isolates of serogroup B and C meningococci (Oliver et al. 2002).

I.2.2. Morphology and Structure

Morphology and staining is similar to *N.meningitidis*, which is a gram-negative diplococci. Colony morphology is also similar but generally, colour is more yellowish (Fig.1.10) compared to a much grayish colour of *N.meningitidis* (Faur, Weisburd, & Wilson 1975).

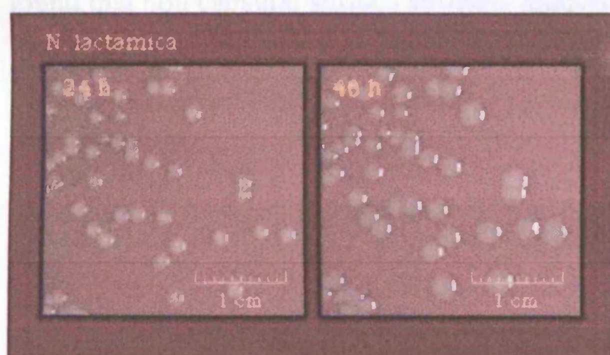

Fig. 1.10. Colony morphology of *N.lactamica*.

At 24 and 48 hours of growth (www.cdc.gov 2006)

Both *N.lactamica* and *N.meningitidis* colonize the nasopharynx of humans but differ in virulence. Where *N.meningitidis* can spread into the blood stream causing meningitis and sepsis, *N.lactamica* is commensal, almost never causing disease. Another key difference between the two species is that *N.lactamica* can utilize lactose to produce acid whereas *N.meningitidis* cannot. The differentiation is of clinical importance and can be marked by simple acid production biochemical tests (Alber et al. 2001;www.cdc.gov 2006)

The structure of *N.lactamica* is essentially the same as *N.meningitidis* but without a capsule. And many surface antigens are the same to *N.meningitidis* but without PorA (Sanchez et al. 2002;Vaughan et al. 2006). Natural immunity to meningococcus is actively induced during life and correlates to the amount of bactericidal activity in humans. This bactericidal activity can be induced by non capsular surface antigens in meningicocci but infants are rarely colonized by *N.*meningitidis. Instead, the commensal *N.lactamica* was found to provide natural immunity in children against meningicocci (Gold et al. 1978). It was also found that non capsular surface antigens induced bactericidal antibodies, which suggests the likelihood that certain surface antigens are shared between *N.lactamica* and *N.meningitidis* (Kim, Mandrell, & Griffiss 1989). Indeed it has been reported that the majority of *N.lactamica* strains shared cross-reactive antigens with *N.meningitidis* (Saez-Nieto et al. 1985). Kim et al used sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting and had identified that it is actually the lipooligosaccharides (LOS) on the surface membrane of *N.lactamica* that contribute

towards developing a natural immunity against *N.meningitidis*. And in this study they found that the pathogenic *N.meningitidis* and *N.gonorrheae* and non-pathogenic *N.lactamica* share LOS epitopes (Kim, Mandrell, & Griffiss 1989).

I.2.3. Vaccine Development

The fight against *N.meningitidis* has been especially difficult in terms of finding a cure to disease. In most bacterial infections, a selection of antibiotics usually kills pathogenic organisms but in the case of *N.meningitidis*, varying ranges of antibiotic resistance is present. The resistance seen is mostly due to highly variable surface proteins and horizontal genetic exchange of parts of the chromosomal genes which are resistant. An example is the sulfonamide-resistant dihydropterate synthase in *N.meningitidis* (Maiden 1998). Horizontal genetic exchange also allows the movement of these antibiotic resistant alleles along different strains of *N.meningitidis*, thus increasing the chance of antibiotic resistance. It may also allow movement into different species of *Neissera*, like *N.lactamica* and a certain degree of antibiotic resistance has been reported (Arreaza et al. 2002).

Thus, naturally, development of a vaccine seems more possible and efficient. A possible route towards developing vaccines are the use of outer membrane vesicles (OMV) of *Neisseria meningitidis*. OMVs can be collected from the surface blebs of the membranes in both *N.meningitidis* and *N.lactamica* and used as a vaccine.

Previous studies have used OMVs as a potential vaccine against the serogroup B meningococcal disease. Efficacy trials have taken place in Cuba, Brazil, and Norway and has shown great efficacy in older children and teenagers but not in infants (Gorringe et al. 2005;Sardinas et al. 2006).

On the other hand, the similarity of surface structures in *N.*lactamica and *N.meningitidis*, epidemiological data demonstrating converse carriage rates between *N.meningitidis* and *N.lactamica*, and results from previous studies confirming the cross reactivity of antibodies against *N.lactamica* and *N.meningitidis*, all point towards a vaccine based on *N.lactamica* surface structures. Amongst the many common surface structures, the OMVs have great potential and have been shown to provide effective protection against many disease causing meningococcal strains (Gorringe et al. 2005;Sardinas et al. 2006).

I.2.4. Genomics

The similarity of *N.lactamica* with *N.meningitidis* is also present in their genomics. It was found that the vast majority of genes present in *N.meningitidis* was also found in *N.lactamica.* The result being, out of a total of 1473 probes to genes on pathogenic *Neisseria*, a total of 1373 probes hybridized to *N.lactamica* (Synder & Saunders 2006). These genes that are shared cannot include virulence genes since *N.lactamica* is non-pathogenic. The genomic regions that are present in *N.meningitis* but absence from *N.lactamica* are likely to be involved in the pathogenic identity, like invasion ability, of the

disease-causing species (Nassif 2002). In detail, to demonstrate the non-existing virulence genes on the *N.lactamica* genome, Synder and Saunders have identified specific virulence genes present in *N.meningitidis* but not *N.lactamica*. Shown in Fig.1.11 and Table.1.1.

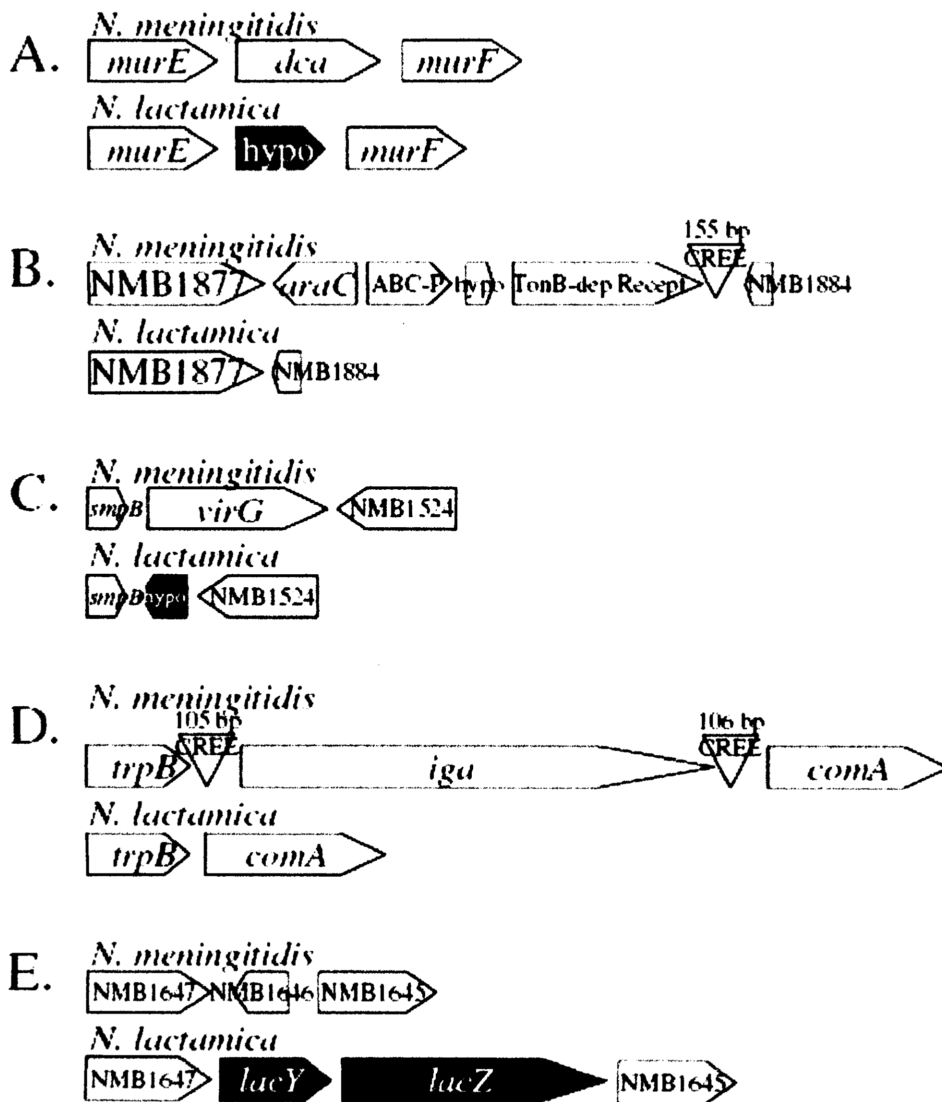

Fig.1.11. The locations of virulence genes present in *N.meningitidis* but not in *N.lactamica*.

Panel	Chromosomal Region in *N.meningitidis*	*N.meningitidis* virulence gene	*N.lactamica* gene
A	Between murE and murF	dca	hypothetical
B	Between NMB1877 and NMB1884	NMB1880 and NMB1882	absent
C	Between smpB and NMB1524	virG	hypothetical
D	Between trpB and coma	iga	absent
E	Between NMB1647 and NMB1645	NMB1646	lacY and lacZ

Table 1.1. Difference in type of genes present at certain locations of the *N.meningitidis* genome.

The complete genome sequencing of *N.lactamica* is currently underway at the Sanger Institute, with currently a theoretical coverage of 99.99% of the genome. This should provide valuable information towards a better understanding of the non-pathogenic *lactamica* (Sanger Institute 2006).

The analysis of the genome of *N.lactamica* that have been produced and currently under way are of one particular strain. But *N.lactamica* exists in many different genotypes and the population diversity is poorly understood (Bennett et al. 2005). 17 genotypes were identified in 26 *N.lactamica* strains (Alber et al. 2001) and a even greater number of 69

genotypes were found in 75 infants (Bennett et al. 2005). This presents further difficulties in choice of representative isolates for use in vaccine development.

I.2.5. Host Cell Interaction with *N.lactamica*

N.lactamica is commensal bacteria which shares the same ecological niche, the nasopharynx, with the pathogenic *N.meningitidis* (SaezNito, Marcos, & Vindel 1998). It is closely related to both pathogenic *Neisseria* species, *N.meningitidis* and *N.gonorrhoeae* but rarely has any pathogenic properties (Bennett et al. 2005). In some exceptional circumstances, it was found to cause meningitis following skull trauma due to cerebral spinal fluid leakage (Denning & Gill 1991), cavitary lung disease following organ transplantation (Zavascki et al. 2006), and septicaemia in an adult with cerebral infarcts (Brown, Ragge, & Speller 1987).

In most cases, however, *N.lactamica* does not cause disease, whereas *N.meningitidis* spreads from the nasopharynx to the bloodstream and meninges causing severe meningitis and sepsis. Upon host cell contact of these two different types of *Neisseria*, changes in certain genes occur. Of the 347 and 285 genes in *N.meningitidis* and *N.lactamica* that were expressed respectively, only 167 genes were common to both species (Grifantini et al. 2002). Thus, different subsets of genes seem to be activated by *N.meningitidis* and *N.lactamica*. This difference may partly explain why one is pathogenic and the other is not.

Also it was found that 40% of the expressed genes encoded surface-associated proteins which suggests both *Neisseria* undergo extensive surface remodeling upon contact with the host cell (Grifantini et al. 2002).

I.3. Inhibitors

I.3.1. MG132 – Inhibitor of Proteasome

Proteasomes are responsible for the majority of protein degradation within the cell (Zimmermann et al. 2001). The specific pathway that is involved in this process is the ubiquitin proteasome pathway. MG132 is a specific inhibitor of proteasome which readily enter cells (Cowan & Morley 2004). Inhibition of this pathway has several results but importantly, it can reduce the production of inflammatory mediators via Nuclear Factor-kappaB (NF-kappaB) inhibition (LaFerla-Bruhl et al. 2006;Lee & Goldberg 1998). The reduction of inflammatory mediators may enable *N.lactamica* or *N.meningitidis* to proliferate or may even reduce disease pathology caused by these mediators and leukocyte adhesion molecules in *N.meingitidis*.

Surfactant proteins on epithelial cells are essential for maintaining normal lung function by controlling innate immune responses in the lung (Scanlon et al. 2005). One pathway that is important for the maintenance of surfactant proteins is the ubiquitin proteasome pathway,

which is responsible for the degradation of major intracellular proteins. Proteasome inhibition by MG132 results in inhibition of surfactant protein gene expression which can ultimately lead to disease in the lungs due to inability to modulate immune responses (Das & Boggaram 2006).

This fact has significant relevance to this project, since the epithelial cells that were used were human alveolar epithelial cells and the MG132 treatment would significantly reduce the ability of these cells to modulate the innate immune system. This 'breakdown' of the immune responses will possibly have implications towards *N.lactamica* adhesion and invasion. The significant reduction of adhesion due to MG132 addition could have important implications in the role of the innate immune response towards *N.lactamica*. Since MG132 inhibits the proteasome function, it could be postulated that epithelial surfactant proteins are highly responsible for the defense against *N.lactamica*.

I.3.2. PD98059 – Inhibitor of MEK

The human cell is subject to many types of stress during infection by pathogenic or sometimes non-pathogenic bacteria. Also, a variety of cytokines are released as part of the innate immune response. These stimuli activate phosphorylation cascades make use of mitogen-activated protein kinases (MAPKs). These MAPKs are also phosphorylated by

MAPK-activating enzymes (MEKs) and these themselves are activated by phosphorylation by MEK kinases (Alessi et al. 1995;Dudley et al. 1995).

PD98059 is a synthetic inhibitor of this MAP kinase pathway. It is a potent non-competitive inhibitor which readily passes through the cell membrane (Bioscource 2006;Pang et al. 1995). In terms of therapeutic potential, it has been highly regarded as a anti-inflammatory agent since it inhibits the synthesis of interleukin-1 (IL-1) and tumour necrosis factor in monocytes induced by bacterial endotoxins (Alessi et al. 1995). Also, PD98059 has been implicated in suppression of T-cell responses which results from the inhibition of interleukin-2 (IL-2) production. Other cytokines associated with T-cell responsiveness were also inhibited such as, tumour necrosis factor-alpha (TNF-α), interleukin-3 (IL-3), and interferon-gamma (INF-γ) (Dumont et al. 1998).

