The role of bacterial infection in the aetiology of the overactive bladder

Thesis submitted by

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For the degree of

DOCTOR OF PHILOSOPHY

In the Faculty of Medicine

University College London

2012

Abstract

The aim of this programme of work was to examine the role of bacterial infection in the aetiology of the Overactive Bladder (OAB). Recent studies in OAB have identified urinary inflammatory exudates occurring despite negative routine urine cultures. Detection of pyuria by microscopy of fresh unspun urine is established as the best surrogate marker of infection and pyuria has been described in over 33% of people with OAB.

The routine methods of urinalysis; dipstick of mid-stream urine (MSU) specimens and MSU culture were scrutinised. New techniques of urine culture were sought by culturing the urinary sediment. Intracellular colonisation of urothelial cells was tested in patients and verified further by using a bladder epithelial cell line. In addition, a cytokine response in the urine was examined as surrogate evidence of an urothelial inflammatory reaction in patients with OAB.

The dipstick test was found to have a low sensitivity and specificity in the context of OAB. In addition, culture of the urine sediment using non-selective culture media enhanced the isolation of bacteria from patients with OAB. The bacterial species isolated were predominantly *Streptococcus spp and Enterococcus spp*.IL-6 was found in higher concentrations in the urine specimens of patients with OAB symptoms and pyuria. Intracellular invasion assays and microscopic methods of identifying intracellular bacteria in this patient group identified adherent and intracellular bacteria.

Declaration

I, Rajvinder Kaur Khasriya, hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated. Professor Malone-Lee, Shozab Khan, Jenny McGlynn (Department of Medicine, Archway Campus, UCL, Highgate Hill, London N19 5LW) helped to collect urine specimens from patients and control volunteers.

Acknowledgements

I would like to thank my supervisors Professor James Malone-Lee and Professor Mike Wilson for their continued support, encouragement and guidance throughout my PhD.

I wish to thank Salim Ismail from the Eastman Dental Institute and Dr. Vidya Mohammad-Ali from the Rayne Institute, UCL for teaching me basic microbiological techniques and ELISA techniques carried out in this thesis and for their helpful discussion.

My thanks also go to all of my friends and colleagues at the Department of Medicine, Archway Campus and the Eastman Dental Institute.

I am extremely grateful to all of the patients and volunteers who provided urine specimens and bladder biopsies for use in this study.

Dedication

For Sharanpal, Maya and Sureya

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List of Abbreviations

ATP	Adenosine Triphosphate
BDGF	Brain derived growth factor
BDNF	Brain derived neurotrophic factor
BLAST	Basic Local Alignment Search Tool
BHI	Brain Heart Infusion
ºC	Degrees Centigrade
CBA	Columbia Blood Agar
CI	Confidence Interval
CM	Centimetres
CO_2	Carbon Dioxide
Cfu	Colony forming units
CNS	Central Nervous System
CSU	Catheter specimen of urine
df	Degrees of freedom
dL	Decilitre
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EEC	European Economic Community
EGF	Epidermal Growth Factor
ESP	Enterococcal Surface Protein
ELISA	Enzyme Linked Immunosorbent Assay
FAA	Fastidious Anaerobic Agar
FimH	Fimbrial Adhesin H
g	Gravitational Force
GBS	*Group B Streptococcus*
h	Hour
hpf	High powered field
ICS	International Continence Society
IL-1β	Interleukin 1-β
IL-1Ra	Interleukin 1 receptor antagonist
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
L	Litre
Log	Logarithmic
LUTS	Lower urinary tract symptoms
mls	Millilitres
mm	Millimetre

µg	Microgram
µl	Microlitre
µmol	Micromolar
MOI	Multiplicity of infection
MSU	Mid-Stream Urine
NCTC	National Collection of Type Cultures
NEAA	Non essential amino acids
NGF	Nerve growth factor
NHS	National Health Service
NICE	National Institute for Clinical Excellence
nm	Nanomole
NPV	Negative predictive value
OAB	Overactive Bladder
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PPV	Positive predictive value
RCF	Relative centrifugal force
ROC	Receiver operator curve
rRNA	Ribosomal Ribonucleic acid
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear Neutrophils
RPM	Revolutions per minute
Sd	Standard deviation
Sec	Second
SEM	Standard error of the mean
Spp.	Species
TAE	Tris-acetate / EDTA
TGFβ1	Transforming growth factor β1
TLR4	Toll like receptor 4
TNF-α	Tumour necrosis factor-α
UPEC	Uropathogenic *E.coli*
UTI	Urinary Tract Infection
WBC	White Blood Cells

List of Publications as a result of this thesis

The Inadequacy of Urinary Dipstick and Microscopy as Surrogate Markers of Urinary Tract Infection in Urological Outpatients With Lower Urinary Tract Symptoms Without Acute Frequency and Dysuria

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J Urol. 2010 May;183(5):1843-7

Chapter 1

Introduction

1.1 Epidemiology of Incontinence

The International Continence Society (ICS) defines urinary incontinence as the ‘complaint of any involuntary leakage of urine.’ The prevalence of all incontinence in women shows great variation in the literature, between 14% to 40.5% (1). The peak ages of prevalence also vary between studies and can be broadly divided into those that report the highest prevalence in old age (2) (3;4) and those that report the highest prevalence at the time of the menopause (5). The prevalence of urinary incontinence has also been investigated in different clinical settings and selected populations. Surveys of female patients visiting their general practitioner have shown 43% were incontinent in a USA based study (1) and 44% in a large study in Sweden (4). Fewer studies have addressed the natural course of incontinence in women. In a retrospective Danish study, yearly incidences between 0.5% and 1.4% were estimated for women aged 20-59 years (6). However, in a prospective community based American study of women aged 60, a yearly incidence of about 20% and a remission of 12% were reported (7).

Some review papers have focused on understanding the problem of estimating prevalence (8-10). In addition to the biases induced by investigating different age groups or selected populations, a significant problem has been defining the term "incontinence". The ICS definition implies an objective demonstration of urine loss, and is rarely used in community based surveys (1). Some population based studies have included institutionalised patients, some have excluded them (4;11). This will obviously

explain some of the differing prevalence's found among older women. Some surveys have taken their samples from lists of patients generated in general practice (12). These surveys usually achieve response rates of around 80%, but they may not be representative of the population as a whole.

Incontinence can be further divided into stress urinary incontinence and urge incontinence. The ICS defines stress urinary incontinence as 'the complaint of involuntary leakage of urine on effort or exertion'. Urge urinary incontinence is the complaint of involuntary leakage, accompanied by, or preceded by, a strong desire to void (13;14)

1.1.1 Risk factors for incontinence

The risk factors for incontinence are thought to be multi-factorial, including ethnicity, pregnancy and childhood enuresis. These are discussed in further detail below.

Ethnic factors influencing the prevalence of urinary incontinence have not been well explored. Most studies report Caucasian women to have increased prevalence of urinary incontinence (7;8;15) whereas some have reported similar rates between white and black women (9;10). The prevalence of stress incontinence seems to be increased in Caucasian women (7) and that of urge incontinence and mixed incontinence increased in the black population(7). Most of the studies of risk factors have included parity, age, and obesity (7). Childhood enuresis and pregnancy and/or delivery seem to

be the most important risk factors for lifetime incontinence in women (16;17). (Table1.1). Few women begin pregnancy with pre-existing urinary incontinence (18). About 30% of new mothers become incontinent after a first vaginal delivery (19;20) (21) and current epidemiological studies suggest that caesarean delivery is partly protective (22;23). Parity is a risk factor for both stress and urge incontinence in women (24); (25).

In most studies, age has been strongly associated with the prevalence of both stress and urge incontinence and it is recognised that some factors contributing to incontinence are increased with age (26). Obesity is a well-established risk factor for stress incontinence and urgency, reported with an increased frequency in overweight women (27) (28;29).

The contribution of oestrogen deficiency post-menopause is not clear. Suspicion may arise through the coincidence of two common conditions of late life; menopause and incontinence. Oestrogen replacement is frequently recommended but has not demonstrated benefit in clinical trials. In fact, one large controlled multicentre study showed that treatment with a combination of oestrogen and progesterone increased the severity of incontinence episodes (30). Oestrogen might have some role in improving lower urinary tract symptoms through achieving greater comfort in the tissues, but is not suggested as a primary treatment (31-33).

Table 1.1 Predictors of severe incontinence in British Women, adapted from "Urinary incontinence in middle aged women: childhood enuresis and other lifetime risk factors in a British prospective cohort. Diana Kuh et al. 1999

	Unadjusted odds ratio (95% CI) p value	***p value***
Enuresis (6 y)		
No	**1.0**	
Yes	**3.5 (1.6,7.7)**	**0.001**
Urinary or kidney infections (15–43 y)		
No	**1.0**	**0.028**
Yes	**1.7 (1.1,2.8)**	
Hysterectomy by (48 y)		
No	**1.0**	**0.005**
Yes	**1.6 (1.2,2.4)**	
Body mass index* (43 y)	**1.5 (1.2,1.8)**	**0.003**
GP consultations (47 y)		
Low	**1.0**	**<0.001**
High	**2.7 (1.7,4.3)**	
Number of Children	**1.2 (1.0, 1.5)**	**0.045**
Educational Level†	**0.77 (0.65,0.91)**	**<0.001**

*Per 5 units (kg/m2).
†Fitted as a continuous variable taking values 0 (no qualifications) to 4 (degree level)

1.2 The Overactive Bladder-Definition and prevalence

The Overactive Bladder (OAB) syndrome has been defined by the International Continence Society as a spectrum of symptoms in which incontinence may or may not overlap with urgency, frequency, and nocturia(1). Urgency, the hallmark of OAB, is defined as the sudden compelling desire to urinate, a sensation that is difficult to defer. Urinary frequency is denoted as voiding eight or more times in a 24-hour period.

Nocturia is delineated as the need to wake one or more times per night to void. OAB is an unpleasant condition with high morbidity, patients consistently reporting a negative effect on the quality of life, particularly, less self-assurance and reduced social activity (4;14;34).

The estimated prevalence of OAB is between 8- 11% (4;35) higher than that of asthma and ischaemic heart disease. The occurrence of this condition increases with age and it affects both sexes equally (35). It is independently associated with falls and fractures in the elderly, urinary tract and skin infections, sleep disturbances and depression. One study estimated the financial burden of OAB in five EEC countries to be in excess of €4.2 billion in 2000 (11).

1.2.1 Aetiology and pathophysiology of OAB

Not everyone is happy with the term “OAB”. The term OAB does not sit comfortably in the lexicon of all clinicians. It is a symptomatic diagnosis in contrast to “detrusor over-activity” which is diagnosed by the use of urodynamic studies. Despite the best efforts of the purists, the two terms have become synonymous in the literature, perhaps because the treatment strategies are the same. The aetiology and natural history are not yet fully described and varying hypotheses are mooted (36). It is assumed by many that a number of different pathophysiological factors contribute (37). Regrettably, much of our current data are gleaned from animal experiments, the preferred models being guinea-pig, rat and pig. Species differences make

extrapolation to the human unreliable. Nevertheless, there are three pathophysiological hypotheses for OAB. In the literature, an etymological error results in them being given the status of theory: The “myogenic theory”, the “neurogenic theory” and the “autonomous bladder theory” (38). The scientific definition of Theory is “A set of statements or principles devised to explain a group of facts or phenomena, especially one that has been repeatedly tested or is widely accepted and can be used to make predictions about natural phenomena”. Colloquially, it is more often used to describe “A proposed explanation whose status is still conjectural” (*see Dictionary.com*)

1.2.2 The Anatomy and physiology of the bladder and the overactive bladder

The lower urinary tract comprises of the urinary bladder and the urethra. The urethra contains both smooth and striated muscles and the bladder can be divided into two parts: the bladder body, above the ureteral orifices, and the base, consisting of the trigone, urethrovesical junction, deep detrusor, and the anterior wall (figure 1.1). The bladder is lined by a mucous membrane and covered on the outer aspect in part by peritoneal serosa and by fascia. The wall is formed of smooth muscle cells, also referred to as the detrusor muscle.

Figure 1.1 Anatomy of the human bladder. Taken from McGraw-Hill

There are three layers of smooth muscle. The cells of the outer and inner layers are mostly oriented longitudinally, and those of the middle layer circularly. In the human detrusor, bundles of muscle cells of varying size are surrounded by connective tissue which is rich in collagen. The bundles are not clearly arranged in distinct layers, but run in all directions (Figure 1.2).