Therefore, it is clear from the evidence that PD98059 can act as an anti-inflammatory agent and when cells which have received this inhibitor treatment, there would be a much lesser degree of immune action by the cells. In this project, PD98059 was used to treat the A549 epithelial cells prior to infection of *N.lactamica*. This should have definite increased effects of *N.lactamica* to colonize these cells.

I.4. Aims

Despite the similarities in ecological niche, surface protein structure and genomics between *N.meningitidis* and, *N.lactamica*, the former causes an extensive range of disease and the latter does not. Therefore it will be of interest to investigate the interactions in terms of adhesion and invasion at the cellular level. The 3 main issues of concern following infection of A549 epithelial cells with *N.lactamica* are:

1) Deduce adhesion patterns of *N.lactamica* by Gentamycin Assay

2) Deduce adhesion patterns of *N.lactamica* by Fluorescent microscopy

3) Deduce adhesion patterns of *N.lactamica* to epithelial cells that have been subject to MG132 and PD98059 inhibitor treatment.

CHAPTER II

MATERIAL AND METHODS

II.1. Epithelial Cell Culture

II.1.1. Epithelial Cell Line

A549 epithelial cells are human alveolar basal epithelial cells. A549 cells fall under the squamous subdivision of epithelial cells and are normally used in vitro as model for a type II pulmonary epithelial cells. In this project, they were used to resemble the epithelial cell lining of the nasopharynx (abcam 2006;Lieber et al. 1976), the niche for both *N.lactamica* and *N.meningitidis*. The cell line was given by Shaoren Wang, Immunology and Infectious Diseases Unit, Institute of Child Health, UCL.

II.1.2. Cell Culture Medium

Dulbecco's modified eagle medium (D-MEM) stored at 4°C.

Containing : 4500mg/l glucose

110mg/l sodium pyruvate

Supplements:

10% Heat inactivated fetal calf serum

1% 100X L-glutamine (200mM)

1% 100X antibiotic solution

(10,000 U/ml penicillin + 10,000 ug/ml streptomycin)

II.1.3. Thawing and Seeding

1. Frozen cells were removed from -80°C freezer and were subjected to rapid thawing in 37°C water bath.
2. Cells were resuspended in 10ml medium and centrifuged at 1000g for 5 mins.
3. Pellet was recovered and supernatant discarded.
4. Pellet resuspend in 5ml medium.
5. Transfered to T-75 (75cm³) flask filled with 5ml medium.
6. They cells were incubated in 37°C humidified incubator with 5% CO2.
7. View under inverted microscope for seeding after 24 hours of incubation.

II.1.4. Feeding

Cells in culture must be 'fed' with appropriate medium containing essential nutrients that the cell needs for growth and survival. Also cells produce waste and cell debris thus medium must be changed every 2/3 days for optimal growth. Feeding or medium change was carried out as follows:

1. Old medium was removed from T-75 flask with pipette.
2. 10 ml sterile PBS (Phosphate Buffered Saline) was added to wash cells.
3. PBS was removed.
4. 10ml fresh medium was added.

II.1.5. Passaging

When cells reach 70-80% confluence, cells can no longer undergo division and grow due to lack of space on the flask. Thus they need to be passaged or replated onto new flasks.

1. Old medium was removed.
2. 10 ml sterile PBS was added to wash cells.
3. PBS was removed.
4. 5ml trypsin-EDTA was added to detach cells from flask wall for 3 mins at 37°C.
5. Wall of the flask was gently tapped to dislodge any remaining cells.
6. Trypsin-EDTA was neutralised with 5ml medium to prevent damage on cells.
7. Re-suspended cells in medium were transferred to conical tube and centrifuged at 1000g for 5 mins.
8. Supernatant removed and re-suspend pellet in 5ml medium.
9. Re-suspended cells were split into four T-75 flasks with 10ml medium for its use within 2 days and split into six T-75 flasks with 10ml medium for use after 3 days.
10. Passaged cells were incubated at 37°C with 5% CO2.

II.1.6. Cell Counting

The number of cells need to be determined in order to effectively replate them onto new flasks or plates.

A cell population of 1x10^6 cells and 2x10^5 cells per well were required to re-plate onto the 6-well and 24-well plate respectively.

The procedure was as follows:

1. 0.5ml of re-suspended cell solution was transferred into 1ml eppendorf.
2. Trypan blue mixture is made. Different dilutions were used depending on cell quantity.

	1:2 dilution	1:5 dilution
Trypan blue	20µl	25µl
PBS	20µl	25µl
Cell Solution	20µl	10µl

3. 16µl in total of the mixture was taken and injected into the Haemocytometer.
4. Viewed Haemocytometer under microscope to count cells.
5. Number of cells in each large square(Fig.2.1) was counted.

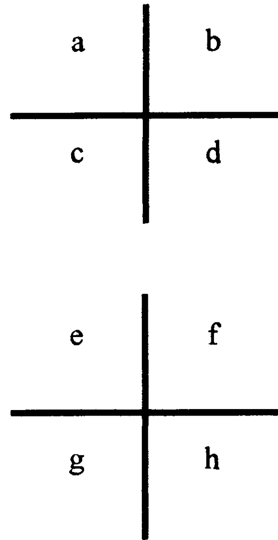

Fig.2.1. Microscopic view of the Haemocytometer.

Number of cells are counted in each quadrant (a, b, c, d, e, f, g, and h).

The total number of cells in the suspension solution can be calculated like below:

(Total haemocytometer cell count / 8) X dilution factor X 10^4

Thus, volume of the cell solution required to plate 1×10^6 cells per well

= 1×10^6 / Total number of cells in suspension

II.2. *N.lactamica* Culture

II.2.1. Growth Environment

N.lactamica selectively grows on Gonococcal (GC) agar with additional nutrients.

N.lactamica was taken from microbeads in horse blood and glycerol that were stored at -80°C and spread onto GC agar plates.

GC agar plates with *N.lactamica* were grown in a humidified incubator at 37°C with 5% CO_2.

GC agar was made up as follows:

1. 37g of powdered GC agar was mixed with 1L of DEPC water in a shaker until completely dissolved.
2. The mixture was autoclaved.
3. Mixture was left to cool in 50°C water bath.
4. 1% Vitox* supplement was added.
5. 20-25ml of agar was poured into Petri-dishes.

*Vitox: a mixture of nutrients and minerals required for *N.lactamica* growth. Details shown in Appendix I.

II.2.2. Subculture

N.lactamica was subcultured every 24 hours.

1. 3-5 random colonies were selected and picked with sterile loop.
2. Spread onto warmed GC agar plates like shown in Fig.2.2.

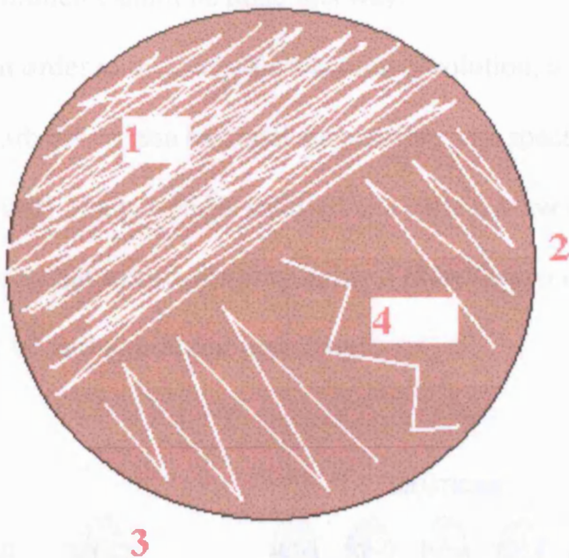

Fig.2.2. *N.lactamica* spreading technique.

1) Spread half the plate back and forth many times to form the 'lawn' area. *N.lactamica* will be harvested from this area later for experimentations.
2) and 3) Spread in a single zig-zag line without going back into the lawn area. This is to dilute down the quantity of bacteria.
4) Spread in a single line to enable picking of single colonies for subculturing.

II.2.3. Quantification

Quantification of bacteria is extremely important for adhesion/invasion assays. The number of *N.lactamica* that are added to the A549 cells must be known in order determine the adhesion or invasion rate of *N.lactamica*. The number of A549 cells can be easily counted with the haemocytometer as shown in II.1.6., but determining the numbers of bugs in solution cannot be done this way.

In order to quantify *N.lactamica* in solution, a spectrophotometer must be used to measure turbidity of the bacterial suspension. The spectrophotometer measures the optical density (OD) of a particular solution at a certain wavelength. The aim will be to obtain near constant colony forming units of *N.lactamica* in solution that gives a OD reading of about 1.00 at 540/600 nm wavelength.

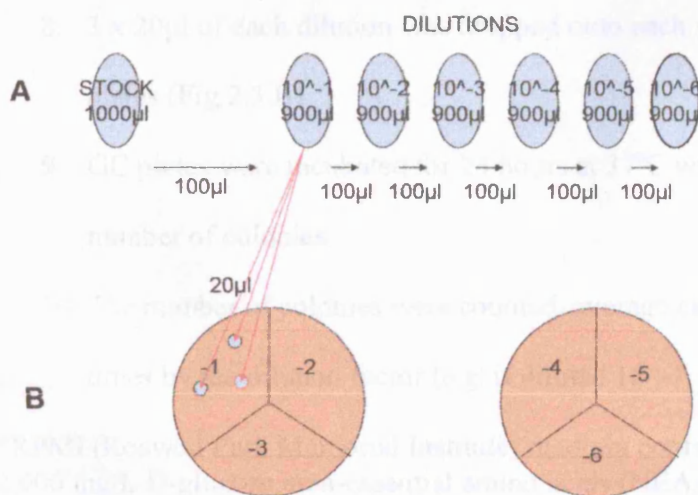

Fig.2.3. Serial dilutions (A) and 20µl drops of dilution solution for counting (B).

The quantification method is as follows:

1. *N.lactmica* was harvested from plates grown for at least 12 hours from the 'lawn' area with a sterile cotton bud.
2. This is suspended in 1.2ml of 3 different types of medium. (RMPI* with 10% FCS, RPMI without 10% FCS, and Mueller Hinton** medium)
3. Spectrophotometer was calibrated to zero with 900μl of medium without *N.lactamica*.
4. OD was measured from 900μl of 10^-1 diluted *N.lactamica* solution at 540nm and 600nm.
5. More medium was added to original stock *N.lactamica* solution for it to reach a OD reading of 1.00
6. Step 5. was repeated 2-3 times in order to obtain a reading near 1.00
7. Serial dilutions of the stock solution was carried out (Fig.2.3.A).
8. 3 x 20μl of each dilution was dropped onto each section of warmed and dry GC agar plates (Fig.2.3.B).
9. GC plates were incubated for 24 hours at 37°C with 5% CO_2 before counting number of colonies.
10. The number of colonies were counted, average calculated, divided by 20μl, and times by the dilution factor (e.g. if diluted 10^-5, dilution factor will be 10^5).

*RPMI (Roswell Park Memorial Institute) medium contains:
2,000 mg/L D-glucose, non-essential amino acids (NEAA), 110 mg/L sodium pyruvate

** Mueller-Hinton (MH) medium contains:
Meat infusion 2.0g/l, casein hydrolysate 17.5g/l, starch 1.5g/l

II.3. Gentamycin Protection Assay

The Gentamycin protection assay is a very useful tool to study adhesion and invasion patterns of bacteria. Gentamycin is an aminoglycoside antibiotic which is used to treat many bacterial infections especially Gram-negative bacteria. It works by binding to bacterial ribosomes rendering them incapable of reading its genetic code correctly [en.wikipedia.org].

Gentamycin was used in this project to evaluate adhesion/invasion properties of *N.lactamica* through the use of the gentamycin protection assay. A schematic diagram is shown in Fig.2.4. to explain how this assay works.

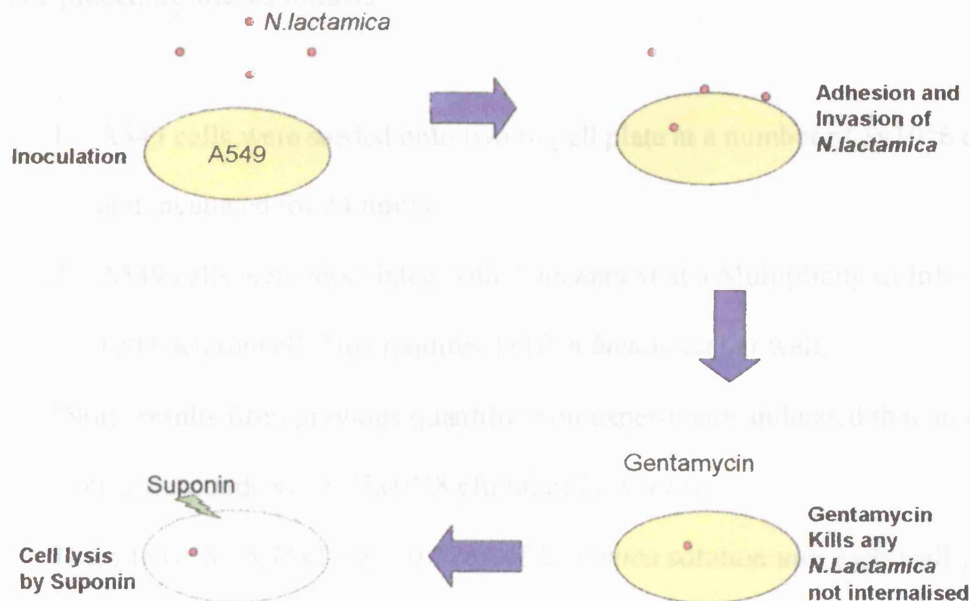

Fig.2.4. The steps in the Gentamycin Protection Assay

Introduction of *N.lactamica* to A549 cells may result in some adhering to the outer membrane of the cell and some invading into the cell cytoplasm. The addition of gentamycin kills all *lactamica* in the surrounding medium and those that have adhered to the outer membrane. Only those that have internalized survive. Following this step, Suponin is added to lyse the epithelial cell, exposing internalized *lactamica*. This solution is spread on a GC agar plate and incubated for 24 hours for colony counting in order to determine the number of colony forming units that have invaded.

It is also important to consider possible variables for the gentamycin protection assay. The variables that were set for optimal adhesion and invasion were, 4 hours of infection incubation and 1ml of 50μg/ml gentamycin used.

The procedure was as follows:

1. A549 cells were seeded onto two 6-well plate at a number of 1x10^6 cells per well and incubated for 24 hours.
2. A549 cells were inoculated with *N.lactamica* at a Multiplicity of Infection (MOI) of 100 bacteria/cell. This requires 1x10^8 *lactamica* per well.

[Note: results from previous quantification experiments indicated that an OD reading of 1.00 corresponds to ~5.75x10^8 cfu/ml of *lactamica*.

Thus, 1x10^8 / 5.75x10^8 = 0.17ml of *lactamica* solution into each well.]

3. Incubate for 4 hours at 37°C with 5% CO2.

4. 2 wells were designated for 'control' cells without inoculation and 3 wells each were designated for 'adhesion' and 'invasion' assays with inoculation.

5. Adhesion:

   a) Remove medium.

   b) Wash x3 with PBS.

   c) Add 1ml of medium without gentamycin.

   d) Incubate for 1 hour at 37°C, 5% CO2.

   e) Remove medium.

   f) Wash x3 with PBS.

   g) Add 1ml of 0.5% suponin and incubate for 30 mins at 37°C for cell lysis.

   h) Scrape off cells using a cell scraper.

   i) Transfer solution into eppendorf.

   j) Vortex vigorously for 30 secs.

   k) Dilute stock down to 10^-1 and spread 100 µl of stock and dilution solution onto 1 GC agar plate.

   l) Incubate GC agar plates at 37°C, 5% CO2 for 24 hours before colony counting.

6. Invasion: Same procedure as '5. Adhesion' was taken except for step c) where 1ml of 50µg/ml of gentamycin was added to kill any *lactamica* adhered or in solution.

7. Control cells: Same procedure as '5. Adhesion' was taken.

II.4. Infection Experiments

II.4.1. Infections 1 to 4.

Prior to the infection experiements, A549 cells were grown on glass coverslips in 24 well plates with fibronectin treatment to allow better sticking of cells to the coverslip. Cells were seeded at 2 x 10^5 cells per well.

Infection experiements no's 1 to 4 were carried out the same way as described below.

1. *N.lactamica* was harvested from GC plates with sterile cotton swab and suspended in RMPI medium with 10% FCS.
2. Turbidity of suspension solution was adjusted to OD of 1.00 and bacterial suspension was subject to quantification.
3. 250µl of *lactamica* solution was mixed with equal volume of FITC and incubated at 37°C for 10 mins.
4. FITC stained *lactamica* was washed and spun down at least 3 times.
5. 34.8µl of this solution was added to each well (OD 1.00 corresponds to ~5.75 x 10^8 CFU/ml and the number of bacteria required was 2 x 10^7 CFU/ml per 2 x 10^5 cells. Thus, 34.8µl of bacterial solution was required in each well to make up an MOI of 100).
6. The experiment was stopped at 2, 4, and 6 hours post-infection. The 24 well plate used is shown in Fig.2.5.

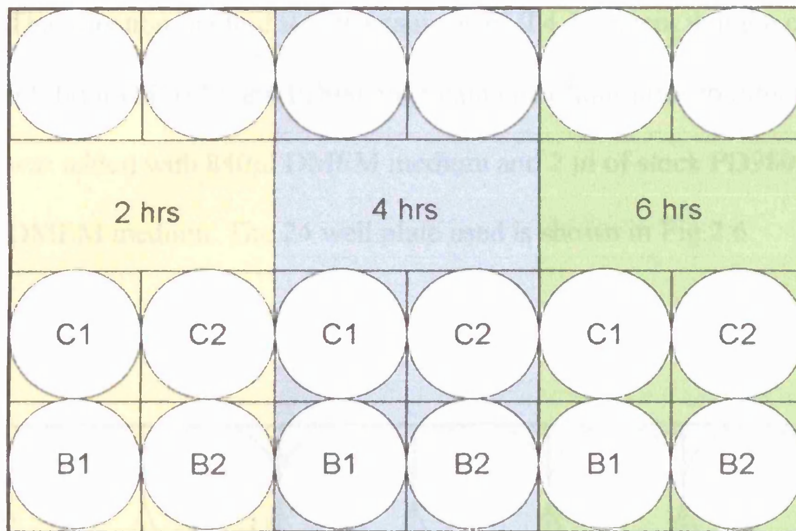

Fig.2.5. 24well plate used for infections 1 to 4.

It indicates content of each well and for how long the infection took place.

C : Control and B : Bacteria.

In addition, the supernatant for Infection 3 and 4 was quantified to determine the number of *lactamica* present.

The quantification method was the same as in II.2.3.

II.4.2. Infection with Inhibitor Treatment

Prior to the infection experiements, A549 cells were grown on glass coverslips in 24 well plates with fibronectin treatment to allow better sticking of cells to the coverslip. Cells were seeded at 2 x 10^5 cells per well.

The infection method was the same as in II.4.1. except that the cells were subject to inhibitor (MG132 and PD98059) treatment 1 hour prior to infections. 1 μl of stock MG132 was added with 840μl DMEM medium and 2 μl of stock PD98059 was added with 541μl DMEM medium. The 24 well plate used is shown in Fig.2.6.

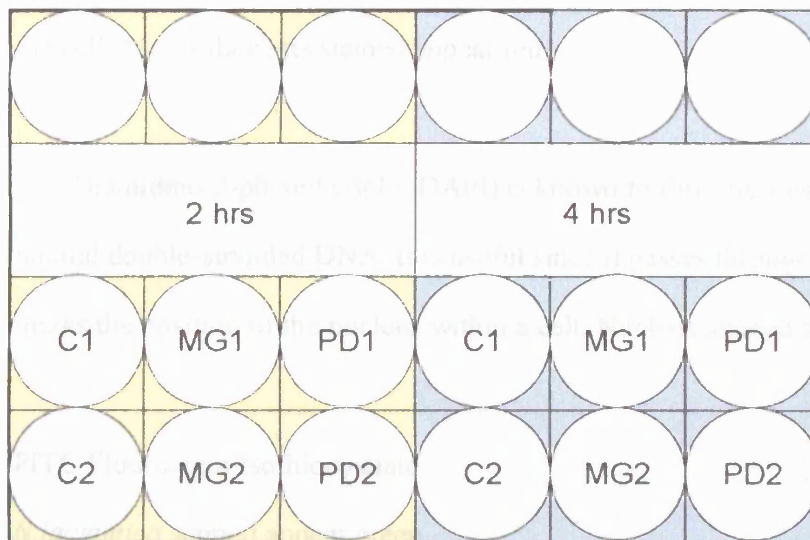

Fig.2.6. 24well plate used for infection with inhibitor treatment.

It indicates content of each well and for how long the infection took place.

C1: Control without Infection (no *lactamica*), C2: Control with Infection (*lactamica* added), MG: MG132, and PD: PD98059

In addition, the supernatant for infection with inhibitor treatment was quantified to determine the number of *lactamica* present.

The quantification method was the same as in II.2.3.

II.5. Staining

3 types of stains were used.

Rhodamine/Phalloidin (Rho/Phal) stain

The phalloidin binds specifically to F-actin and does therefore stain the actin stress fibers in the cell. Microfilaments stained appear red.

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA. It is useful since it passes through the cell membrane and marks the position of the nucleus within a cell. Nucleus stained appear blue.

FITC Fluorescein Isothiocyanate

N.lactamica stained appear green.

After the infection had been stopped at certain times set (2, 4, or 6 hours), coverslips containing A549 cells and FITC stained *lactamica* was washed 3 times in PBS.

The stages below were followed accordingly.

1. Wash coverslips 3 times with PBS.
2. Fix with 150µl of 4% Paraformaldehyde (PFA) and leave for 10 mins at room temperature.
3. Wash 3 times with PBS.
4. Permeabilse with 400µl of 0.5% Triton X100 in PBS and leave for 5 mins at room temperature.
5. Wash 3 times with PBS.
6. Block with 400 µl of 1% BSA and leave at 37°C for 30 mins.
7. 150 µl of 1:100 Rhodamine/Phalloidin in 1% BSA was added and incubated in the dark for 30 mins at room temperature.
8. Wash 3 times with PBS.
9. 150 µl of 1:1000 DAPI in PBS was added and left for 5 mins at room temperature.
10. Coverslips were mounted onto glass slides using Vectorshield.
11. Coverslips were sealed onto glass slides with clear nail varnish.

II.6. Fluorescent Microscopy

All images were viewed and taken at X63 magnification with oil immersion under a standard fluorescent microscope. DAPI, FITC, and Rho/Phal filters were used to view fluorescence stained A549 cells and *N.lactamica.*

Images taken under each filter were merged. Example shown in Fig.2.7.

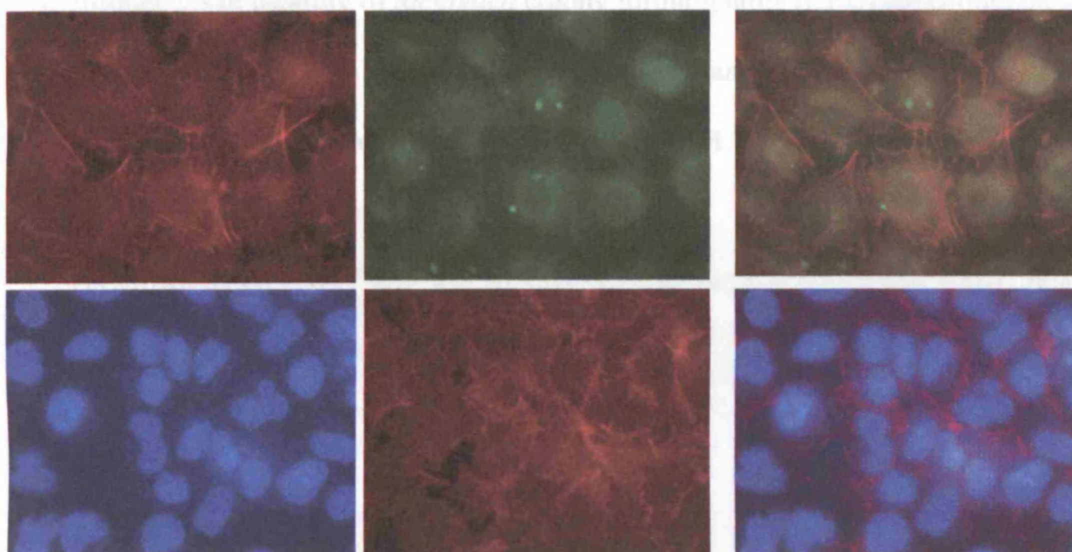

Fig.2.7. Merging of microscopic images.
Top 3 images show the merging of Rho/Phal and FITC images and the bottom 3 images show DAPI and Rho/Phal image merging. DAPI: blue, Rho/Phal: red, and FITC: green.

The number of *N.lactamica* was counted in 4 random fields. For those fields that had too much *lactamica* and thus was impossible to count, number was noted as 100.

CHAPTER III

RESULTS

III.1. Quantifications

The aim of the quantification experiments were first to establish whether there were any differences in quantity of *lactamica* colony forming units (CFU) depending on what type of medium was used. The second objective was to obtain a CFU/ml value for *N.lactamica* that corresponds to a spectrophotometer OD reading of 1.00 which will be used as a reference point for gentamycin and infection experiments.

As shown by Fig.3.1, There was no significant difference in CFU/ml value obtained between RPMI and Mueller Hinton (MH) medium.

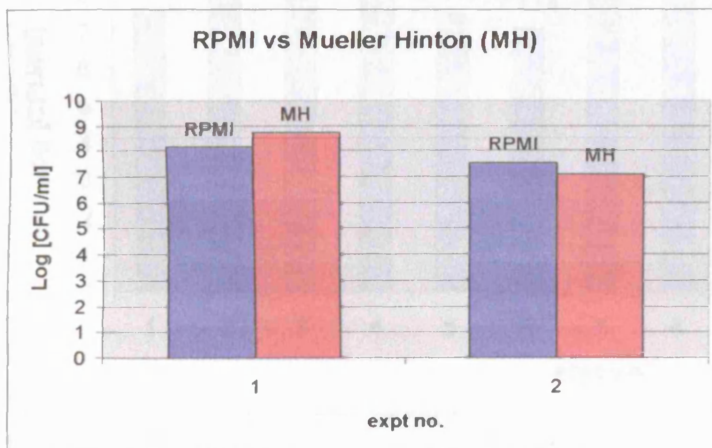

Fig.3.1. RPMI vs Mueller Hinton medium, CFU/ml values

Also, more bacteria clumping was observed when using RPMI without 10% Fetal Calf Serum (FCS).

Therefore, the medium used was RMPI with 10% FCS for suspending *N.lactamica* harvested from incubated GC plates.

The quantification results are shown in Fig.3.2 with each individual values marked. The results are not significantly different and the average value of these results were used for future experiements.

Thus, an OD reading of 1.00 of a *lactamica* suspension solution corresponded to

5.75 x 10^8 CFU/ml

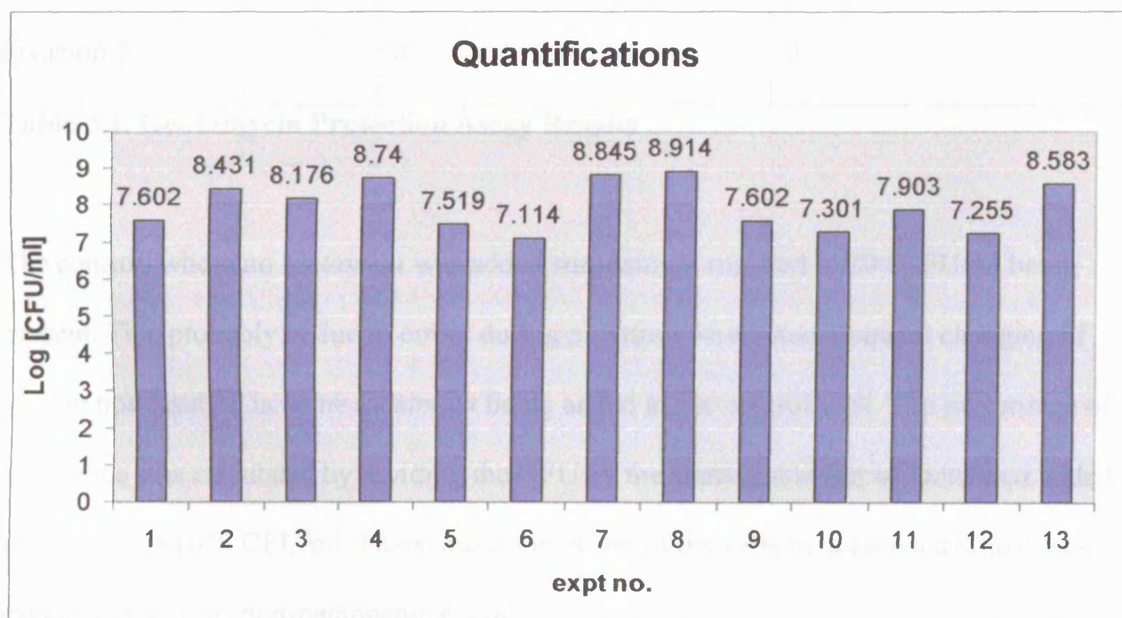

Fig.3.2. Quantifications Results

III.2. Gentamycin Protection Assay

The gentamycin assay is a useful tool to discriminate between adhered and invaded *lactamica* and also enables viable *lactamica* to be quantified, since the supernatant is spread onto GC agar to count the number of colonies after 24 hours of growth. The results are shown in Table.3.2.