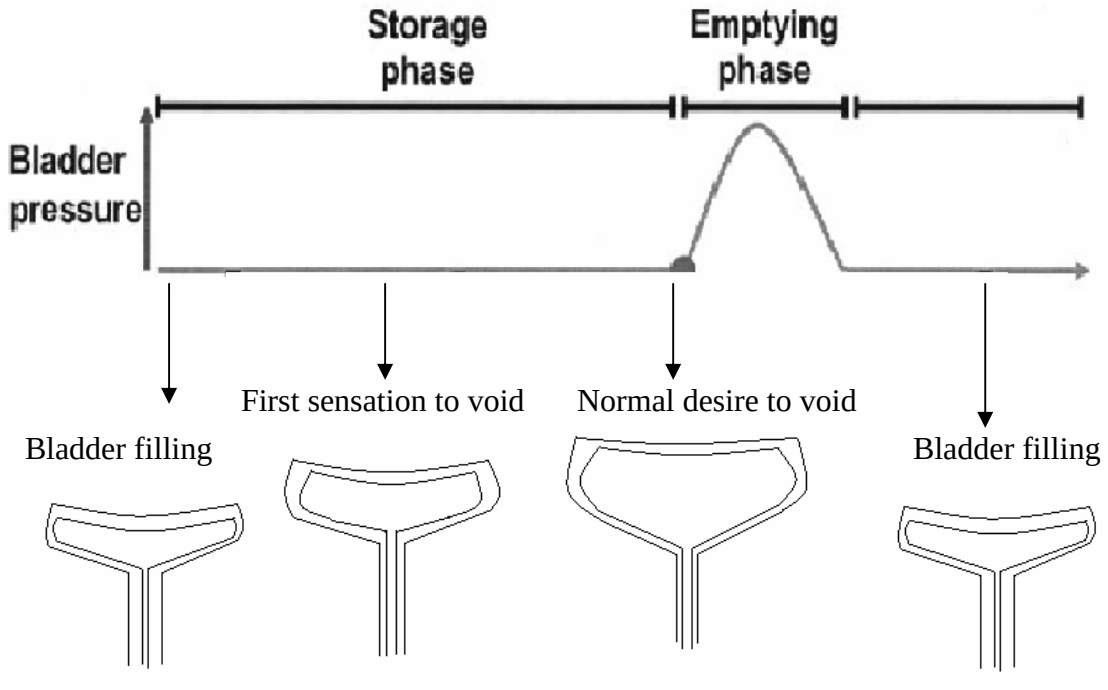
Figure 1.2 The lumen of the urinary bladder showing the detrusor muscle layer. Taken from www.iupucanatomy.com/chapter

Within the main bundles, the smooth muscle cells may exist in groups of small functional units, or fascicles (39).The orientation and interaction between the smooth muscle cells in the bladder are important as this determines how the bladder wall behaves and what effect activity in the cells have on the intraluminal pressure.

1.2.3 Micturition and the myogenic theory

The storage and expulsion of urine during micturition, involve a complex interaction between the elements of the urinary tract and nervous control systems. During filling of the urinary bladder, the smooth muscle cells relax and elongate. Micturition is a highly synchronised process of detrusor contraction. Synchronisation of each part of the urinary tract musculature is mediated by specific transmitters which are released from nerves or generated locally to activate downstream second messenger cascades (Figure 1.3).

Figure 1.3 The micturition cycle. The diagram depicts the micturition cycle under normal physiological conditions. Adapted from "Pathophysiology of Overactive Bladder" (Chu et al. 2006).



The myogenic theory (Brading et al) (40;41), suggests that denervation of the detrusor smooth muscle leads to increased cell excitability leading to involuntary contractions of the detrusor muscle which manifests as urinary urgency and urgency incontinence. This is most applicable to patients with bladder outlet obstruction, in which chronic increases in intravesical pressure, cause partial neurological denervation of the bladder smooth muscle. Spontaneous action potentials can be propagated from cell to cell in the bladder via gap junctions. When the smooth muscle is denervated, there is an increase in the number of spontaneous action potentials and in the ability of the action potentials to propagate from cell to cell which is also supported by increased gap junction density (42). As a result repeated small contractions of

the detrusor muscle also stimulate afferent receptors providing feedback to the CNS and cause the symptoms of OAB.

1.2.4 Neural and hormonal control and the autonomous theory.

Various efferent and afferent neural pathways and neurotransmitters are involved in the storage of urine and emptying of the bladder. Central neurotransmitters, such as glutamate, serotonin, and dopamine, are thought to play a role in urination. For example, serotonergic pathways facilitate urine storage. Dopaminergic pathways may have both inhibitory and excitatory effects on urination. Dopamine D1 receptors appear to have a role in suppressing bladder activity, whereas dopamine D2 receptors appear to facilitate voiding.

Acetylcholine, which is the predominant peripheral neurotransmitter responsible for detrusor contractions, is released from the parasympathetic nerve terminal and binds to muscarinic receptors on the detrusor muscle. Five muscarinic receptor subtypes have been identified (43). The M3 muscarinic receptor appears to be responsible for detrusor contractility in the normal bladder. The M2 receptor is negatively coupled to the adenyl cyclase mechanism and may act to suppress sympathetic effects (44). Its influence appears to be much less overt than that of the M3 receptor. Data from small studies demonstrate up-regulation of the M2 receptor in obstruction and spinal-cord injury and may contribute to detrusor overactivity in those conditions (44;45). Binding of acetylcholine to the M3 receptor activates

phospholipase C via coupling with G proteins. This action causes the release of calcium from the sarcoplasmic reticulum and contraction of the bladder smooth muscle. Increased sensitivity to stimulation by muscarinic receptors has been mooted as a mechanism leading to OAB. Leakage of acetylcholine from the parasympathetic nerve terminal may lead to small contractions of the detrusor, which may activate sensory afferent fibres, leading to the sensation of urgency. This is the basis of the autonomous theory of the OAB (46).

Unsolicited activation of sensory afferent nerves may also play a role in OAB, especially in neurological disorders. Neuroreceptors such as vanilloid, purinergic, neurokinin A, and nerve growth factor receptors are associated with these afferents and are mediated by neurotransmitters including nitric oxide, calcitonin gene-related protein, and brain-derived neurotropic factor.

1.2.5 The neurogenic Theory

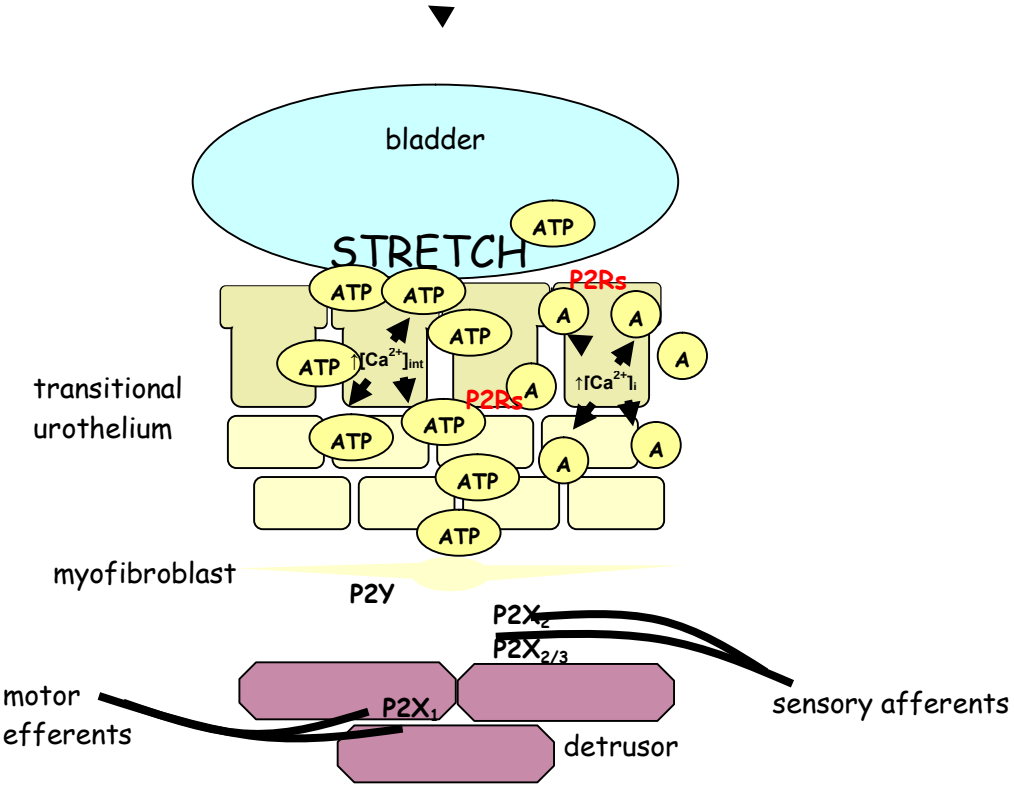
De Groat (47), describes decreased suprapontine inhibition of the micturition reflex, following, for example, a cerebrovascular accident as a cause of the OAB. Other neurogenic aetiologies of OAB proposed include damage to axonal paths in the spinal cord, loss of peripheral inhibition, and enhancement of excitatory neurotransmission in the micturition reflex pathway such as seen in stroke, spinal cord injury and multiple sclerosis.

1.2.6 The role of the urothelium in OAB

Although once thought to be a simple water resistant epithelial lining, it is now thought that the urothelium performs an important sensory role in bladder function and may be implicated in OAB (Figures 1.5). The urothelium is thought to exhibit neuron-like properties by releasing several neurotransmitters in addition to expressing receptors for other messenger molecules (47). Moreover, there is direct communication with sub-urothelial afferents. Ferguson et al showed that when mouse detrusor muscle is stretched, flux of calcium intracellularly results in the release of ATP on the serosal aspect of urothelium (48). It is now known that ATP is released by both surfaces of the urothelium (49). This ATP activates the purinergic receptors, $P2X_3$ and/or $P2X_{2/3}$, on suburothelial afferent nerves and relays information to the central nervous system to produce a sensation of bladder

fullness and urgency (50). Thus, purinergic signalling has a function in the mechano-sensory transduction in the bladder (51;52) (figure 1.4).

Figure 1.4 P2 receptors in the bladder; response to stretch.



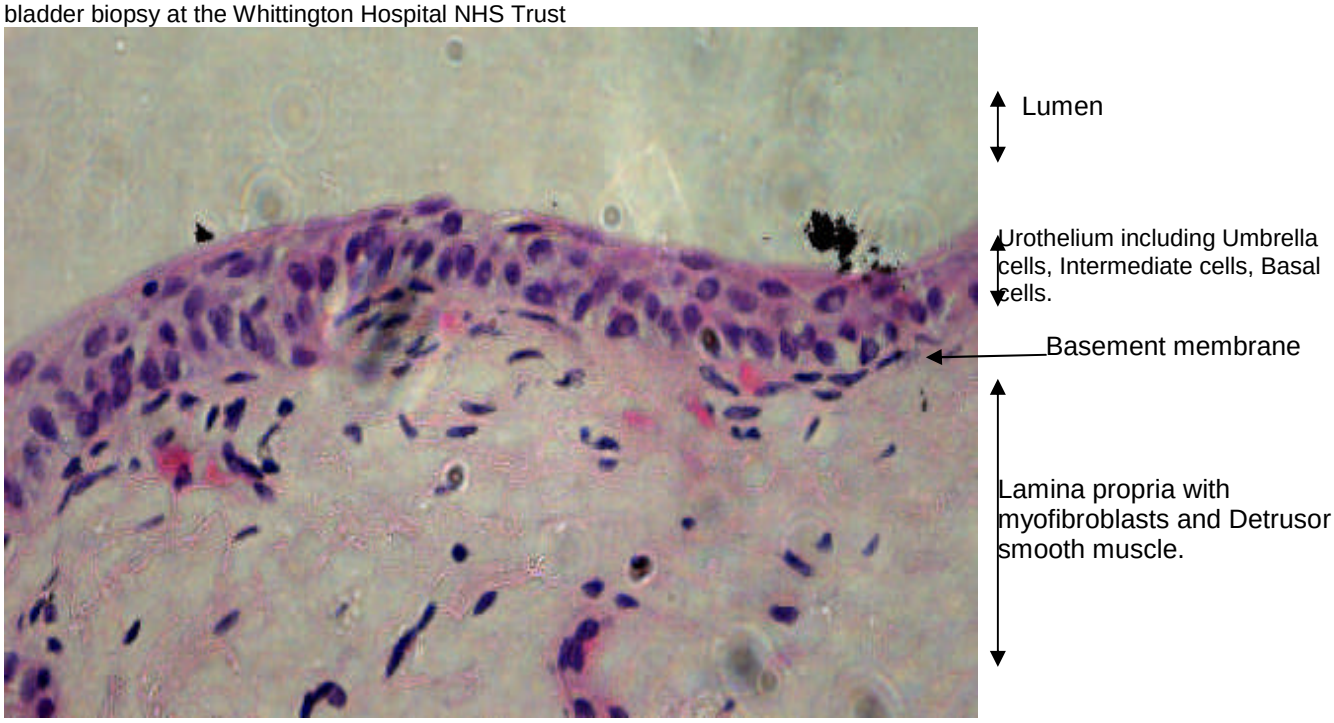
P2X3 receptor knock- out mice display reduced pain related behaviour in response to chemical irritation of the bladder with formalin and ATP injection. Furthermore, these mice also exhibit marked bladder hyporeflexia characterized by a decreased micturition frequency and an increased bladder capacity under normal bladder pressures (53). In another study that also made use of the P2X3 knockout mice, ATP release was shown to be proportional to the extent of bladder distension. It was also noted that gradual bladder distension resulted in a progressive increase in the activity of afferent

nerves (54). In one study, intravesical ATP was demonstrated to induce detrusor overactivity when the urothelial barrier was compromised by chemical treatment with protamine sulphate to increase its permeability at pH 6.0. This was a very interesting finding as previous studies have shown exogenous ATP to induce bladder overactivity but at much lower pH's. The authors concluded that an increased permeability of the urothelium is required for exogenous ATP to induce detrusor activity via the P2X receptor (55).