	CFU/ml	% Adherence / Invasion
Control	200	-
Adhesion 1	390	3.9 x 10^-4 %
Adhesion 2	20	2.0 x 10^-5 %
Invasion 1	0	0
Invasion 2	0	0

Table.3.1. Gentamycin Protection Assay Results

The control, where no *lactamica* was added surprisingly resulted in 200 CFU/ml being present. This probably is due to errors during pipetting where non-frequent changing of pipette tips resulted in some *lactamica* being added to the control well. The percentage of adherence was calculated by dividing the CFU by the starting number of *lactamica* added, which was 1x10^8 CFU/ml. There was no invasion of the cells by *lactamica* which was expected due to its non-pathogenic nature.

In conclusion, the gentamycin protection assay is a very sensitive and difficult assay to perfect and thus needs much technical experience as well as multiple runs of experiments to exactly determine viable counts.

III.3. Infections

A549 epithelial cell infection with *N.lactamica* was carried out 4 times in total with an additional infection experiment with inhibitor treatment. The 4 infection experiements were carried out for a total of 6 hours and experiements stopped at 2, 4, and 6 hours post-infection. The results of each experiment carry a common theme in terms of *N.lactamica* adhesion patterns but differ in number which may be due to experimental errors rather than actual differing adhesion kinetics.

It is difficult to identify by standard fluorescent microscopy on whether *lactamica* has actually invaded the cells. Thus, based on previous Gentamycin assay, where no internalized or invading *lactamica* was found, all *lactamica* found in fluorescent microscopy are considered adhered.

First, Fig.3.3. shows a light microscope image of the A549 epithelial cell without infection with *N.lactamica*. This is to demonstrate what the cells look like before infection with

N.lactamica. At x40 magnification, the cells can be seen as confluent and densely packed with close proximity between each cell. Although it is difficult to see the boarders of the cells, at x63 magnification, the individual nuclei (in black circle) and chromatin (red circles) can be seen.

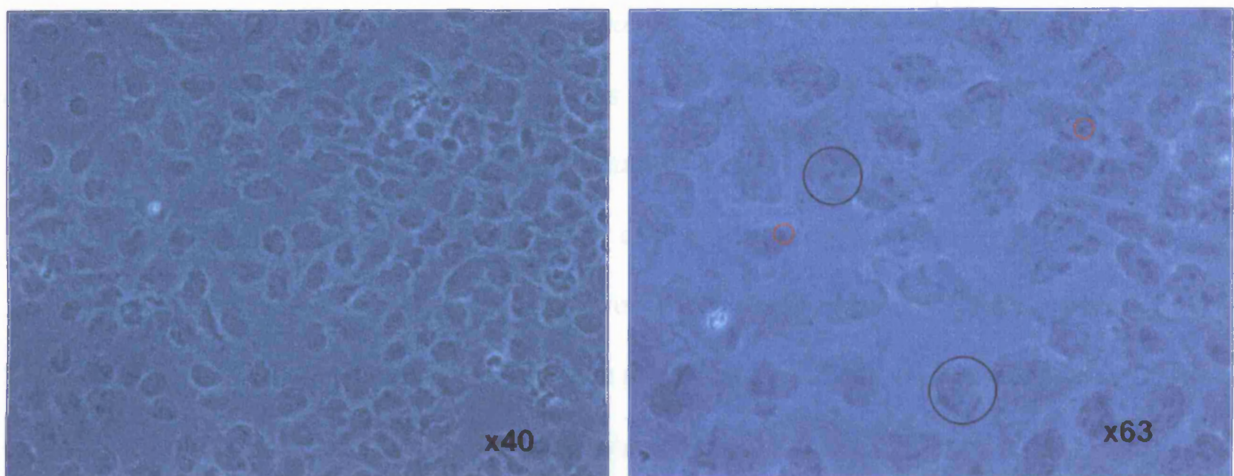

Fig.3.3. A549 epithelial cells shown by light microscopy at x40 and x63 magnification.
Black circles show the nucleus of the cells and red circles show the dark chromatin.

III.3.1. Infection 1

The number of *N.lactamica* that was added to the A549 cells were determined by quantification method described in section II.2.3.

At 10^-6 dilution, average number of colony forming units were 8.67 CFU in 20μl.

Thus, quantity added was 4.33x10^8 CFU/ml.

III.3.1.1. Adhesion Patterns

Control cells were not infected with *N.lactamica* thus did not show any FITC stained bacteria at 2, 4, and 6 hours (Fig.3.4.A and B).

2 hours post infection resulted in two differing patterns of colonization by *N.lactamica*. Fig.3.4.C shows extensive colonization by *N.lactamica* where as in Fig. 3.4.D, there are only a few *lactamica* present, indicated by circles.

Although, it is not possible to verify whether *lactamica* has internalized or adhered, their relative positions within the 3D cellular structure can be distinguished. As shown in Fig.3.4.E, F, and G, 4 hours post-infection, different *lactamica* is seen at different positions. These images were taken from the same field but at different focuses. Focus increases as we move from E to G, moving from the base towards the outer membranes of the cell. This is true since the coverslips are upside-down and focus is moving from the base to the outer structures. Another interesting finding is that at the base of the cell, *lactamica* was found to adhere in-between the cells (Fig.3.4.E) whereas towards the top surface of the cell, *lactamica* is seen to adhere possible to the sides and top of the cells (Fig.3.4.F and G) without any discrimination towards adhering site of cells. Fig.3.4.H. is also an image from 4 hours post-infection where just a few cells are present.

At 6 hours-post infection, extensive colonization by *lactamica* can be seen from Fig.3.4.I. Colonisation is not random but appears to occur in clusters or groups around the cell.

III.3.1.2. Adhesion Numbers

The result from the *N.lactamica* count per 1000 cells reflects microscopic images above. As seen by Fig.3.5. A, the number of *lactamica* per 1000 cells differ significantly between the 2 wells(B1 and B2) at 2 hours and 6 hours post-infection.

III.3.1.3. Conclusion

The differing number of *lactamica* present after 2 hours of infection between the two wells may due to human error when adding the bacteria into the wells. This is again reflected at 6 hours post infection where in the second well, *lactamica* could not be detected. In terms of numbers of *lactamica* that have adhered, Fig.3.5. again represents the abnormal number of *lactamica* at 2 hours post-infection. There could have been a lower concentration of *lactamica* in the B2 wells of the infection to start with or they may have been accidentally washed away during the PBS washing step.

It seems that from Fig.3.4.F, G, and I, *lactamica* does not have a preference to adhere to certain cells or microscopic cellular structure. Further molecular studies need to be conducted to confirm this.

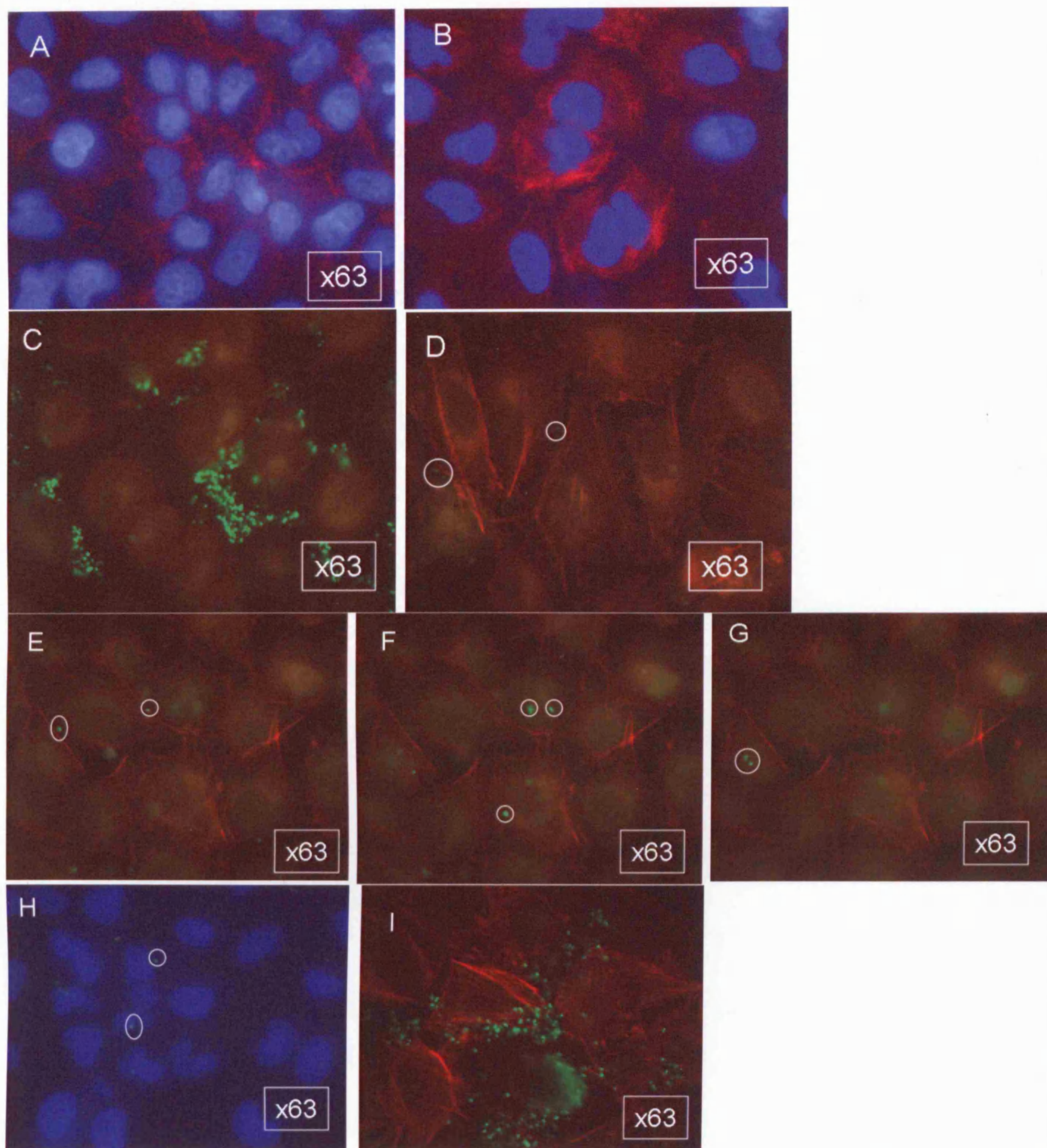

Fig.3.4. Infection 1. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*

A and **B** (control); **C** (**2 hrs**, B1); **D** (**2 hrs**, B2); **E**, **F** and **G** (**4 hrs**, B1); **H** (**4 hrs**, B2); **I** (**6hrs**, B1). DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica*.

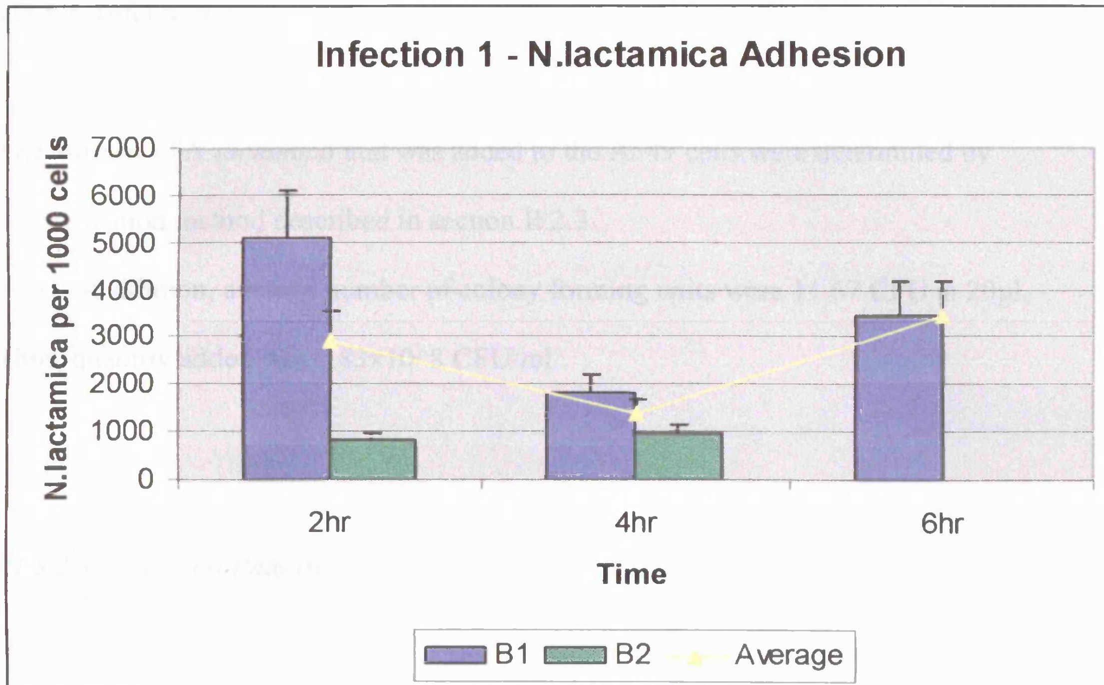

Fig.3.5. Infection 1. Number of *N.lactamica* adhesion per 1000 cells at 2, 4, and 6 hours.

III.3.2. Infection 2

The number of *N.lactamica* that was added to the A549 cells were determined by quantification method described in section II.2.3.

At 10^-6 dilution, average number of colony forming units were 11.67 CFU in 20μl. Thus, quantity added was 5.83x10^8 CFU/ml.

III.3.2.1. Adhesion Patterns

Control cells with DAPI and Rho/Phal staining does not show any *lactamica* under DAPI/Rho/Phal filter (Fig.3.6.A) and FITC filter (Fig.3.6.B).

Fig.3.6.C, D, E, and F, shows individual and clusters of *lactamica* adhered to the cell surface at 2 hours post-infection. As focus increases (i.e. from D to F) moving from the base to the apex of the cell, again *lactamica* was found at different positions within the same visual field (indicated by white circles). Interestingly, *lactamica* that has adhered at the base of the cell are present as single diplococci (Fig.3.6.D) and as the focus moves up the cell, numbers seem to increase represented by the larger area of fluorescence (Fig.3.6.E). Eventually, as focus reaches the apex of the cell, *lactamica* is found in clusters.

At 4 hours post-infection, the number of *lactamica* increases considerably which can be seen visually in Fig.3.6.G, H, and I. Again, in most cases, *lactamica* seem to prefer to adhere or colonize in clusters, shown by circles.

There is an even more colonization at 6 hours post-infection (Fig.3.6. J and K). The numbers are uncountable and the positions are not random but appear in clusters around the position of the nucleus.

III.3.2.2. Adhesion Numbers

The numbers of adhered *lactamica* are similar between the two wells (B1 and B2) at 2, 4, and 6 hours post-infection. There is an overall increase in the average number with the increase in infection time. A marked increase in numbers from 2 hours to 4 hours and a less greater increase from 4 hours to 6 hours can be seen (Fig.3.7.).

III.3.2.3. Conclusion

The clustering of *lactamica* towards the apex of the cell may be due to more space being available. Since at the base of the cell, intercellular connections are present and the spread-out nature of the cells create a tighter area thus making it more difficult for *lactamica* to adhere.

The greater increase in *lactamica* adhesion from 2 hours to 4 hours than 4 hours to 6 hours post-infection suggests that after 4 hours of infection, *lactamica* acquires greater ability, via various endogenous or exogenous compounds, to adhere to epithelial cells. Also, 4 hours of infection may be the optimum time for *lactamica* to interact with and modify the cellular membrane components to facilitate its adherence.

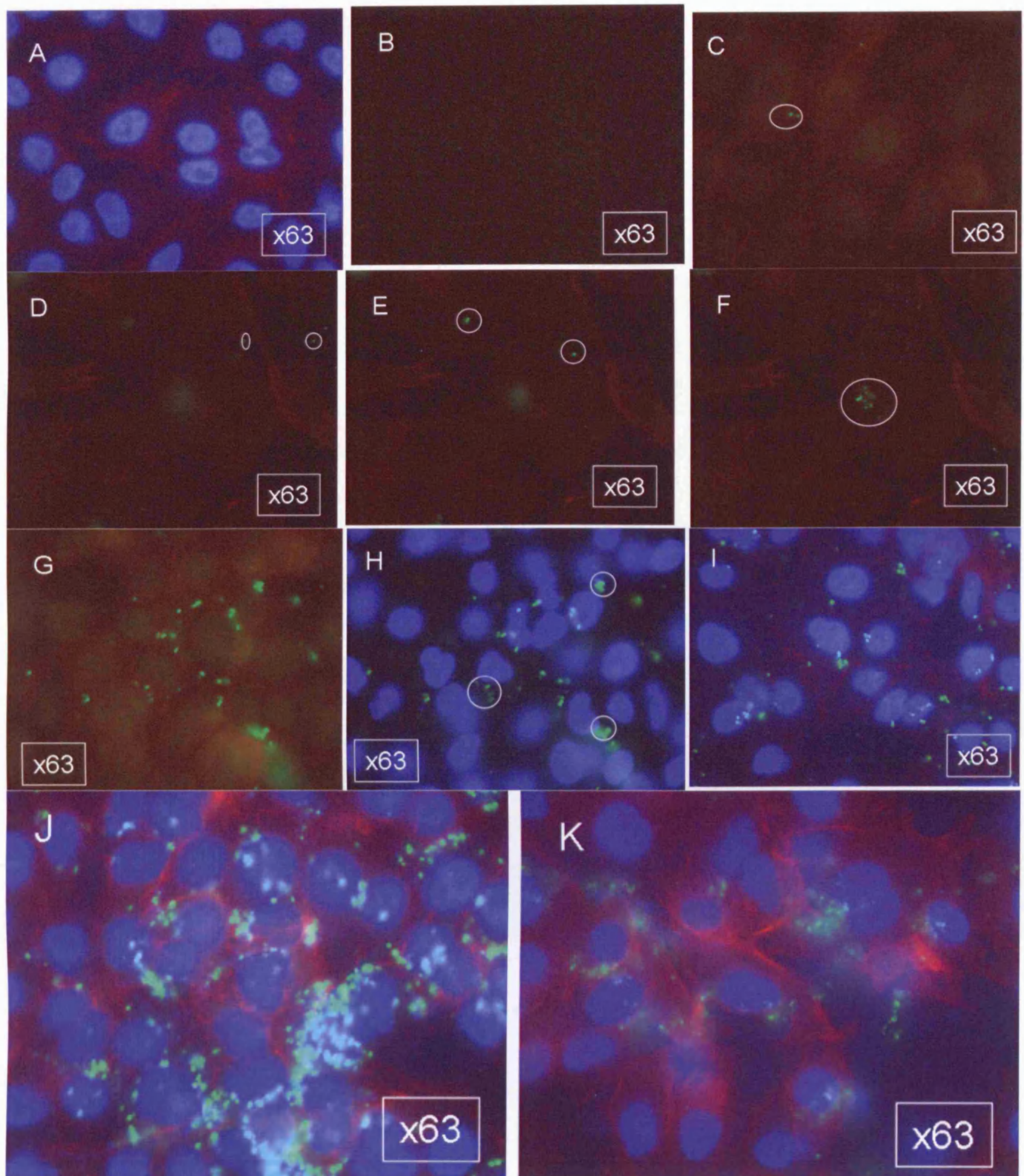

Fig.3.6 Infection 2. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*

A and **B** (control); **C** (**2hrs**, B2); **D**, **E** and **F** (**2hrs**, B1); **G** and **H** (**4hrs**, B1); **I** (**4hrs**, B2); **J** (6hrs, B1); **K** (**6hrs**, B2)

DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica*.

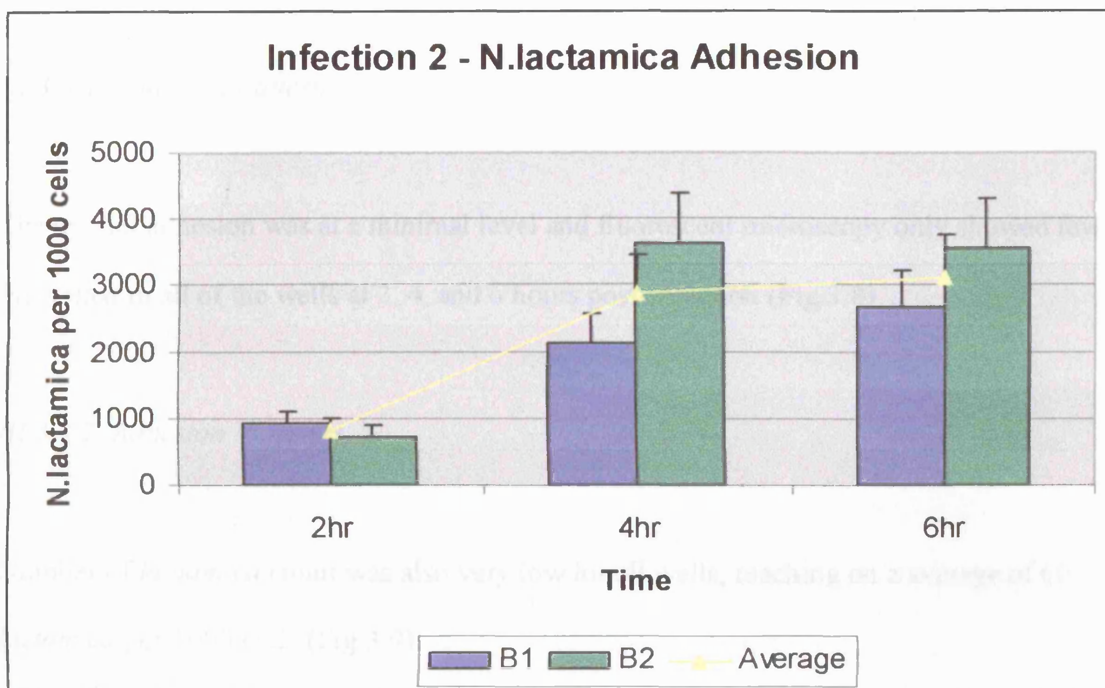

Fig.3.7. Infection 2. Number of *N.lactamica* adhesion per 1000 cells at 2, 4, and 6 hours.

III.3.3. Infection 3

The number of *N.lactamica* that was added to the A549 cells were determined by quantification method described in section II.2.3.

At 10^-6 dilution, average number of colony forming units were 11.67 CFU in 20μl.

Thus, quantity added was 5.84x10^8 CFU/ml.

III.3.3.1. Adhesion Patterns

The overall adhesion was at a minimal level and fluorescent microscopy only showed few *lactamica* in all of the wells at 2, 4, and 6 hours post-infection (Fig.3.8).

III.3.3.2. Adhesion Numbers

Number of *lactamica* count was also very low for all wells, reaching on a average of 60 *lactamica* per 1000 cells (Fig.3.9).

The supernatant of the well solution after removing the coverslip for staining was analysed to quantify the amount of *lactamica* present. All CFU numbers counted were at 10^-5 dilution and thus were multiplied by a dilution factor of 10^5. The results are shown in Table.3.2.

Number of CFU	B1	B2
2 hours	0.72×10^8	0.65×10^8
4 hours	1.2×10^8	1.0×10^8
6 hours	1.9×10^8	1.9×10^8

Table.3.2. Number of *N.lactamica* present in the supernatant of B1 and B2 wells at 2, 4, and 6 hours post-infection.

The number of *lactamica* within the supernatant increases with respect to time. The numbers roughly doubles from 2 to 4 hours and from 4 to 6 hours.

III.3.3.3. Conclusion

The reason for this poor adhesion rate in infection 3 may be because of several reasons. First, *lactamica* may not have been stained properly with FITC or its fluorescence may have faded. This is probably not true since not many *lactamica* can be seen through the DAPI filter either (Fig.3.8.B, C, D, E, F, and G). If there are abundant amounts of *lactamica*, it should be distinguishable through the strong DAPI filter as evident in Fig.3.11.H, Fig.3.14.D, and Fig.3.15.D. The second reason may be because *lactamica* have been washed away during the PBS washing stage or poor mounting technique onto the microscope slides. The third reason is the less-than-desired amount of *lactamica* being put in for infections. The abundance of *lactamica* in the supernatant implies that sufficient amount of *lactamica* was added to the cells. Thus, together with results from the supernatant quantifications, poor adhesion due to washed away *lactamica* is the most reasonable answer.

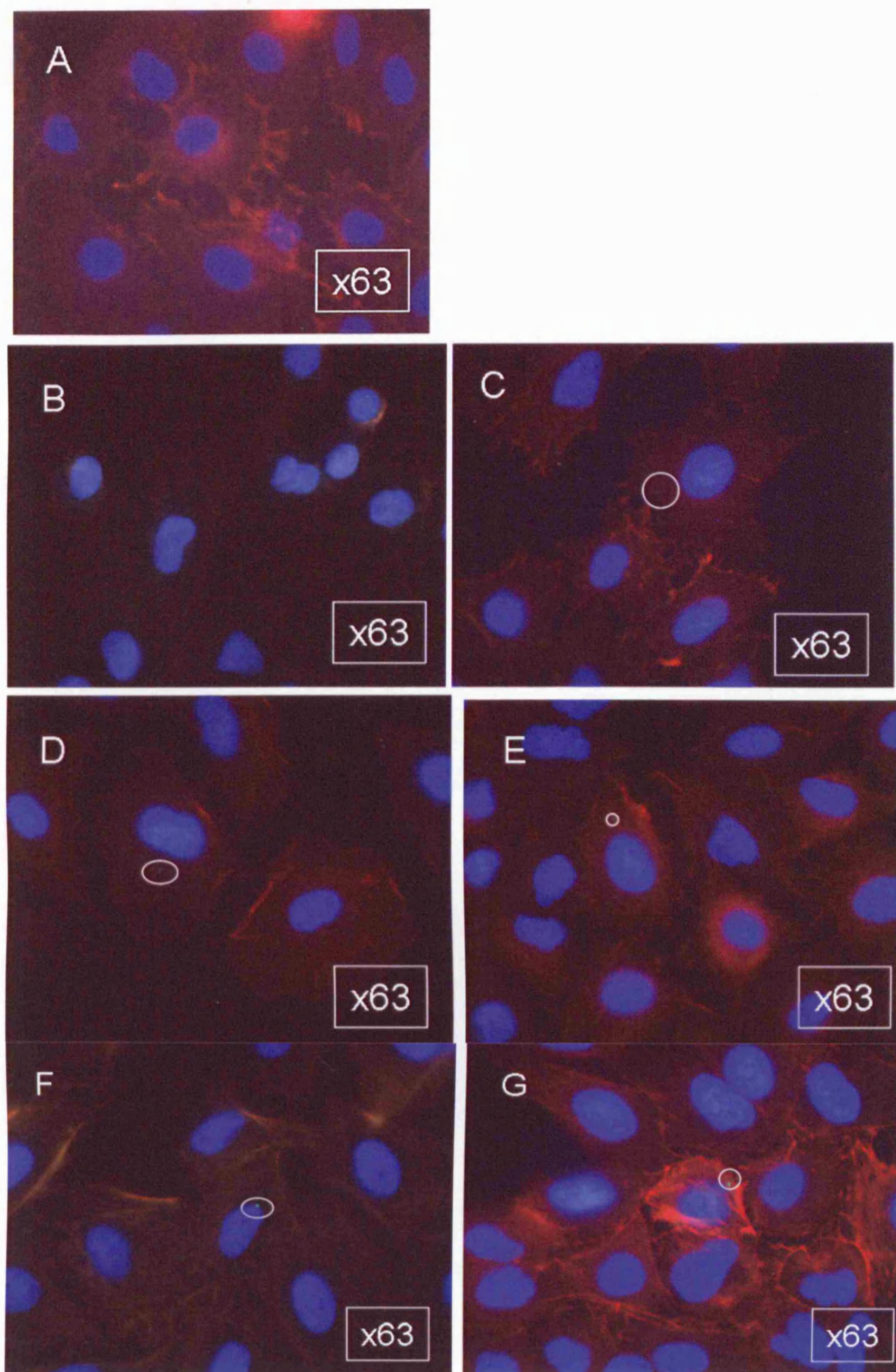

Fig.3.8 Infection 3. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*

A (control); **B** (**2hrs**, B1); **C** (**2hrs**, B2); **D** (**4hrs**, B1); **E** (**4hrs**, B2); **F** (**6hrs**, B1); **G** (**6hrs**, B2)

DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica*.