ATP has been shown to play a role in inflammation where an increased release has been described (56). Consistent with this observation, an augmented release of ATP in response to stretch as well as an increased urinary secretion of ATP has been demonstrated in patients with interstitial cystitis in which bladder inflammation is described during cystoscopy (56). Inflammation of the urothelium may play a part in the pathophysiology of OAB by not only disrupting the integrity of the urothelial barrier but also by leading to an increased release of ATP by the urothelium.

An aberrant ATP response induced by an inflammatory reaction may play a role in the pathophysiology of OAB by increasing sensory nerve excitation.

Figure 1.5 Photomicrograph showing the normal human urothelium (H&E staining) taken from a bladder biopsy at the Whittington Hospital NHS Trust



1.3 The Diagnosis of OAB

Lower urinary tract symptoms (LUTS) described by OAB patients overlap with those caused by other lower urinary tract syndromes such as interstitial cystitis, painful bladder syndrome and acute urinary infection. The National Institute of Clinical Excellence (NICE) states that the clinical history should be sufficient to diagnose and differentiate between lower urinary tract syndromes. There are many validated urgency scores in the literature which help to diagnose OAB (57-60). It is important to understand that the exclusion of infection is the cornerstone investigation in the diagnosis of OAB (1).

In support of this, the International Consultation on Incontinence guidelines on LUTS emphasise the need to exclude urinary tract infection (UTI) and recommend dipstick testing as a screening method (61). NICE also recommends the use of urinary dipstick for screening patients with lower urinary tract symptoms (62). However, there is no published data to support dipstick as a valid screening tool for patients with non- acute, non dysuric symptoms. NICE further recommends a mid-stream urine (MSU) culture of urine if the dipstick is positive for leukocyte esterase or nitrite. Similarly, there is no evidential justification for this.

1.3.1 The exclusion of urinary tract infection (UTI) in patients presenting with symptoms of the Overactive Bladder

As described, two techniques are commonly deployed to exclude UTI. An MSU is submitted for culture, or urinalysis by dipstick is performed. The latter seeks surrogate evidence of infection, either pyuria by detecting leukocyte esterase activity; or bacterial splitting of urea by measuring nitrite.

1.3.2 Urine Culture

The diagnosis of UTI from culture of an MSU has rested on criteria described by Kass et al (1957) in his seminal paper (63). He reported that the MSUs of 25 patients with systemic symptoms (chills, fever, flank pain) and dysuria had grown more than 10^6 bacterial colony forming unit's ml^{-1} (cfu ml^{-1}). After studying the MSU in asymptomatic women, he established that 10^5 cfu ml^{-1} of a known urinary pathogen should be the threshold between true bacturia and contamination. Kass never claimed to define a threshold for use with "cystitis", i.e. localised symptoms of frequency/dysuria. Nevertheless, 10^5cfu ml^{-1} has been widely adopted in clinical practise and the surrogate tests of a dipstick have been validated against this standard.

In 1982, Stamm (64) published work on women presenting with acute dysuria. He concluded that a culture result of 10^2 cfu ml^{-1} was a more appropriate threshold for diagnosis of bacterial cystitis. There are no data on a threshold applicable to patients with other lower urinary tract symptoms, notably frequency, urgency and incontinence.

As a result of setting standard laboratory methods (64) for analysing MSUs at a threshold of 10^5cfu ml^{-1} instead of 10^2cfu ml^{-1} , Hooton & Stamm have demonstrated that laboratory MSU cultures miss over 50% of genuine infections in women with frequency/dysuria (65).

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It must also be acknowledged that the culture methods assume dominant pathogenicity from the *Enterobacteriaceae* species, notably *Escherichia coli*. Because of this assumption, the MSU culture is performed on a selective medium (chromogenic medium) for *Enterobacteriaceae* under aerobic conditions. No anaerobic bacteria are sought and some aerobic bacteria can be missed because of the choice of medium. Whilst this might be appropriate for diagnosing acute infection, there is no evidence that the same bacteria are implicated in chronic infections or patients presenting with non-dysuric lower urinary tract symptoms such as in the overactive bladder. Thus, there are no data to inform the selection of a suitable gold standard for diagnosing UTI in this context.

1.3.3 Urine Dipstick Test

There have been two meta-analyses of urinary dipsticks, used to assess acute frequency/dysuria, in adults (66;67) and one in children (68). Hurlburt and Littenberg (67) concluded that dipsticks cannot exclude infection reliably in most clinical settings. Deville et al (66) reported leukocyte esterase sensitivity of 76% (95% CI 60% to 98%) and specificity 46% (95% CI 32% to 68%) for the diagnosis of urinary infection and a nitrite sensitivity of 49% (95% CI 38% to 62%) and specificity of 85% (95% CI 73% to 100%) in primary care.

Leukocyte esterase is used as evidence of pyuria, which in turn is considered to be a surrogate for infection. Therefore, in validating the leukocyte esterase test there are two potential gold standard references, the results of urine culture and the urine microscopic white cell count.

The Nitrite test uses the Greiss reaction to detect nitrite as a marker for urea-splitting urinary bacteria. The chemical basis of this reaction is that in an acid environment nitrite reacts with an aromatic amine (sulfanilamide) to form a coloured diazonium salt that in turn reacts with hydroxybenzoquinolone to provide a pink colour (69). It requires the presence of bacteria that can convert nitrate in the urine to nitrite. The published data on the nitrite dipstick has used the 10^5 cfu ml^{-1} threshold as the gold standard for diagnosing infection, and reported sensitivities ranging from 18% to 71% and specificities from 82% to 100% (70).

1.3.4 Urine microscopy and the quantification of pyuria

An alternative method of diagnosing urine infection is the identification of significant pyuria. The determination of the excretion rate of white blood cells in urine was first described by Hottinger in 1893 (71). However, the technique used is usually attributed to Addis, who published his method in 1925, referred to as the "Addis count" (72). Hamburger in 1950 (73), and Houghton and Pears in 1957 (74) described modifications of the Addis count which made it simpler but reliable, although a catheter was used to collect the urine. The leukocytes were enumerated by examining the urinary sediment in a haemocytometer.

Hamburger, Houghton and Pears obtained the sediment by centrifugation of 10 ml of urine at 1500 rpm (radius 12 cm) for three minutes. 9 ml of supernatant was removed and the 1 ml of sediment re-suspended with a Pasteur pipette before spreading on the haemocytometer chamber to count the leukocytes. Having used this technique in volunteers, they concluded that the normal rate of leukocyte excretion varied between 18,000 and 196,000 per hour.

The upper limit of the normal leukocyte excretion rate per hour was established as 20,000 wbc h^{-1} to 200,000 wbc h^{-1} by Houghton and Pears in 1957 (74) . Hutt, Chambers and MacDonald in 1961 (75); Osborn and Smith in 1963 (76), Little in 1962 (77) demonstrated excretion rates of >400,000 wbc h^{-1} in patients with proven, symptomatic urinary infection. In 1968, Mabeck

attempted to simplify evaluation by counting the white cells per high powered field (x40 objective x10 optical = x400: wbc hpf^{-1}) using the re-suspended, stained, sediment. All patients showing three or more wbc per high powered field (X400 , hfp) excreted more than 400,000 wbc h^{-1}, but fewer wbc hpf^{-1} did not exclude excretion rates of >=400,000 wbc h^{-1}. The spun sediment underestimated the leukocyte excretion (78;79).

Mond (1965) examined unspun fresh urine and counted the white cells using a haemocytometer, finding that >=10 wbc mm^{-3} were noted in all patients with symptomatic acute cystitis and bacteriuria (10^5 cfu ml^{-1}) (80). In 1968 Gadeholt compared the examination of spun and unspun specimens and showed that the calculated excretion rates on uncentrifuged specimens showed higher values (81;82). This confirmed earlier suspicions that centrifugation caused loss of cells. Gadeholt correlated unspun urine with >=10 wbc mm^{-3} excreted >400,000 wbc h^{-1}; This was confirmed by Mabeck in 1968 (79) and by Baerheim et al (1989) (83), all of whom implicated centrifugation. Thus the finding of >=10 wbc mm^{-3}, of unstained, unspun urine examined on a haemocytometer, became established as the most effective method for diagnosing urinary infection.

Latham and Stamm reported that the detection of pyuria (>=10 wbc mm^{-3}) was the most accurate and efficient method of identifying urinary tract infections in ambulatory women with symptoms of acute cystitis (84). Whilst Stamm has achieved much in clarifying the diagnostic criteria, useful with classical symptoms, there remains a problem with the lower urinary tract

symptoms not typical of acute cystitis, including those of OAB. It will be argued that it is no longer reasonable to rely on the reassurances of bacteriuria of $>10^5$ cfu ml^{-1} without due validation.

OAB could reflect genuine urinary infection, undetected by traditional analysis. One study (85) has reported the results of a catheter specimen of urine (CSU) taken from 864 women at the time of urodynamic investigation. Laboratory reports of pyuria and bacturia were found to occur more often in patients with OAB compared to those with stress incontinence.

1.4 Acute urine infection

The American Foundation for Urological Diseases reports that acute bacterial cystitis affects 8–10 million Americans a year and most of these patients are women. The National Institute of Diabetes and Digestive and Kidney Diseases notes that acute cystitis prompts about 9.6 million doctor visits annually; $4.5 billion is spent on 11 million antibiotic prescriptions. Occurring in otherwise healthy young women, acute, symptomatic, uncomplicated urinary tract infection (UTI) is the most common infection (86) (87), next to the common cold. 25 to 40% of females will experience an acute UTI in their lives, and up to 6% of women will have one or more UTIs in a given year (87;88).

UTI is frequently caused by organisms which are normal commensals in the distal urethra and adjacent sites. The most common route of infection is by ascension. The gender difference in the prevalence of UTI is thought to be

related to the short length of the female urethra and the uropathogens involved are usually part of the normal faecal microbiota. These bacteria colonise the perianal region and then ascend in women to the *introitus vaginae* which is a reservoir for several uropathogens. Colonisation spreads to the periurethral area, urethra, and bladder,this is also dependant on sexual activity. The role of the normal vaginal microbiota in the defence against genital colonisation with potentially pathogenic adhering *E. coli* has been demonstrated in several studies. The reported vaginal colonization rate of *E. coli* varies from 6 to 26% (89).

Defensive properties of the commensal microbiota against colonisation by bowel organisms include production of inhibitory substances against pathogens, colonisation of epithelial surfaces, and competition with potential pathogens for sites of adhesion. The relationship of vaginal *E. coli* load with phases of the menstrual cycle also indicates hormonal determinants of vaginal colonisation with *E. coli*. Local trauma, such as sexual intercourse or urethral massage, promotes invasion of the urinary tract. A vaginal pH of 5 or less protects against vaginal colonisation and urogenital infections. *Lactobacillus spp* usually colonise the vagina and generate an acidic vaginal pH which interferes with the adhesion of *E. coli*, one of the most common uropathogens in otherwise normal women. It is also likely that use of soaps to clean the genital tract alters the pH and normal microbiota. In addition, the use of diaphragms, cervical caps, or spermicides for contraception is associated with a higher incidence of UTI (89). Spermicides have been shown to increase colonisation of the vagina with uropathogens independently of

sexual intercourse. Most spermicides contain Nonoxynol-9, which appears to be active against hydrogen producing lactobacillus in the vagina, which may be protective against colonisation of uropathogens (65;90).

In females with recurrent UTI, hormonal factors are thought to influence bacterial attachment to epithelial cells. Genitourinary mucosal cells have oestrogen receptors (91;92). Adherence changes during the menstrual cycle and is maximal during peak oestrogen stimulation. Oestrogen deficiency in postmenopausal women is associated with a higher risk of UTI. The mucosa atrophies, lactobacilli disappear from the vaginal microbiota, vaginal pH increases, and the vagina is then predominantly colonized by *Enterobacteriaceae*, especially *E. coli*. The Cochrane review 2008, looked at nine randomised control trials, in which postmenopausal women received any kind of oestrogen therapy for recurrent UTI compared to placebo. They found two studies (Eriksen 1999 and Raz 1993) (93;94) showing vaginal oestrogens; a cream and a ring pessary to be superior to antibiotics for treatment. *Raz et al* (94) reported both a lower vaginal pH in the oestrogen treatment group and a higher proportion of vaginal *Lactobacillus spp.* as compared to controls.

1.4.1 Causative Organisms

Escherichia. coli

The predominant causative organism of acute UTI in women is reported to be *Escherichia. coli* (*E. coli)* (88) (Figure 1.6). The prevalence of *E. coli* in symptomatic patients with positive urine cultures is between 50-77% (95;96). Other pathogens of importance are *Staphylococcus. saprophyticus, Streptococcus spp., Enterococcus. faecalis, Klebsiella. pneumoniae* and *Proteus. mirabilis*.

E. coli belongs to the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultative anaerobic Gram-negative rods. Physiologically, *E. coli* is very well adapted to its habitat. *E. coli* colonizes the gastro intestinal tract within hours or a few days after birth. There are over 700 antigenic types (serotypes recognized based on O, H, and K antigens. Serotyping is still important in distinguishing pathogenic strains (95).

Uropathogenic *E. coli* (UPEC) cause most of the UTIs in anatomically normal urinary tracts. The bacteria colonize from the faeces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14-times more common in females than males by virtue of the shortened urethra (97). UPEC differ from non-pathogenic *E. coli* and from other *E. coli* pathotypes by the production of specific virulence factors, which enable the bacteria to adhere to uroepithelial cells and to establish UTIs. In addition to cell adherence factors,

toxins, ‘modulins’, capsules, iron uptake systems and other bacterial products contribute to the mechanism of virulence.

*Figure 1.6 E. coli with fimbriae (*Transmission Electron Micrograph x17,250). Taken from Dennis Kunkel Microscopy, Inc.

Staphylococcus. saprophyticus

Staphylococcus. saprophyticus is second to *E. coli* as the most frequent causative organism of uncomplicated UTI in women (Figure 1.7). The vast majority of infections with this organism occur in young sexually active women. Wallmark et al (98) isolated *S. saprophyticus* from the urine of 22% of consecutive female patients found to have bacturia. The highest rate of *S. saprophyticus* infection was 42.3% among women aged between 16-25 years in the study. Gupta et al. reported a prevalence of 8% among 665 women with UTI (99).

The gastrointestinal tract is the major reservoir of *S. saprophyticus*. Rectal, vaginal and urethral colonisation with *S. saprophyticus* is also associated with UTI caused by other organisms (100).

Figure 1.7 Electronmicrograph of S.saprophyticus. Taken from Dennis Kunkel Microscopy, Inc.

Group B Streptococcus

Group B *Streptococcus* (GBS) is a leading cause of UTI (Figure 1.8). There are particular GBS capsular serotypes that are disproportionately associated with colonization and disease; serotypes Ia, III, and V cause the majority of invasive infections in the elderly (101). Multiple serotypes of GBS also cause UTIs, asymptomatic bacturia, pyelonephritis, urethritis, and urosepsis (102;103). GBS asymptomatic bacturia is particularly associated with immunocompromise, such as the elderly, pregnant women, patients with diabetes mellitus and chronic renal failure (104;105). However, the underlying mechanisms of pathogenesis that lead to acute GBS UTI are unknown. A recent study suggests that binding of uropathogenic GBS to the uroepithelium and induction of the inflammatory cascade with interleukin 1α represents the initial stages of GBS urinary tract infection (106).

Figure 1.8 Electronmicrograph of Group B Streptococcus. Taken from Dennis Kunkel Microscopy, Inc.

Enterococcus. faecalis

Enterococcus. faecalis, while normally a gut commensal, is a frequent cause of many serious infections, including urinary tract infections (Figure 1.9). *E. faecalis* is responsible for approximately 110,000 cases of UTI each year. *E. faecalis* is especially troublesome to treat because of its frequent resistance to multiple antibiotics, including vancomycin (107).

Both rat and mouse model systems have been used to study factors involved in the pathogenesis of *E. faecalis* in the urinary tract. Studies by Guze (108) showed that *E. faecalis* has a growth advantage over other *Enterococcal* species in rat kidneys and that pyelonephritis caused by *Pseudomonas aeruginosa* was aggravated by coinfection with *E. faecalis*, determined by histological changes in the kidney. In a bladder catheterization model of urinary tract infection, the Esp (enterococcal surface protein) adhesin was

found to increase persistence in the bladder of mice, although no histological changes were observed (109).

Figure 1.9. Pseudocolor low-voltage field emission scanning electron micrograph of Enterococcus faecalis biofilm microcolony cultured on a submerged cellulose membrane (40,000× magnification). Taken from "Development and Use of an Efficient System for Random mariner Transposon Mutagenesis to Identify Novel Genetic Determinants of Biofilm Formation in the Core Enterococcus faecalis Genome." ***Kristich et al 2008***

1.4.2 Defence against infection

The first defences against bacterial infection and adhesion to the urothelium are the physical and chemical properties of the urine. Invading bacteria stimulate micturition. Voiding washes out bacteria from the bladder and urethra and urine dilutes the bacterial load. Bacterial growth is impeded by low pH and the high urea and organic acid concentrations and the extremes of high and low osmolality deter some less adapted bacteria (110;111). Nevertheless, aggressive bacterial species have evolved mechanisms to overcome these obstacles.

The role of bacterial infection in the aetiology of the overactive bladder

There are a number of interrelated anatomical/ physiological factors which influence the maintenance of bacterial numbers in urine once infection is established. The flow rate of ureteric urine into the bladder; the volume of residual urine remaining in the bladder after micturition and the frequency with which micturition occurs.

The flow rate of ureteric urine into the bladder is inevitably variable, but averages at about 1 ml/min during the day and at about 0.25 ml/min during the night (112). The flow rate is increased by high fluid intake and diuretics so the urine produced may be dilute and less suitable for supporting bacterial growth. Providing the ureteric urine is not carrying bacteria from a focus of infection in the kidney, the influence of urine flow on bacterial numbers in bladder urine is a dilution effect. However, this dilutional effect also influences the immune response, by diluting immune cells at the site of infection. Furthermore, post micturition residual urine can vary enormously in the infected bladder. Studies by Shand have suggested that a normal residual of 1ml may increase enormously and even modest increases (> 1 to 10 ml) may by association render UTIs more difficult to treat (Shand et al 1970) (113). Mackintosh et al. (1975) (114) have constructed a mathematical model to explain this phenomenon. Their calculations predict that, in conditions in which the mean generation time of bacteria is 40 min, the ureteric urine flow is 1 ml per minute and the bladder is emptied hourly, a residual bladder volume of 8.5 ml would prevent bacterial numbers from falling, even in the unlikely event that all the bacteria are evenly distributed in the urine and fully affected by washout forces. The interval between episodes of micturition will fluctuate

during the day and vary with fluid intake. Most people micturate only once, or not at all, during the night, but urinary infection may alter this habit. In acute cystitis frequency of micturition is a common presenting system. This may be invoked as a natural defence mechanism until the resolution of infection. In addition, higher residual volumes are also associated with urinary tract infection, particularly in the post-menopausal group.

As a result of normal kidney function, the variability in the chemical characteristics of urine such as pH and osmolality can facilitate or suppress bacterial growth. Any reduction in the bacterial growth rate will, of course, be to the patient's advantage, but this does not necessarily equate to elimination of infection. The situation may be complicated by the fact that the bacteria may fix themselves to the surface of the bladder mucosa or reside in foci within the urinary system where they are unaffected by hydrokinetic forces (fig 1.10).

Figure 1.10 Scanning electron micrograph of an infected bladder superficial facet cell with E.coli. Taken from “A murine model of urinary tract infection.” Scott J Hultgren et al 2009

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The importance of 'bound' organisms in the persistence of infection has been investigated, through mathematical modelling, by Mackintosh et al. (1975) (114). They calculated that if 10 'bound' bacteria continuously seed the urine with fresh organisms, infection would be maintained at around the 10 bacteria per ml level, even in the most favourable washout conditions in which a patient with a normal residual bladder volume micturates every hour.

This synthetic data is supported by clinical studies which have shown that, infection will persist in a proportion of women despite adequate diuresis and any impairment of the efficiency of the washout mechanism will exacerbate the situation (115).

1.4.3 The Inflammatory Response

Urothelial damage by infection leads to the release of inflammatory mediators including platelet derived growth factor (PDGF), tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 8 (IL-8). *E. coli* infection has been well studied (116) (117) (118), in which a rapid cytokine response is observed, particularly with adhering, P- or type 1-fimbriated *E. coli*.

Fimbria-mediated adherence is one of the virulence factors needed to stimulate the mucosal barrier (fig 1.11). Recent studies with human urinary epithelium have confirmed this concept (119). Adherence is an important first step to establish tissue contact, in addition, the molecular interactions

between bacteria and host trigger a host response (120) (118). In urinary tract cell lines, epithelial cell activation by fimbriated *E. coli* requires primary recognition receptors for fimbrial adhesins and Toll-like receptor 4 (TLR4) for transmembrane signalling (121). Human urinary tract epithelial cells express both glycosphingolipid and mannosylated surface glycoprotein receptors, which recognize the P fimbrial adhesins (122) and the type1 fimbriae, respectively (123).

Figure 1.11 Transmission electron micrographs of UPEC expressing different fimbriae. Taken from "Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney." Mobley et al. 2007

1.4.3.1 The role of cytokines and other inflammatory markers

Cytokines play a key role in the innate defence (123). Almost all cells produce cytokines when appropriately stimulated, especially lymphocytes. Cytokines perform an almost hormonal role by allowing communication of epithelial cells with local and distant host cells. Recently, there has been much interest in other inflammatory markers in OAB, particularly for use as biomarkers for the

disease. Neurotrophins such as Nerve growth factor (NGF) and Brain derived growth factor (BDGF) and prostaglandins have been studied in OAB.

1.4.3.2 The role of cytokines in the overactive bladder

Urinary cytokines are elevated in patients with UTI (124)(125). Murine UTI studies have identified epithelial cells as early producers of cytokines (123) but the epithelial cytokine response of the human mucosa *in vivo* has not been investigated. IL-6 is known to cause fever and trigger the acute-phase response in response to bacterial infections and in addition, it has been demonstrated that IL-6 plays a critical role in the formation of chronic inflammation, including in the bladder (126), while chemokines such as IL-8 recruit inflammatory cells to the site of infection. Uroepithelial cell lines constitutively make IL-6 and respond with elevated IL-6 production to exogenous stimuli like bacteria or cytokines (120;124). Studies of patients with UTI have shown rapid increases in urine IL-6 levels after the onset of infection or instillation of bacteria into the urinary tract (124;127) but serum IL-6 levels are only elevated in those with acute pyelonephritis (128).
There is very little and conflicting data on the expression of inflammatory mediators in OAB. Other lower urinary tract syndromes such as Interstitial Cystitis and painful bladder syndrome have been linked to an inflammatory aetiology. Interstitial cystitis and the painful bladder syndrome are associated with increased mast cells on bladder biopsy and raised inflammatory markers in the urine including histamine, methylhistamine and IL-6, thought to result

from the degranulation of granules from the mast cells(129;130). Erickson et al. found elevated levels of IL-6 and Epidermal growth factor (EGF) in patients with interstitial cystitis (131). Transforming growth factor β1 (TGFβ1) is upregulated in interstitial cystitis and a three-fold increase in IL-10 has been demonstrated in patients with OAB (132). Bouchelouche et al. showed that IL-1β and and TNFα stimulate the secretion of IL-6 in cultured human detrusor smooth muscle cells (133). The regulatory effect of cytokines in inflammation has been shown in animal models, particularly the stimulation with bacterial endotoxin lipopolysaccharide leading to the secretion of IL-6 (134). Heinrich et al (135) showed an upregulation in the gap junction proteins connexin 43 and 45, secondary to stimulation by IL-6. They suggested that this modification of cell-cell communication by IL-6 could be pivotal in the pathophysiology of the overactve bladder and interstitial cystitis. Conversely, Ghoniem et al. (136) have shown a down regulation of IL-6 in the urine of patients with OAB, however, they do not describe the minimum level of detection of the cytokine in their micro-array.