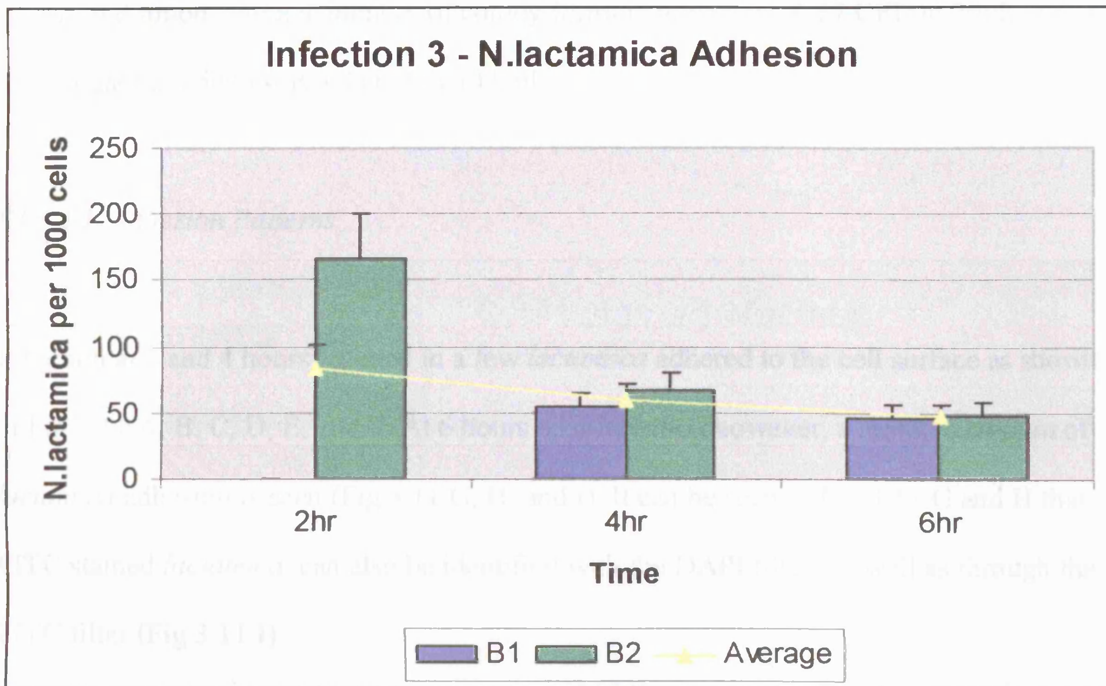

Fig.3.9. Infection 3. Number of *N.lactamica* adhesion per 1000 cells at 2, 4, and 6 hours.

III.3.4. Infection 4

The number of *N.lactamica* that was added to the A549 cells were determined by quantification method described in section II.2.3.

At 10^-6 dilution, average number of colony forming units were 6.67 CFU in 20µl.

Thus, quantity added was 3.3x10^8 CFU/ml.

III.3.4.1. Adhesion Patterns

Infection at 2 and 4 hours resulted in a few *lactamica* adhered to the cell surface as shown in Fig.3.11.A, B, C, D, E, and F. At 6 hours post-infection however, a massive amount of *lactamica* adhesion is seen (Fig.3.11.G, H, and I). It can be seen in Fig.3.11.G and H that FITC stained *lactamica* can also be identified with the DAPI filter, as well as through the FITC filter (Fig.3.11.I).

The enormous number of *lactamica* colonizing the cells are spread out in a certain fashion. The area circled by a white line in Fig.3.11.G and H indicates that *lactamica* does not adhere randomly but adheres around the nucleus. Also, in Fig.3.11.I, *lactamica* is seen to colonize nearly the whole area except the region of the nucleus, circled by a white line. In 3D, this area corresponds to areas at the side of the cell rather than on top of or at the base of cells. Due to the nucleus, the cell is not completely flat but has an apex region and *lactamica* preferentially avoids this area as well as areas at the base of the cell. A schematic figure of this adhesion arrangement is shown in Fig.3.10.

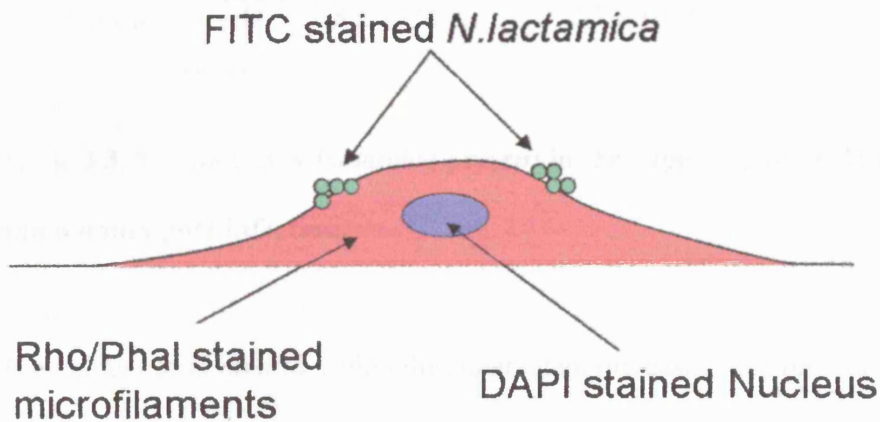

Fig.3.10. Schematic figure of *N.lactamica* adhered to the sides of the cell.

III.3.4.2. Adhesion Numbers

As shown by Fig.3.12., there isn't much adhesion until 4 hours post-infection. But at 6 hours, there is a sudden great increase in adhesion. This suggests that the optimum time for greatest number of adhesion is at 6 hours post-infection.

The supernatant of the well solution after removing the coverslip for staining was analysed to quantify the amount of *lactamica* present. All CFU numbers counted were at 10^-5 dilution and thus were multiplied by a dilution factor of 10^5. The results are shown in Table.3.3.

Number of CFU	B1	B2
2 hours	0.23×10^8	0.33×10^8
4 hours	0.78×10^8	0.6×10^8
6 hours	1.8×10^8	2.7×10^8

Table.3.3. Number of *N.lactamica* present in the supernatant of B1 and B2 wells at 2, 4, and 6 hours post-infection.

The number of *lactamica* within the supematant increases with respect to time. The numbers roughly triples from 2 to 4 hours and quadruples from 4 to 6 hours.

III.3.4.3. Conclusion

First, although it is difficult to see the pattems of adhesion for single or small-scale colonization, there seems to be a distinct pattem of adhesion for large colonizations. They seem to be mostly around the sides of the cells and not on top of or at the base of cells. Also, from Infection 4 experiment *lactamica* count data, optimum adhesion happens at 6hours post-infection. Supematant analysis also provides evidence that *lactamica* is growing and dividing within the medium during the infection process, thus giving a result of tripled and quadrupled numbers from 2 to 4 and 4 to 6 hours post-infection respectively.

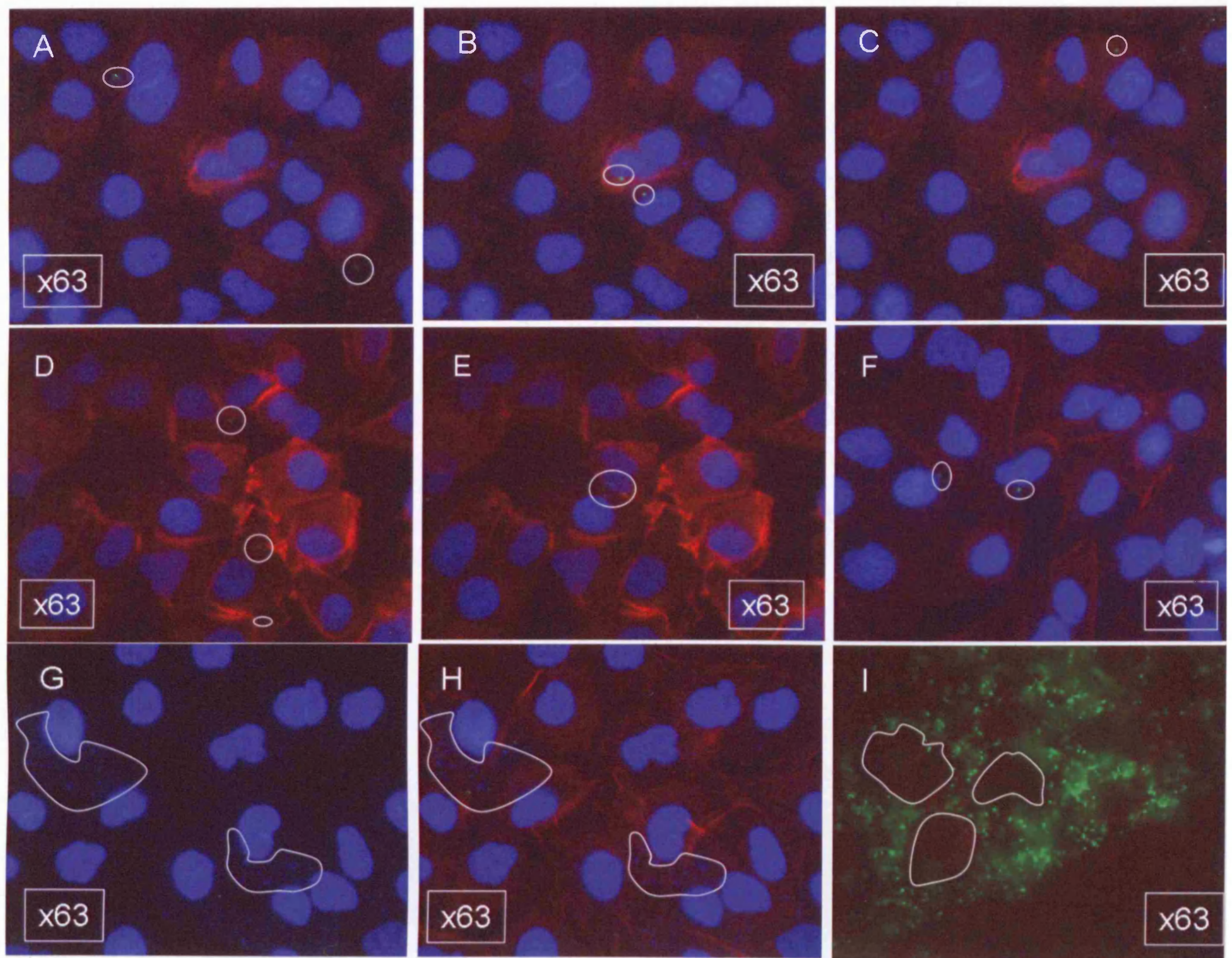

Fig.3.11. Infection 4. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*

A, **B**, and **C** (**2hrs**, B1); **D** and **E** (**4hrs**, B1); **F** (**4hrs**, B2); **G** and **H** (**6hrs**, B1); **I** (**6hrs**, B2)

DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica* (except in **I** where it represents areas occupied by the nucleus).

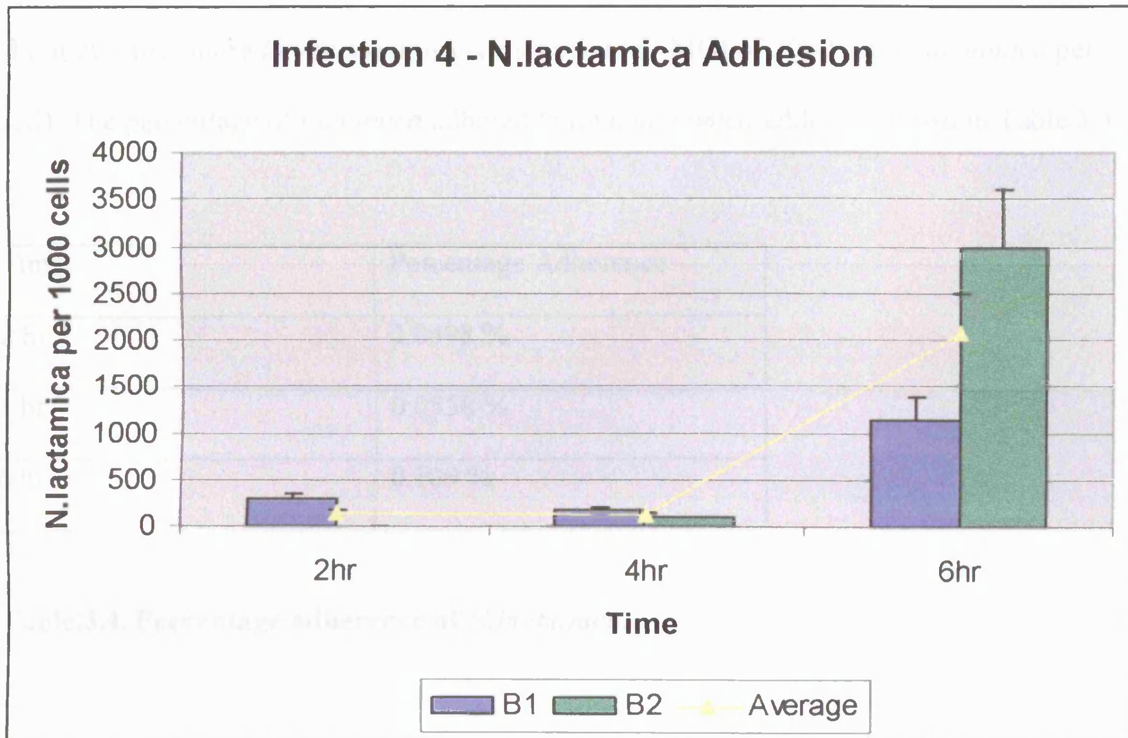

Fig.3.12. Infection 4. Number of *N.lactamica* adhesion per 1000 cells at 2, 4, and 6 hours.

III.3.5. Total

The total number of *lactamica* adhered at 2, 4, and 6 hours was averaged and plot on a graph to demonstrate the general trend in change in adhesion numbers (Fig.3.13). Consistent with results from Infection 4, there was a great increase at 6 hours. Indicating again that optimal conditions for adhesion are present at 6 hours post-infection.

The number of *lactamica* added to 2x10^5 cells averaged to 4.82x10^8 CFU. This is a higher number than the planned 2x10^7 CFU based on the original MOI of 100. This is about 20 times more *lactamica* thus giving us a 'real' MOI of 2000 (2000 *lactamica* per cell). The percentage of *lactamica* adhered to total *lactamica* added is shown in Table.3.4.

Time	Percentage Adherence
2 hr	0.0498 %
4 hr	0.0558 %
6 hr	0.109 %

Table.3.4. Percentage adherence of *N.lactamica*.