Tyagi et al. (137) studied IL-5, IL-6, IL-10, IL-12p70/p40, IL-1 receptor antagonist (IL-1Ra), Epidermal growth factor (EGF) and soluble IL-2 receptor a (sIL-2Ra) in the urine of patients diagnosed with OAB compared to control volunteers. They found a significant increase in IL-10, IL-12, EGF and IL-12p70/p40. They suggested a relationship with inflammation in OAB, possibly secondary to irritation or stress. An increased IL-8 has been shown in Interstitial Cystitis but not in the overactive bladder (121;125;127).

1.4.3.3 The role of neurotrophins in the overactive bladder

NGF and BDGF are both increasingly studied in OAB. In animal models, NGF is released in high amounts from smooth muscle cells and the urothelium in OAB models (138) and has been shown to reduce bladder capacity and increase bladder reflex contractions (139). In human studies, increased levels of NGF have been found in the urine of patients with OAB and IC (140;141). Interestingly, it has been suggested that urinary NGF concentration in OAB patients correlates with urgency intensity (142).

Little is known about the role of Brain derived neurotrophic factor (BDNF) in bladder function. After chronic bladder inflammation or spinal cord injury, the synthesis of BDNF in the urinary bladder is increased (143). Antunes-Lopes et al. (144) assessed urinary levels of BDNF in healthy volunteers to investigate if there was a physiological pattern of secretion and to discern any gender differences. In healthy volunteers, BDNF/creatinine ratio was low, irrespective of gender or time of urine sampling. In contrast, urinary BDNF/creatinine ratio was significantly higher in OAB patients compared to controls.

1.4.3.4 The role of prostaglandins in the overactive bladder

The role of prostaglandins in OAB is very controversial. They are locally synthesised in the bladder muscle and urothelium, triggered by detrusor muscle stretch, bladder nerve stimulation, bladder mucosa damage, and inflammation (145) . They are involved in the micturition reflex by decreasing the thresholds of the stimuli necessary to trigger bladder contraction through activation of the capsaicin-sensitive afferent nerves (145) Activation of

prostaglandin EP3 receptors exerts an excitatory effect on urinary bladder function through modulation of bladder afferent pathways (146). Kim et al. found urinary levels of PGE2 and PGF2*α* in patients with OAB were significantly increased compared to a control group (145). In addition, an inverse correlation was found between urinary PGE2 and the volume to first desire to void and the maximum cystometric capacity. However, in contrast Liu et al. measured urinary levels of PGE2 in patients with OAB wet, OAB dry, IC, and controls and did not find significant differences (147).

The literature certainly supports a role for inflammation in the pathophysiology of the overactive bladder with the presence of many inflammatory markers described in OAB as above. However, there is little suggestion as to the cause of the inflammation, and more a move to investigate a novel biomarker for the disease.

1.4.4 A mouse model of urine infection

Hultgren et al. have mapped out the probable sequence of events involved in UTI by means of a series of experiments conducted on mice as described below, (Fig1.12). Hultgren et al. used the mouse model due to the similarities in the bladder epithelium between mouse and human. The urothelium in both species is comprised of small, relatively undifferentiated basal and intermediate epithelial cells underlying a single layer of highly differentiated, large and often-binucleate superficial facet cells. These cells are coated on the apical side with semi-crystalline arrays of four integral membrane proteins known as uroplakins. The primary sequence and biochemical properties of the uroplakins as well as the ultrastructures of the asymmetric unit membrane they form are highly conserved among many mammalian species, including human and mouse (148).

(1) UPEC possess adhesive, fibrous organelles on their surface, which are called pili. The type-1 pilus adhesin FimH binds to mannosylated uroplakins on the surface of urothelial umbrella cells (149).

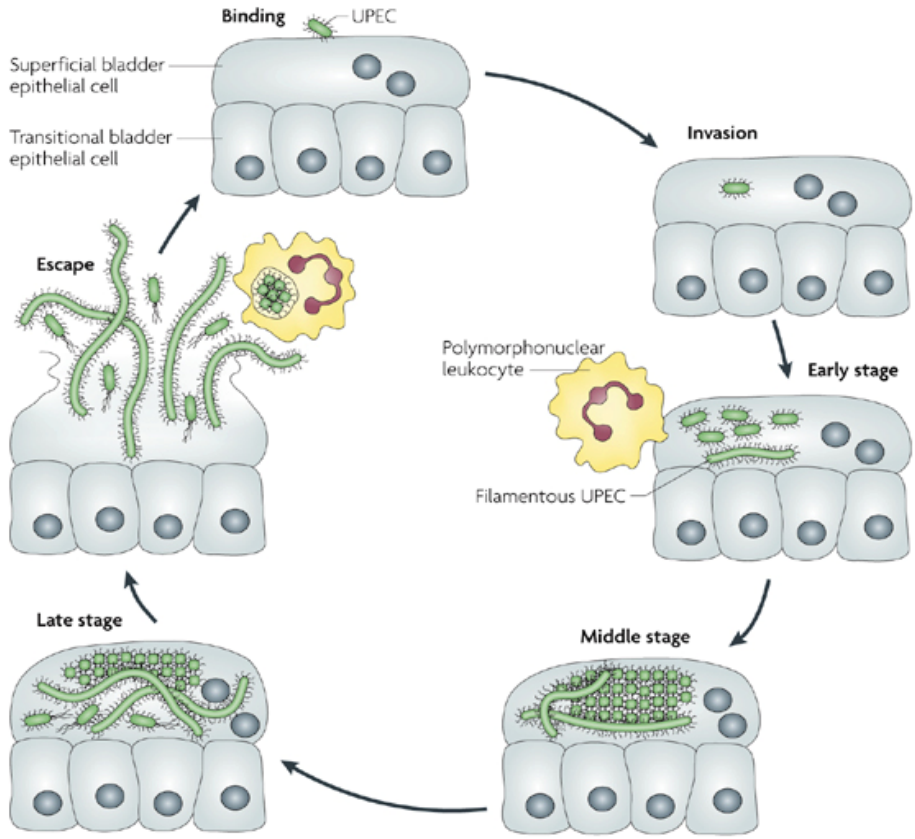
(2) The UPEC then invade the umbrella cells and can be detected in the cytoplasm.

(3) Following invasion the bacteria enter replication phase (150) that begins with the rapid growth of rod-shaped bacteria in an amorphous colony. Hultgren et al. have called these “Bacterial factories” (151). The bacteria exhibit a 30-minute doubling time during this period.

(4) After 6 to 8 hours the bacteria form organised collections of coccoid bacteria. Growth is slowed to a doubling time in excess of 45 minutes. Eventually these colonies come to occupy most of the cytoplasm (151).

(5) At the periphery of the colonies, some of the *E. coli* regain their rod shape, detach and swim in the cytoplasm. If the umbrella cell becomes perforated, these bacteria will swim out into the urine and attach to other uninfected umbrella cells. Hultgren et al. referred to this as “Bacterial fluxing” (151).

Figure 1.12. A murine model of UTI *taken from Hultgren et al 2009.*



In response, the urothelium attempts to eliminate the pathogens with a, bipartite, innate immune response (152). The first is mediated through Toll-like receptor 4 (TLR4) a transmembrane lipopolysaccharide receptor. Activation causes the release of antimicrobial peptides, inflammatory cytokines and chemokines, and co-stimulatory molecules that initiate the innate immune response to common Gram-negative bacteria. The signalling cascade is activated by UPEC lipopolysaccharides binding to TLR4 on the urothelium, which eventually recruits Polymorphonuclear Neutrophils (PMN). However, as a great deal of the bacterial load is intracellular, multiplication/growth can continue while evading the action of PMNs as well

as antibiotics and other defence mechanisms. The filamentous bacteria also appear to be resistant to phagocytosis by PMNs although the mechanism is unknown (150;153). Even if the umbrella cells exfoliate with their bacterial load, mobile, flagellated rods are released into the urine. These can then bind to and migrate into exposed transitional epithelial cells. Through this manoeuvre, a chronic infection can establish. It appears that the bacteria that are able to invade these transitional cells are biologically quiescent, as a superficial umbrella cell layer is able to re-establish itself (150;152).

In the murine model, chronic infection can serve as a nidus for recurrent infections that are resistant to short-term (10-day) treatment with trimethoprim-sulfamethoxazole (154). This recapitulates a common pattern seen in human infection, where very protracted courses of urinary antibiotic are required to clear pyuria and the associated symptoms.

Hultgren et al. have discovered that in their murine model, populations of *E. coli* can persist in the bladder for months on end, during which time they exist as a quiescent reservoir (151).

1.4.5 Evidence for bladder pathogens in Patients with LUTS without acute frequency and dysuria.

The diagnosis of OAB relies on the exclusion of infection using conventional methods; however, there are a few early publications which have sought bacterial pathogens using enhanced culture techniques, in patients with LUTS.

In 1988, Payne et al. studied 88 patients attending for urodynamic studies. 37 of 42 women with detrusor instability and 14 of 17 women with stress urinary incontinence showed evidence of infection with aerobic or fastidious bacteria before investigation (155). They suggested that urinary symptoms may be caused by infection, the urodynamic findings being secondary to this.

Maskell et al., in 1983, (156) conducted a two-year study of 51 women with chronic urinary symptoms. They showed a highly significant relation between high counts of fastidious organisms in the urine and the presence of symptoms.

1.4.6 Fastidious bacteria in the bladder

In the context of UTI, fastidious bacteria can be defined as those which require culture conditions other than overnight incubation in air on a primary isolation medium. Culture on additional media, or prolonged incubation in an atmosphere containing carbon dioxide or anaerobic conditions, may be necessary for their detection (156).

Before the practice of examining MSU samples and the application of the Kass threshold of greater than 10^5cfu ml^{-1} of a known urinary pathogen, as diagnostic of UTI, such organisms were found in catheter specimens of urine from patients with clinical evidence of UTI (157). The importance was then placed on aerobic incubation and high bacterial counts and techniques for detecting other organisms were no longer applied. Since that time, published studies of UTI refer only to the isolation of aerobes. Some suggest we reconsider the possible role of fastidious organisms in the pathogenesis of UTI (156).

Another reason for reconsidering the role of fastidious bacteria is the growing body of published evidence that such organisms can be isolated from the urine of many patients with LUTS and from patients with indicative symptoms but seemingly sterile pyuria. Such organisms require a reduced oxygen tension and are usually present, as natural commensals, occupying the sites where the aerobes that cause ascending UTI are also found. There is increasing acknowledgment that organisms, otherwise lacking pathogenicity,

may become secondary pathogens as a result of selection by antibacterial treatment or by other changes in the natural environment such as hormonal involution.

It has been shown that certain fastidious bacteria, such as *Streptococcus* spp., (158) *Gardnerella vaginalis*, (159) *Haemophilus influenzae* (160) some *Corynebacterium sp (161)* and *Lactobacillus* spp. *(162)* may be associated with infections of the bladder and upper urinary tract. These organisms have been isolated from urine collected by bladder catheterisation, by suprapubic aspiration or by ureteric catheterisation, and by culture of bladder biopsy tissue (159), from patients with objective evidence of bladder or kidney infection. They have also been isolated from bladder urine of symptom-free pregnant women who, however, later developed symptoms more often than those with sterile bladder urine (163;164). Maskell et al.(156) describe culture of *Lactobacillus spp*. in many of their study group. They assume pathogenicity of the organisms they have cultured, despite other literature that suggests *Lactobacillus spp* to be protective against colonisation of the urogenital tract with known urinary pathogens, particularly *E.coli* (89;165).

1.4.7 The role of Chlamydia and other sexually transmitted infections.

In men, the role of these organisms in the aetiology of urinary symptoms is well understood. They are implicated in urethritis and epididymitis. Evidence for chlamydial infection as a cause of urinary symptoms in women is conflicting. Stamm et al. (166) reported that chlamydial infection may be

associated with dysuria and frequency in young women. They did not, however, seek the presence of fastidious bacteria in the urine of these patients and they did not explain the presence of bladder pyuria. Others have failed to prove chlamydial infection in women with urinary symptoms (167). Maskell et al. (168), considering the question from the reverse direction, questioned women with proven chlamydial infection for the presence of urinary symptoms. 70% of them denied any such symptoms. Of the 30% who admitted to them, all had positive MSU cultures, two with *E. coli* and one with *G. vaginalis*. This supports the hypothesis that women with chlamydial infection and urinary symptoms may have more than one infection.

1.4.8 Evidence for an Inflammatory Response in the Overactive bladder

There are several clinical conditions, affecting patients, which are characterised by objective evidence of inflammatory disease, including pyuria, for which no aerobic causal organism is found. Furthermore, comprehensive explorations for causes of inflammation other than UTI are typically unsuccessful. These include interstitial cystitis, urethral syndrome and acute abacterial cystitis. Many patients with these conditions have pyuria; others have clinical evidence of inflammation, for example a tender prostate or paraurethral glands, as well as symptoms which are clinically identical to those of patients with aerobic pathogens and acute UTI. As we have seen,

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several studies have shown an inflammatory cytokine response in patients with Interstitial Cystitis.

Malone-Lee et al. (169) identified evidence of pyuria in 58% to 74% of patients presenting with overactive bladder symptoms, despite a negative routine urine culture. They suggested that an occult bacterial cystitis may be responsible for this inflammatory marker in the urine. They also showed that patients with pyuria had worse 24 hour frequency and incontinence in comparison to patients without pyuria.

Taking into account the studies described above, it is entirely plausible that OAB could in part be due to an occult chronic urine infection that has evaded detection. This may be due to inadequate microbiological techniques and intracellular colonisation of bacteria not detected because of the low sensitivity of current clinical tests.

1.5 Hypotheses to be tested

1. The presence of pyuria ≥ 10 wbc μl^{-1} in patients with OAB is associated with other evidence of an inflammatory response.
2. The traditional diagnostic methods deployed to exclude urinary infection in OAB patients are missing genuine infection.
3. OAB is associated with intracellular bacterial colonisation of uroepithelial cells by pathogenic bacteria

1.5.1 Aims of the project

1. To revalidate the routine clinical methods used to exclude infection in patients with OAB.
2. To discover whether patients with overactive bladder with pyuria harbour bacteria which are undetected in current clinical practice.
3. To discover whether any bacteria isolated from patients with OAB are intracellular bacteria parasitizing uroepithelial cells.
4. If such bacteria are present, to identify and quantify them.
5. To determine whether bacteria isolated from patients are able to invade uroepithelial cells.

Chapter 2

Patient recruitment and routine methods for analysis

2.1 Ethical Review

The study was approved by the Moorefield's and Whittington Hospitals Research Ethical Committees. (REC reference number 07/H0704/74).

2.2 Patient recruitment

Women and men newly presenting to the Incontinence Clinic at The Whittington Hospital were approached. They were characterised as having OAB by the use of a validated questionnaire (see appendix 1) (170). There are many validated questionnaires that can be used to characterise patients with OAB and express symptom severity such as the OAB-ss (171) , OAB-q (172),OAB-SCS (173) and the UPS (174). However, most of these scores were developed to look at symptom improvement during drug trials and some look at symptom bother and quality of life. These aspects of the disease were not relevant to my studies and for this reason I used a simple 10 point scale showing symptom severity.

A medical history was taken. Previous negative urine culture results, reported by the referrer, were used for purposes of diagnosis. Pregnant women were excluded from the study as were those with a known anatomical or functional abnormality of the urinary tract.

2.3 Patient data

Patient details and symptom questionnaires were recorded onto the hospital database “Artemis”, which is a secure database that is backed-up by the hospital IM&T department each day. It is defended by the NHS and Hospital Firewall.

The patient demographics and symptom scores are shown in table 2.1 for each study conducted.

Table 2.1 Patient demographics and symptom profile for patients used in the studies throughout this thesis.

Study	Chapter	Patient number	Male: Female ratio	Mean age patients (Sd)	Mean age controls (Sd)	Median frequency 24hrs patients (interquartile range)	Median urgency score patients (interquartile range)	Median Incontinence episodes 24hrs patients (interquartile range)	Comment
MSU vs CSU	2	40	0:40	60 (18)	N/A	9.5 (7-12)	2.5 (1-6.5)	1 (0-2)	
Dipstick analysis experiment 1	4	615	89:526	51 (19)	34 (11)	8 (6-11)	2 (0-5)	1(0-3)	
Dipstick analysis experiment 2	4	607	0:607	57 (18)	34 (11)	8 (5-11)	2 (0-5)	1 (0-2)	
Cytokine study	5	104	0:104	56 (17)	N/A	9 (6-11)	1 (1-1)	2 (0-5)	
Enhanced culture study	3	194	0:194	57 (18)	N/A	7.5 (5-12)	2.5 (1-5.5)	1 (0-5)	
Sediment culture study	3	89	0:89	56 (17)	29 (12)	10 (8-12)	3 (0-6)	0.5 (0-5)	
Bladder biopsy study	3	11	0:11	56 (19)	N/A	8 (5-11)	2.5 (0-5)	1.5 (0-6)	All patients used in this study were also part of the sediment culture study
Urothelial intracellular invasion study	6	23	0:23	56 (17)	29 (12)	7.8 (5-8)	2 (0-5)	2.5(0-5)	All patients in this study were also part of the sediment culture study
Urothelial intracellular invasion study- microscopy	6	24	0:24	56(17)	29 (12)	8.75 (5-9)	2 (1-6)	0.9(0-3)	All patients in this study were also part of the sediment culture study and urothelial intracellular invasion study

The methods outlined in this chapter were used throughout the project. Methods used for a specific chapter are outlined at the beginning of the relevant chapter.

2.4 Diagnosing Urine infection in clinical Practice

2.4.1 Urine microscopy

A disposable pipette was used to load a clean haemocytometer chamber with a sample (fig 2.1). This preparation was examined using an x20 objective with an x10 optical (magnification x200). The leukocyte count (wbc mm^{-3}) was enumerated by counting cells in five large squares and doubling the result. If a cell overlapped a ruled line engraving, it was counted "in" if it overlapped the top or right ruling; ignored as "out", if it overlapped the bottom or left ruling (fig 2.2).

Fig 2.1.A haemocytometer grid

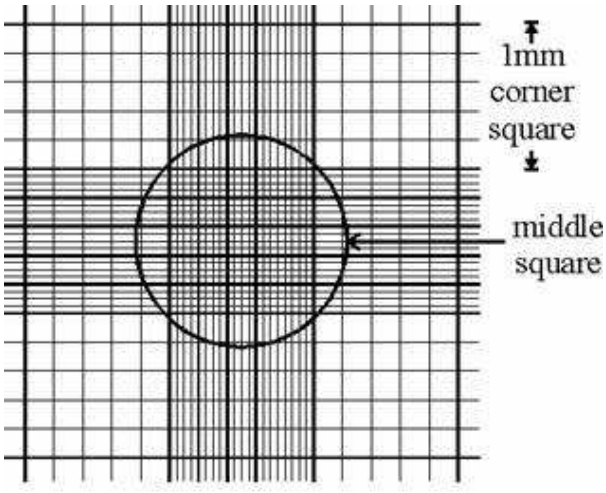
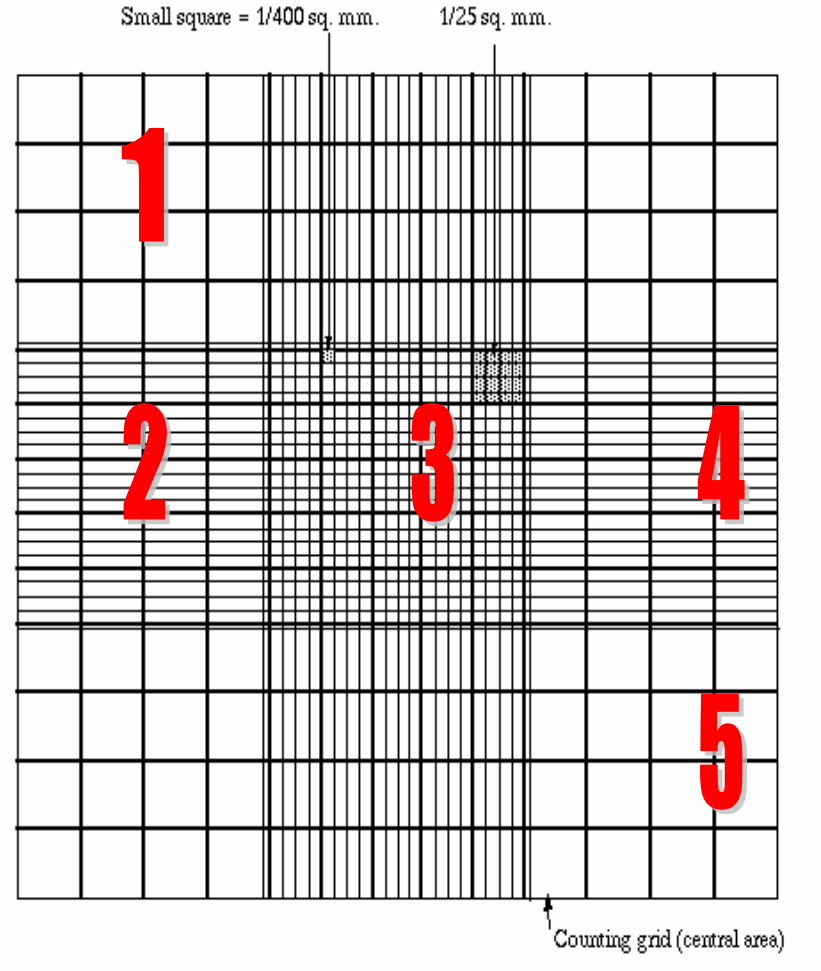


Figure 2.2 A haemocytometer grid. The numbers indicate the sequential order in which the large squares were viewed under the microscope (Adapted from Caprette 2000).



2.4.2 Urine Dipstick examination

Nitrite Test: The urine was dipped by a Multistix® 8 SG and read by a Clinitek Status colorimeter (Bayer Healthcare). The nitrite test pad sensitivity of the dipstick was stated to be 13-22 µmol/L (0.06-0.1 mg/dL) nitrite ions. Therefore if the urine sample contained nitrite ion in the range specified, the pad would turn pink indicating a positive result.

Leukocyte Esterase Test: The urine was dipped by a Multistix® 8 SG. The leukocyte esterase pad sensitivity of the dipstick was stated to be 15 wbc μL^{-1} when “trace positive” and this level was considered to be positive.

2.4.3 Urine Sampling

2.4.3.1 Mid-stream urine collection

Written or verbal instructions were given to the patient. Samples were obtained by the midstream clean-catch method. The patients began urinating into the toilet or urinal. After the urine had flowed for a few seconds, a sterile container was placed into the stream and approximately 60 ml was collected without interruption of flow (see appendix 2).

2.4.3.2 Obtaining a catheter specimen of urine

The procedure was explained to the patient and verbal consent was obtained. Hands were washed using soap and water. Disposable gloves were worn to minimise the risk of cross-infection. The perineum was wiped with sterile normal saline solution. A Lofric (in and out) size 12 Fr catheter was inserted into the urinary bladder under aseptic conditions with a “no touch technique”. The urine was collected into a sterile container. The urine was transferred into a sterile 20ml container which was labelled with the patients details. Hands were washed using soap and water.

2.5 What is the best method of sampling urine from female patients?

2.5.1 Hypothesis

Clean-catch MSU samples are unacceptably contaminated by extrinsic white cells when compared to samples collected by the catheter specimen of urine (CSU) method.

2.5.2 Background

The diagnosis of UTI is based on urine sampling, which requires a high quality specimen, free of vaginal, perineal or faecal contamination with organisms or inflammatory cells.