The percentage of adherence rates seen here differ significantly from the percentages from the gentamycin protection assay (Table.3.2). The difference may be due to loss of bacteria during the cell scrapping and transfer to eppendorf stage or during the extensive PBS washes

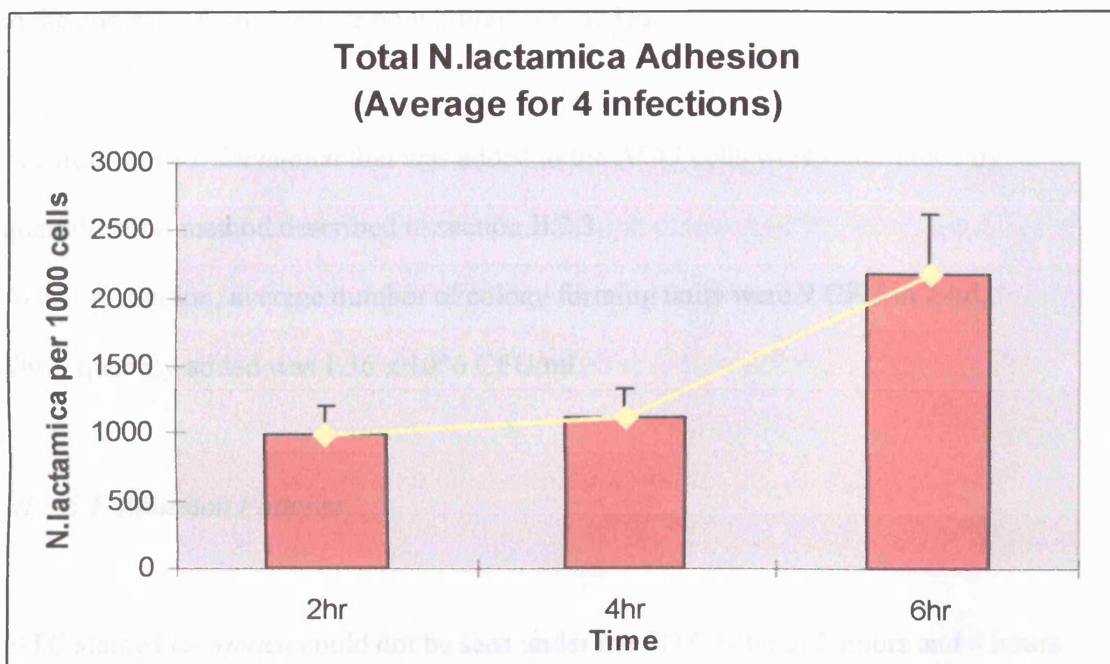

Fig.3.13. Average for Infections 1, 2, 3, and 4. Number of *N.lactamica* adhesion per 1000 cells at 2, 4, and 6 hours.

III.3.6. Infections with Inhibitor Treatment

Both MG132, a proteasome inhibitor, and PD98059, a MEK inhibitor, dampens down the innate immune response in different ways. Thus, a marked difference is expected compared to the control infection where no inhibitor was added.

The number of *N.lactamica* that was added to the A549 cells were determined by quantification method described in section II.2.3.

At 10^-6 dilution, average number of colony forming units were 9 CFU in 20µl.

Thus, quantity added was 1.36 x 10^6 CFU/ml.

III.3.6.1. Adhesion Patterns

FITC stained *lactamica* could not be seen under the FITC filter at 2 hours and 4 hours except for PD2 at 4 hours post-infection (Fig.3.15.E). Instead, *lactamica* could be viewed under the DAPI filter on both occasions and there are clear *lactamica* like structures seen in Fig.3.14 and Fig.3.15 indicated by white circles. Thus, *lactamica* counting was somewhat difficult and two different sets of counting was performed, one under the FITC filter and the other under the DAPI filter. These yielded two different results.

The general outcome is that, visually, there are greater adhesion numbers at 4 hours post-infection compared to 2 hours. This may be due to the anti-inflammatory properties of the inhibitors that were added.

III.3.6.2. Adhesion Numbers

The results from the FITC filter count (Fig.3.16) are somewhat obscure and a clear pattern cannot be observed, except that there was greater adherence of *lactamica* in inhibitor treated cells compared to the control (infection without inhibitor treatment).

In comparison, the results from the DAPI filter count (Fig.3.17) represents a clear increase in adhesion from 2 hours to 4 hours post-infection.

At 2 hours post-infection, MG132 and PD98059 treated cells did not present any difference towards adhesion rates of *lactamica* and adhesion numbers for control, MG132, and PD98059 were similar. But when it reaches 4 hours, adhesion of PD98059 treated cells were more prone to adhesion by lactamica. A marked increase in adhesion was also seen for Infections without inhibitor treatment but the sharp increase was at 6 hours post-infection. Thus the PD98059 MEK inhibitor may function to shorten the time need for optimal adhesion by *lactamica*.

The supernatant was also quantified, the results are shown in Table.3.5. All CFU numbers counted were at 10^-5 dilution and thus were multiplied by a dilution factor of 10^5.

Number of CFU	MG1	MG2	PD1	PD2
2 hours	0.67 x 10^5	0.67 x 10^5	1.33 x 10^5	0.33 x 10^5
4 hours	2.33 x 10^5	2.33 x 10^5	8.67 x 10^5	9.67 x 10^5

Table 3.5. The number of *N.lactamica* present in the supernatant of MG1,2 and PD1,2 for 2 and 4 hours post-infection.

The increase in quantity of *lactamica* in the supernatant reflects lactamica growth and doubling over the course of the experiment.

The smaller number of *lactamica* compared to previous infection experiements is firstly because a lower amount of *lactamica* was added to the cells. The quantity of *lactamica* added in Infection 3 and 4 was 5.84x10^8 CFU/ml and 3.3x10^8 CFU/ml respectively. In this infection experiment, 1.36 x 10^6 CFU/ml was added.

The greater rate of increase in number reflects the effect of the inhibitors on the growth of *lactamica*. Number of *lactamica* quadruples for MG132 treated cells and numbers increase by more than 8 fold for PD98059 treated cells. This indirectly reflects the greater degree of adhesion of *lactamica* to PD98059 treated cells (Fig.3.17). Thus, these inhibitors inhibit the immune response elements that the cell normally releases into the extracellular space, resulting in a higher growth rate of the *lactamica*. Also, the results from the quantification also supports the result from the DAPI filter count in that many more *lactamica* are adhered to the cell, resulting in a less amount of *lactamica* in the supernatant.

III.3.6.3. Conclusion

The absence of FITC stained *lactamica* from the visual field may indicate that the bacteria was not stained with FITC properly. This may be due to a shorter incubation period with FITC or the FITC itself being out of date. Another reason could be that *lactamica* stained with FITC lost its fluorescence over a long period of time thus being undetectable under fluorescent microscope.

The count under the DAPI filter should provide sufficient results of the degree of colonization. The relative positions and morphology of the structures seen under DAPI, highly resembled FITC stained *lactamica* and the numbers adhered also represented an increase in adherence with respect to time, which corresponds to previous experiments.

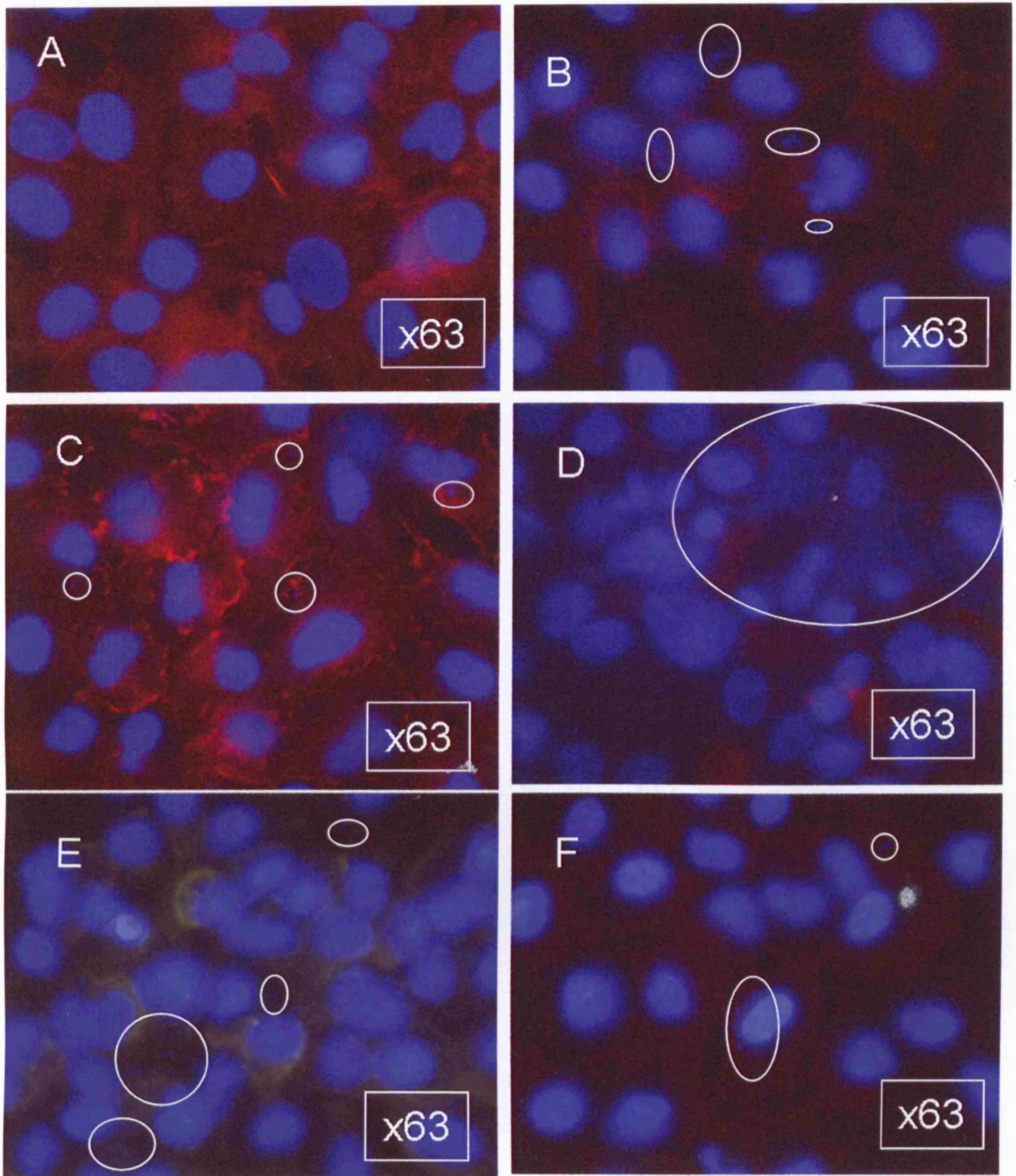

Fig.3.14. Infection with MG132 (MG) and PD98059 (PD) inhibitors. 2 hours post infection. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*

A (control 1, no *lactamica*, no inhibitors); **B** (control 2, with lactamica, no inhibitors); **C** (MG1); **D** (MG2); **E** (PD1); **F** (PD2)

DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica*.

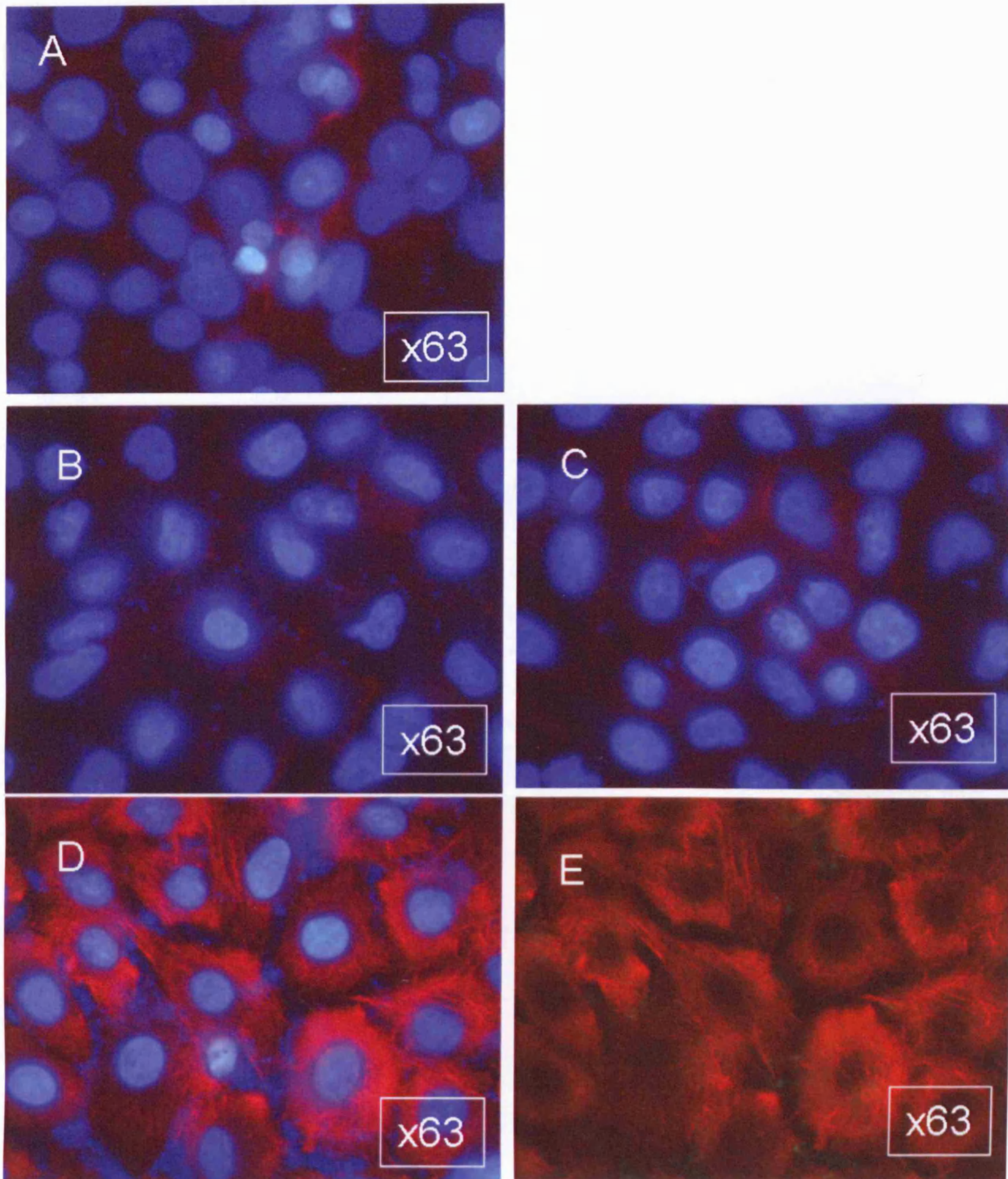

Fig.3.15. Infection with MG132 (MG) and PD98059 (PD) inhibitors. 4 hours post infection. Fluorescent Microscopy of DAPI, Rho/Phal, FITC stained A549 cells and *N.lactamica*
A (control 2, with *lactamica*, no inhibitors); **B** (MG1); **C** (MG2); **D** and **E** (PD2)
DAPI stains cell nucleus blue, Rho/Phal stains cell microfilaments red, and FITC stains *N.lactamica* green. White circles indicate FITC stained *N.lactamica*.

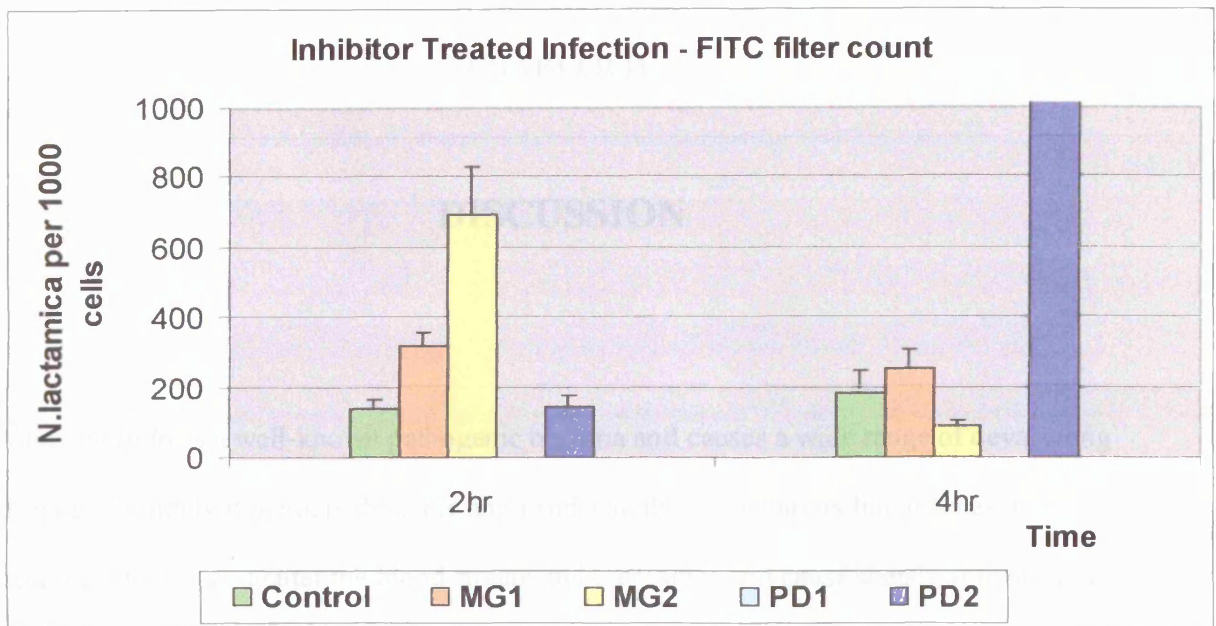

Fig.3.16. Infection with Inhibitor Treatment. Number of *N.lactamica* adhesion per 1000 cells at 2 and 4 hours. MG: MG132 and PD: PD98059

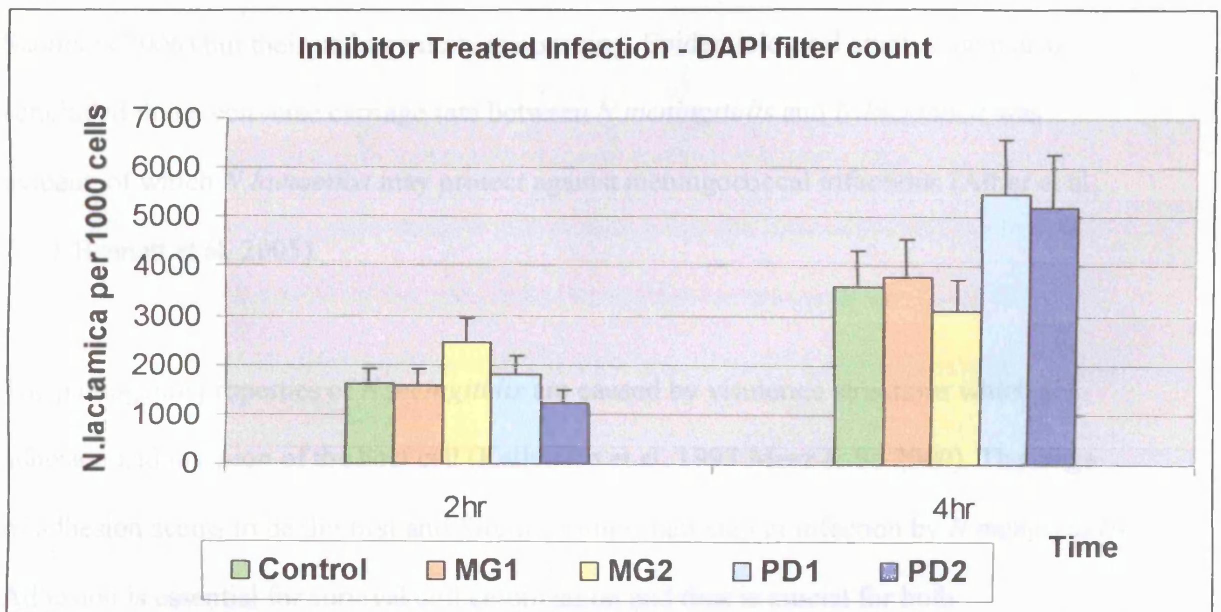

Fig.3.17. Infection with Inhibitor Treatment. Number of *N.lactamica* adhesion per 1000 cells at 2 and 4 hours. MG: MG132 and PD: PD98059

CHAPTER IV

DISCUSSION

N.meningitidis is a well-known pathogenic bacteria and causes a wide range of devastating disease. Normally it is not pathogenic and resides in the nasopharynx but at times it can become invasive and enter the blood stream and meningies and cause spetsis or meningitis (Morley & Pollard 2002).

In comparison, not much is yet known about the commensal *N.lactamica*. It is non-pathogenic and hardly ever causes disease. Both these species have similar outer membrane structures (Sanchez et al. 2002;Vaughan et al. 2006) and share majority of genes (Synder & Saunders 2006) but their pathogenicity is opposing. Epidemiological studies have also concluded that a converse carriage rate between *N.meningitidis* and *N.lactamica* was evident, of which *N.lactamica* may protect against meningococcal infections (Alber et al. 2001;Bennett et al. 2005).

The pathogenic properties of *N.meingitidis* are caused by virulence structures which aid adhesion and invasion of the host cell (Kallstrom et al. 1997;Merz & So 2000). The stage of adhesion seems to be the first and foremost important step in infection by *N.meningitidis*. Adhesion is essential for survival and colonization and thus is crucial for both *N.meningitidis* and *N.lactamica*. The aim was to investigate the adhesion numbers of

N.lactamica with respect to time and whether there was significant numbers that had adhered at a certain time point. The gentamycin protection assay and fluorescent microscopy was used to validate the results.

Massive adhesion of *N.lactamica* in some cases (especially at 6 hours post infection) were seen which could be implicated for the survival of these commensals. Overall there was a pattern of adherence that resembled a sudden increase in adhesion at 6 hours post-infection. This could be due to certain morphological changes in surface bound protein or configuration of the host cell membrane for optimum adherence at 6 hours. Further experiments for long periods of time (i.e. 0 to 12 or 0 to 24 hours) will help determine at which time is the peak for adhesion rates for *lactamica*.

Fluorescent microscopy analysis of adhesion patterns of *N.lactamica* revealed that it mostly adhered to the sides of the cells and not the base or towards the apex. This may simply reflect the structure of the outer surface of the cells in that the base of the cells are more tightly bound together leaving few grooves or spaces for *lactamica* to adhere to. Or, it may suggest more complex receptors or proteins available at certain locations across the cellular surface. It is known that *N.meningitidis* infections have a distinct pattern of adhesion. After a few hours of infection, microcolonies of 10-100 *N.meningitidis* adhere first the apical cellular membrane (Merz & So 2000), which differs from *N.lactamica* adhesion patterns. This difference is no conclusive however, due to the fact that the model epithelial cells used

in this project were alveolar epithelium, which may have different consequences for adhesion. Also, the fact that this in vitro model does not have a mucous barrier, it does not fully simulate the in vivo adhesion pattern of *N.lactamica*.

In comparison to adhesion rates of *N.meningitidis* from Shaoren Wang (Appendix II), where the same MOI and infection environment was used, there was relatively less *lactamica* adhesion than *meningitidis* adhesion. This may reflect the more effective mechanisms or the presence of certain adhesion aiding structures (typeIV pili) in *meningitidis* for efficient adhesion to host cells (Merz & So 2000).
It must be noted that there are clear limitations of counting by 4 visual fields. Many *lactamica* could be missed and important numbers could not be included in the results. Thus in future experiments, counts from 6 or more visual fields is desirable.

Analysis of the supernatant of the wells after removing coverslips with cells and adhered lactamica, revealed an increase in *lactamica* number in the surrounding supernatant. This was surprising since with an increase in adherence to the cell, theoretically there should be less numbers of *lactamica* in the supernatant. There appears to be active growth of *lactamica* within the DMEM medium required for cell survival. And in fact, the high number of *lactamica* adherence at 6 hours post-infection, may reflect the additional number of increased *lactamica* due to growth.
But why isn't there a similar pattern of adhesion at 4 hours-post infection? Despite the doubling or tripling of number of *lactamica* in the supernatant from 2 hours to 4 hours post-

infection, there isn't a large increase in adhesion numbers. This may be due to differing growth and adhesion properties of *N.lactamica*. Growth or doubling of *lactamica* may be relatively linear on a timescale of 0 to 6 hours but adhesion rates may peak between 4 and 6 hours. The exact adhesion dynamics and mechanics of *N.lactamica* are not well known but it seems that at 6 hours post-infection, optimum configuration of the A549 cells by *lactamica* enables great adhesion rates.

All in all, there is a need for further experiementation in order to make more clear of the results encountered in this project. Some examples of further study that need to be carried out are as follows.

- Invasion / Adhesion assay on whole tissue cultures, possibly with a mucosal barrier
- Confocal microscopy to distinguish between adhesion and invasion.
- Mastering the gentamycin assay. Since, many trials leads to improvement in technique and results.
- Analysis of the supernatant to see whether certain inflammatory mediators or bacterial products are present.

The findings from adhesion and invasion studies may provide valuable knowledge on the dynamics of *N.lactamica* colonization compared to those of *N.meningitidis*. But this is only part of the large picture in elucidating why *N.lactamica* does not cause disease and its role in protection against *N.meningitidis*. Advanced molecular biology will hopefully help

discover previously unidentified receptors or molecules that *N.lactamica* uniquely posess and the completion of the genomic sequence will also provide valuable information on certain proteins involved in colonization.

A better understanding of a possible relationship between immunosuppression, nasopharyngeal colonization, and invasive disease may determine the real importance of *N.Lactamica* as an adult human pathogen. Also, an improved understanding of the population biology of *N.lactamica* will provide valuable insights for investigating the role that *N.lactamica* plays in inducing immunity against the meningococcus and in choosing isolates and subcellular components that might be suitable for meningococcal vaccines.

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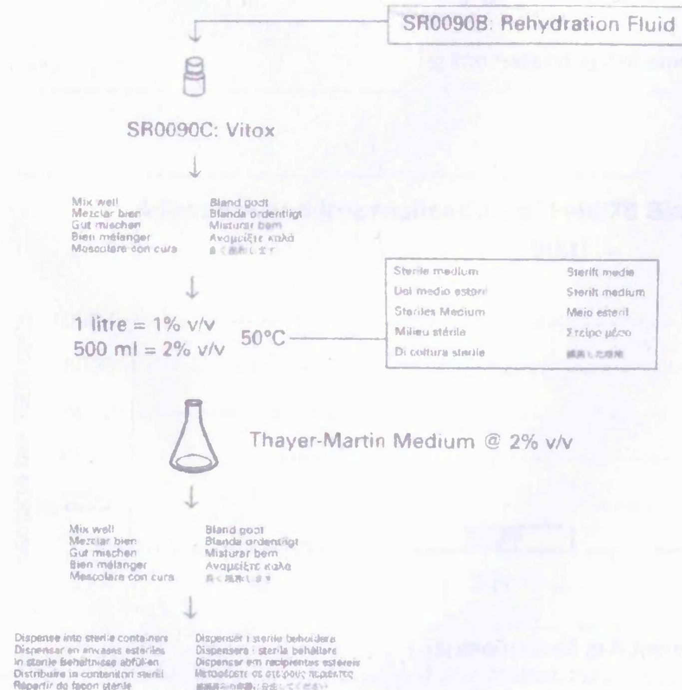
Appendix I

Vitox Supplement information

Vitox Supplement
SR0090A

5 x SR0090B: Rehydration Fluid			
Distilled water	10.0 ml	Glucose	1.0 g
5 x SR0090C: Vitox			
Vitamin B_{12}	0.1 mg	NAD (Coenzyme I)	2.5 mg
Adenine SO_4	10.0 mg	Cocarboxylase	1.0 mg
L-glutamine	100.0 mg	Ferric nitrate	0.2 mg
Guanine HCl	0.3 mg	Thiamine HCl	0.03 mg
p-Aminobenzoic acid	0.13 mg	Cysteine HCl	259.0 mg
L-cystine	11.0 mg		

Precautions: Use sterile techniques at all times. Do not use beyond stated expiry date.
Precauciones: Manipular asépticamente durante todo el procedimiento. No utilizar una vez caducado.
Vorsichtsmassnahmen: Nur unter aseptischen Bedingungen arbeiten. Nicht nach Ablauf des Verfalisdatum verwenden.
Précaution: Travailler à chaque fois de manière stérile. Ne pas utiliser après la date d'expiration.
Precauzioni: Operare sempre in condizioni di sterilità. Non utilizzare dopo la data di scadenza.
Forholdsregel: Anvend steril teknik til hver en tid. Må ikke anvendes ud over den angivne udløbsdato.
Försiktighet: Använd alltid sterila tekniker. Använd inte produkterna efter deras utgångsdatumt.
Precauçoes: Utilizar sempre a técnica estéril. Não utilizar depois de ter caducado o prazo de validade indicado.
Προφυλάξεις: [illegible]
注意：常に無菌処理を行ってください。記載されている使用期限を過ぎたものは、絶対に使用しないでください。



Appendix II

Adhesion number counts from infections reactions of two strains of *N.meningitidis*.

From Shaoren Wang,

Infectious Disease and Microbiology Unit, Institute of Child Health, UCL.