Urine specimens from women are particularly at risk of contamination due to anatomical factors; proximity of urethral meatus to the vulva and labia and due to compliance factors in collecting the sample.

Urine can be sampled by suprapubic aspiration, catheter insertion or mid-stream urine collection. Contamination by vaginal, perineal and faecal flora is inherent in MSU sampling. Contamination rates as high as 30% have been reported (175) (176) (177).

The role of bacterial infection in the aetiology of the overactive bladder

Most sample collections are unsupervised and although instructions on collection can be given to patients to reduce contamination, this is difficult to achieve in a busy, unfamiliar clinical environment. In addition, physical constraints such as old age or urinary pathology such as overactive bladder can make compliance difficult (175;176). It has also been found that conventional sampling of the MSU with additional cleansing procedures of the perineum does not significantly alter culture outcomes or levels of contamination (178) (179).

The presence of true infection can be confirmed by urethral catheterisation or suprapubic aspiration. Although suprapubic aspiration is free of contaminating organisms, it is an invasive procedure and not suitable for routine diagnosis. A novel device, which collects a MSU sample by excluding the initial low-flow portion of the urinary stream and with no interruption of flow, has been studied and shown to reduce contamination rates in urine samples. However, this device is not yet commercially available (180).

In order to collect a sample that is of sufficient quality to be studied with confidence, particularly without contamination by vaginal cells, a catheter specimen of urine is the preferred option. This delivers a specimen approaching the quality of a suprapubic aspiration (181). The method is remarkably well tolerated in women and proves painless.

As I was to investigate patients with OAB symptoms and pyuria, the aim was to obtain a urine specimen which was uncontaminated by skin and vaginal

white cells and pathogens. One method of investigating any potential contamination was to compare a MSU specimen with a catheter specimen (CSU) in the same patient for white cells.

2.5.3 Methods

40 newly presenting, female patients with symptoms of OAB were recruited and gave consent. The patient demographics are described in table 2.1. They provided an MSU sample and up to three days later, during which time they went untreated, they provided a CSU. The sequence was used to avoid CSU introduction of new infection. The sample was analysed immediately on collection. The leucocyte count (wbc μL^{-1}) was enumerated by microscopy (2.4.1) The investigators were blinded to the previous results.

2.5.4 Statistics

Two statistical tests were used. In the first, the null hypothesis was that the proportion of pyuria positive samples was identical whether collection was by MSU or CSU. A clinically significant difference in proportions was estimated as 0.33 (specifically, 0.66 versus 0.33). A sample size of 40 pairs of data was calculated to have power of 85.3% to yield a statistically significant result. The data were collated and analysed. The between method differences in proportions of positive (pyuria) and negative (no pyuria) samples were assessed by the Chi squared test. The between method differences in white cell counts were compared by the paired t-test. In the second, in agreement with the current literature, it was assumed that the CSU is a superior method

of sampling and so the sensitivity and specificity of the MSU was calculated using the CSU as the gold standard test.

2.5.5 Results

40 women were recruited. Their mean age was 60 (sd=18). The patient demographics are described in table 2.1. The results from the different sampling methods are illustrated in table 2.2

Table 2.2 The comparison of pyuria detection between sampling by midstream urine collection (MSU) and catheter specimen (CSU) collection in the same patient

Method of sampling	No pyuria detected	*Pyuria detected*
MSU	3	*37*
CSU	*24*	*16*

37 (93%) of the MSU samples showed pyuria but only 16 (40%) of CSU samples ($X2 = 25$, $df=1$, $p<0.001$). The differences in absolute white blood cell count between sampling methods is illustrated in Figure 2.3.

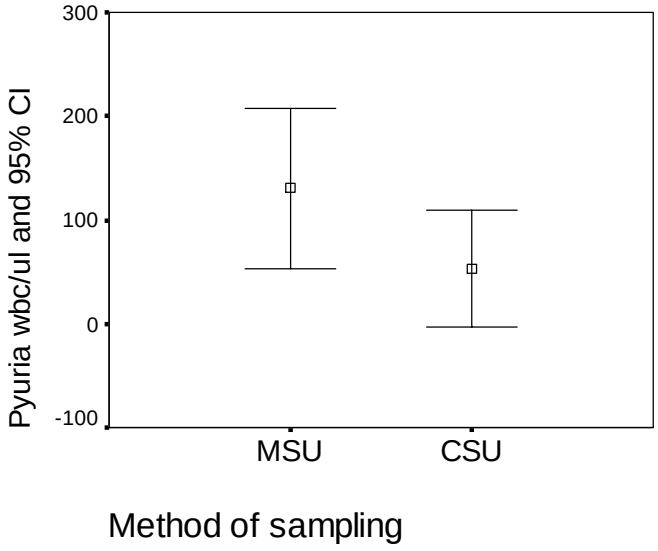
If the CSU should be treated as the gold standard reference for the analysis of pyuria, then the sensitivity of the MSU is 1 (95% CI = 0.7 to 1.0) but the specificity is 0.1 (95% CI = 0.04 to 0.4). See Table 2.3.

Table 2.3 The comparison of pyuria detection between sampling by midstream urine collection (MSU) and catheter specimen (CSU) collection in the same patient, showing the sensitivity and specificity of MSU when compared to CSU

	CSU Neg	CSU Pos	Totals
MSU Pos	21	16	37
MSU Neg	3	0	3
Totals	24	16	40
Sensitivity 1 (95% CI 0.75-1), Specificity 0.081(95% CI 0.037-0.37)			

These data imply substantial contamination in the MSU.

Figure 2.3 Comparison of absolute pyuria counts obtained from midstream urine (MSU) and Catheter urine (CSU) samples obtained from the same patients.



There was a significant difference in mean pyuria count between the two groups with higher counts evident from MSU samples (95% CI of difference = 18 to 144, p=0.013, t=2.6).

2.5.6 Discussion

These data support the hypothesis that MSU samples may be contaminated by extraneous white blood cells, possibly from the vagina. This could lead to over-diagnosis of urinary infection and studies that involve the analysis of pyuria in association with symptoms must take this into account. This was a blinded study but the method did not randomise the sampling because of concerns that a catheterisation of the bladder might introduce infection or perturb the bladder in some way. It may be that the difference in counts could be explained by spontaneous resolution during the time between sampling. Given the numbers, it is not probable but nevertheless, despite the risk of catheter contamination, these data will require verification in a trial using a randomised order for sampling. These data suggest that in the case of women, a CSU is a much more reliable sampling method for enumerating pyuria and potentially avoiding contamination from bacteria and cells also from the vagina.

2.6 Routine Laboratory Urine Culture

An aliquot of all CSU specimens collected was sent to the Whittington Hospital NHS Trust microbiology laboratory, as is routinely done, for urine microscopy, culture and sensitivity. The sampled urine was treated fresh, or after overnight storage at 4° C at the hospital laboratory. 1µl of unspun urine was transferred by loop to a chromogenic media, CPS ID2 (bioMerieux). The plate was incubated aerobically for 24 hours at 37°C. Bacterial colonies were identified by colour change and size as below. The result was taken as positive if greater than 10^5 colonies were generated per ml after 18-24 hours culture.

2.6.1 Chromogenic Agar

Some of the organisms found in UTI produce enzymes either for the metabolism of lactose or glucosides or both. Other organisms produce none of these enzymes. For example, *E.coli* contains enzymes for lactose metabolism but is β-glucosidase negative. These enzymes can be used to identify microorganism phenotype in culture.

The principle of this medium is the use of chromogenic substrates revealing metabolic enzymes. The medium is composed of peptone, yeast extracts and agar and a special chromogenic mixture. It enables the presumptive identification of *E. coli, Enterococci, Klebsiella-Enterobacter group*, and *Proteus group*.

Table 2.3 Bacterial identification using chromogenic agar according to colour change

SPECIES COLONY	COLOUR SENSITIVITY
E.coli	Red
Klebsiella spp, Citrobacter spp	Metallic blue
Enterococcus spp	Turquoise blue
Proteus mirabilis	Clear with brown halo
Staphyloccus saprophyticus	Pink opaque
Staphylococcus aureus	Colourless opaque
Candida spp	Creamy

2.7 Bladder biopsy

Well characterised, suitable patients with refractory OAB symptoms were provided with an information leaflet summarising the cystoscopy procedure and bladder biopsy (see appendix 3). Patients were consented for the procedure on a follow up visit. All cystoscopies were performed under sterile conditions in the main theatre complex at the Whittington Hospital NHS trust. The perineum of patients was cleaned with Iodine solution and all patients were draped with sterile drapes. A flexible cystoscope or size18 rigid cystoscope was used. Two biopsies were taken from the dome of the bladder and immediately placed in sterile normal saline or gluteraldehyde solution for

routine pathology. The biopsy placed in the normal saline was transported to the Eastman Dental Institute.

2.8 Control urine catheter specimens

Female patients attending The Whittington Hospital for day surgery procedures under the care of the Gynaecology department were approached. Any patient requiring the routine emptying of the bladder during surgery was asked to fill out a bladder symptom questionnaire (appendix 1). Patients with any lower urinary tract symptoms were excluded. Patients without symptoms were asked to donate urine removed during the procedure. Typically, patients attending for a laparoscopy gave permission for the use of their urine specimen. The specimen was collected as above (2.4.3.2) prior to their surgery. The urine was collected into a sterile container and patient details were entered into the Artemis database (2.3). An aliquot of urine was subject to microscopy, dipstick, and routine culture and enhanced culture as above (2.4.1, 2.4.2, 2.6, 2.9.1). An aliquot of urine was transported to the Eastman Dental Institute and subject to sediment culture (2.9.2)

2.9 Laboratory Methods- Culture Techniques

2.9.1 Enhanced Culture Technique

The urine sample was mixed by gentle inversion and the top of the container was removed aseptically. With a calibrated sterile 200µL pipette, urine was dropped onto the edge of the ChromID plate (CPS Biomerieux).The inoculum was spread over the entire surface of the plate with a spreading spatula taking care to avoid the edges of the plate. In a similar way, a second aliquot of specimen was inoculated onto a blood agar plate (Oxoid). The blood agar plates were placed into anaerobic jars (AnaeroGen, Oxoid) and the manufacturer's instructions were followed to generate the anaerobic environment using a gas pack. The ChromID plates were transferred to racks and transported to together with the "gassed out" anaerobic jars to the microbiology laboratory at the Whittington Hospital. The chrome plates were incubated aerobically at 35-37°C for 18 hours. The blood agar plates were incubated anaerobically for 5 days at 35-37°C. The colonies were counted and identified visually.

2.9.2 Sediment Culture

The samples were transported to the Eastman Dental Institute from The Whittington. The volume of the urine was recorded. The entire volume of the

urine was centrifuged at 8000rcf for 5 minutes at room temperature using an Eppendorf 5804R centrifuge. The supernatant was removed gently and the sediment was kept. The sediment was re-suspended in 400µL of normal saline. Serial dilutions were performed to 10^{-3} and 100µL of each solution (50µL on each half) was placed on a Columbia Blood Agar (CBA) plate (E&O laboratories) and Fastidious Anaerobic Agar (FAA) plate (E&O laboratories). 10µL of the neat solution was placed on a double frosted slide for Gram stain. The CBA plates were placed in a CO_2 incubator at 37°C for 48hrs. The FAA plates were placed in an anaerobic incubator at 37°C for 7 days.

2.9.3 Colony counts and sub-culturing

After the respective incubation periods, colony descriptions were recorded following the colony description guide as below. The colonies were counted and recorded. A single colony of each micro-organism was streaked onto a fresh CBA plate or FAA plate and incubated as above. The plates were examined after the incubation periods. If the culture appeared to be a pure growth, biochemical identification tests were carried out as below (3.2). Subcultures were repeated until a pure culture was obtained.

2.9.4 Colony Description

Taken from "Western Michigan University Microbiology Lab Procedures"

http://homepages.wmich.edu/~rossbach/bios312/LabProcedures.html

Size: A ruler was held under a plate and used to measure the diameter of several representative colonies; these measurements were then averaged and recorded.

General Shape: The shape of a colony was described as round, irregular and spreading, filamentous, rhizoid or curled when viewed from above

Margin: The margin was observed from above and represented the outside edge of the general shape. The margin was described as entire, undulate lobate or filamentous

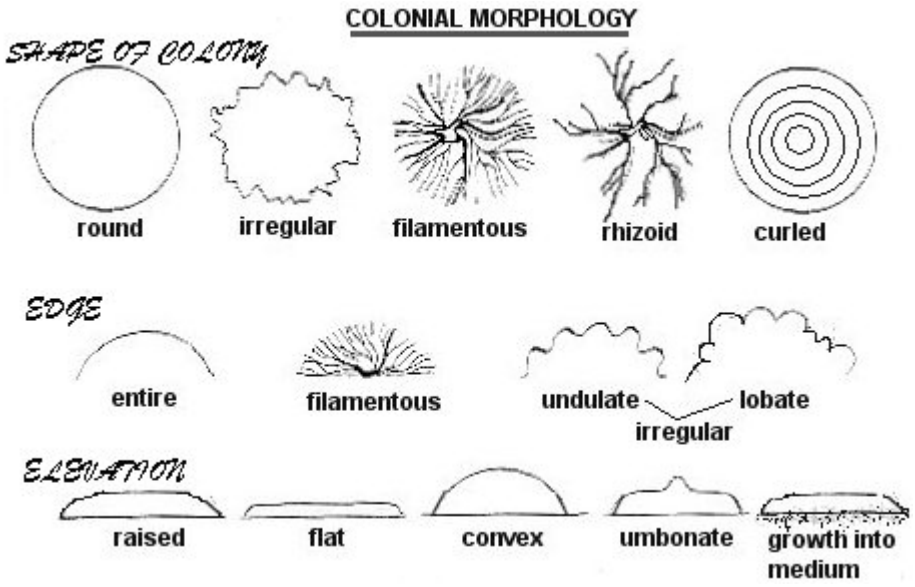
Elevation: The elevation of a colony was observed from the side of the plate and was characterized in the following ways; effuse (like water on a flat surface), flat (discrete colony with a flat top), convex (water on wax paper), umbonate (with a nipple in the centre), or umbilicate (sunken centre)

Surface: The surface of the colony was observed from the top and was described as smooth, filamentous, powdery, wrinkled or ringed.

Density: If the colony was opaque or translucent

Pigment: (1) Non pigmented colonies were described as colourless, white, or off-white, (2) Non-diffusible pigment described the colony as pigmented, while (3) Diffusible soluble pigment coloured the agar around the colony

Figure 2.4 Colony Morphology



2.10 Methods- Biochemical Identification Tests

2.10.1 Catalase Test

Using a sterile disposable loop, growth from the centre of a colony was transferred to a clean glass slide. 20µL of 3% hydrogen peroxide (Sigma) was added to the colony. The sample was observed for the production of bubbles. A sustained appearance of effervescence was reported as positive.

2.10.2 Oxidase Test

A solution of tetramethyl-p-phenylenediamine (Sigma) was prepared in 1ml of sterile distilled water. A sterile swab was placed into the solution and soaked. The cotton bud was then touched onto the surface of the test colony. A positive reaction was taken as the development of a purple colour within 20 seconds. *Pseudomonas. aerogenosa* (NCTC 10662) was used as a positive control.

2.10.3 Carbohydrate Oxidation- Fermentation Tests

To determine the products of sugar fermentation, a carbohydrate fermentation medium was prepared at pH 6.6-7.0. This medium contained three essential ingredients: glucose, nutrient medium, and the pH indicator bromothymol

blue. The test organisms were inoculated into 20ml sterile tubes containing 10ml of the medium by stabbing the centre of the medium to greater than half the depth with a sterile loop. The medium was incubated at 37°C for 48 hours. A duplicate tube was inoculated with the same organism and covered with 3ml of paraffin oil to create an anaerobic environment. A bright yellow colour indicated the production of enough acid products from oxidation/ fermentation of the sugar to drop the pH (182). *E. coli* (NCTC 10418) was used as a positive control strain.

2.10.4 Bacterial Motility

A motility medium was prepared containing brain heart infusion broth, gelatine, potassium nitrate and agar. 20ml sterile tubes containing 10ml of the agar were inoculated with a pure culture by stabbing the centre of the column of the medium to greater than half the depth. The tubes were incubated for 24-48 hours at 37°C in an aerobic atmosphere. After incubation, the tubes were observed for growth in relation to the stab line. Non-motile organisms grew only along the line of inoculation, while motile organisms spread out from the line of inoculation. *Pseudomonas. aeroginosa* (NCTC 10662) was used as a positive control.

2.10.5 Gram stain

20µl of the neat sediment solution was placed on a glass slide. In addition, a single pure colony was picked with a sterile loop and mixed on a glass slide with 20µl of PBS. The solutions were allowed to dry in air. Crystal violet stain (Sigma) was added over the slide. This was allowed to stand for 30 seconds. The slide was then gently rinsed with a stream of water. Iodine solution (Sigma) was added on the smear, enough to cover the slide. This was allowed to stand for 30 seconds. The iodine solution was rinsed off the slide with running water. A few drops of acetone (Sigma) were added so the solution trickled down the slide and rinsed off with water after 5 seconds. A counter stain of 1% neutral red solution (Sigma) was added for 30 seconds. The solution was washed off with water. The slide was air-dried after blotting off excess water.

2.10.6 Identification based on biochemical Tests

Identification was carried out using standard tables from Cowan and Steel's Manual for the Identification of Medical Bacteria, third edition (183).

2.11 Laboratory methods-16s rRNA gene typing

After biochemical microbiological identification techniques had been carried out, DNA was extracted from the bacterial isolates, which was used as a template to amplify the 16S rRNA gene using global primers (27f and 1492r, Genosys, Sigma, UK) via the polymerase chain reaction. Sequences were analysed using the Basic Local Alignment Search Tool (BLAST). DNA was submitted for sequencing at the Cambridge University DNA sequencing facility. They carry out sequencing on an Applied Biosystems 3730xl DNA Analyser.

2.11.1 Extraction of genomic DNA

The DNA extraction kit was obtained from Invitrogen. A single bacterial colony from a fresh culture was suspended in 200µL of phosphate buffered saline (PBS). The sample was centrifuged at 3000rcf for 5 minutes. The supernatant was expelled and the pellet obtained re-suspended in 200µl PBS. 5µl of Lysozyme was added and mixed using a vortex. The specimen was incubated at 37°C for 15 minutes to digest the cell walls. 200µl of binding buffer (guanidinium hydrochloride) was added to increase DNA binding. 40µl of proteinase K was added and the sample was then incubated at 70°C for 15 minutes to digest protein. In addition, proteinase K rapidly inactivates nucleases that might degrade the DNA. 100µl of propan-2-ol was added to the sample and mixed by vortexing to precipitate the DNA. The whole mixture

was then applied to a filter tube provided by Invitrogen. Nucleic acids bind selectively to glass fibres pre-packed in the filter tube. After centrifuging the sample at 8000rcf for 1 min, the supernatant was discarded and 500µl of inhibitor removal buffer was added. The sample was centrifuged again at 8000rcf for one minute and the supernatant discarded. 500µl wash buffer was then added and the sample centrifuged again at 8000rcf for one minute. The supernatant was discarded and this step was repeated. The filter tube was then applied to a 1.5ml Eppendorf tube and 200µl of elution buffer was added to the filter tube. This releases the DNA from the glass fibres. The sample was then centrifuged at 8000rcf for 1 minute and the supernatant containing the eluted DNA was stored at 4°C until used.

2.11.2 Polymerase Chain Reaction (PCR) protocol

The total reaction volume was determined as 50µl. This provided the basis for the calculations concerning buffer solution (1X concentration), $MgCl_2$ (2.5mmol), deoxynucleoside triphosphates (dNTP's) (200µM) and DNA polymerase enzyme used; Taq polymerase (1.5µl per reaction). The 16S gene primers used for the PCR reactions were 27f (5' AGAGTTTGATCMTGGCTCAG 3' and 1492r 5' TACGGYTACCTTGTTACGACTT 3') (184). The PCR programme, using the Biometra T3000 Thermocycler, was as follows: 94ºC for five minutes to ensure denaturing of the template DNA. This was followed by 29 cycles of 94 ºC for 1 minute, 54 ºC for 1 minute and 72ºC for 1minute 30 seconds. After cycling, an extension of 72 ºC for 5 minutes was followed by cooling to 4ºC

until the samples were analysed. Standard techniques were used for agarose gel electrophoresis and PCR purification(184). The supernatant containing the purified DNA was stored at 4°C until used. 10µL of the purified DNA was sent for sequence analysis.

2.11.3 Storage of bacterial isolates

Bacterial isolates were inoculated into Brain Heart Infusion (BHI, Oxoid Ltd.) broth. Sterile glycerol (BDH Chemicals, Poole, UK) was added to the broth to give a final concentration of 10% glycerol. One ml aliquots were then stored at -70°C as frozen glycerol 10% v/v) stocks.

Chapter 3

Using an enhanced culture method to detect urinary infection in patients with overactive bladder and the use of urine sediment culture and bladder biopsy culture to enhance the detection of bacteria in patients with OAB

3.1 Hypotheses

This study tested two hypotheses and this chapter is therefore split into two parts.

1. The first hypothesis tested was that a more inclusive culture method and a diagnostic threshold of 10^2 cfu ml^{-1} would demonstrate more infections than routine clinical laboratory methods applying the 10^5 cfu ml^{-1} criterion.
2. The second hypothesis tested was that OAB is associated with intracellular bacterial colonisation of uroepithelial cells by pathogenic bacteria.

3.2 First hypothesis

3.2.1 Methods

Female patients with symptoms of OAB gave their consent to participate. Their symptoms were recorded using a validated questionnaire (appendix 1) and their antibiotic consumption noted. A CSU was obtained by inserting a Lofric 12 Fr catheter into the urinary bladder under aseptic conditions (2.4.3.2). The urine was collected into a sterile container and processed immediately as per 2.9.1. An aliquot of each CSU also underwent routine analysis at the threshold of 10^5 cfu ml^{-1} (2.6). An aliquot of urine was also taken for enumeration of white blood cells using a light microscope and haemocytometer (2.4.1).

3.2.2 Statistics

Because I wished to include patients taking antibiotics, it was estimated that a clinically significant effect would be the observation of a positive diagnostic bacterial culture in 10% of routinely processed samples and 20% of those using the study method. It was calculated that a sample size giving 400 pairs would have a power of 97.8% to yield a statistically significant result.

3.2.3 Results

194 women with OAB symptoms were recruited with a mean age of 57 (sd=18). They yielded 378 urine samples collected by CSU. Some women were sampled twice according to their attendance at clinic during the study period. The patient demographics are described in table 2.1.

The routine laboratory cultures reported positive results in 46 (12%) samples whereas the study culture methods isolated bacteria in 114 (30%) samples ($X2=100$, $df=1$, $p<0.001$, 95% CI of difference 17% to 26%). Figure 3.1 describes the pyuria counts according to culture result for the two methods. The study method identified bacteria in 43 (94%) samples positive on routine culture. More Significantly, 71 (21%) of those positive with enhanced culture did not grow bacteria with the established laboratory method. The concomitant consumption of antibiotics had no effect on these differences. The different bacteria cultured are shown in figure 3.2. Four per cent of

patients grew anaerobic bacteria. The prominent bacteria in the OAB patients were *E.coli*, *Enterococcus sp*., *Pseudomonas sp* and *Streptococcus sp.*

Figure 3.1 The mean white cell count and 95% CI comparing the routine culture results and study culture results

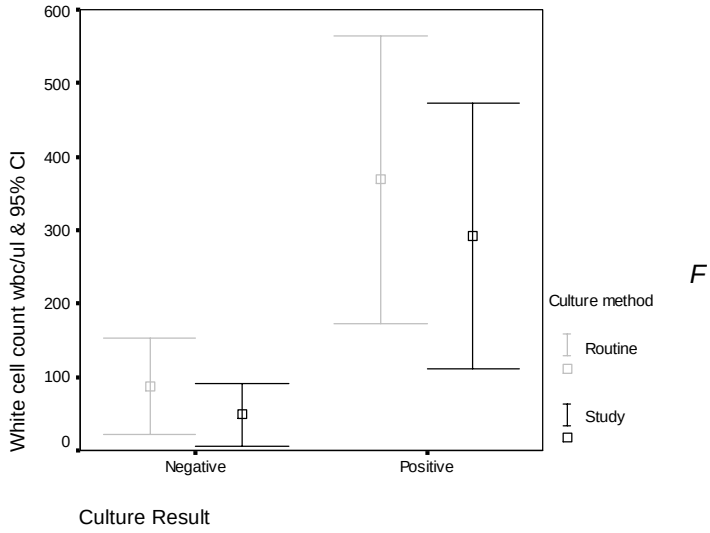


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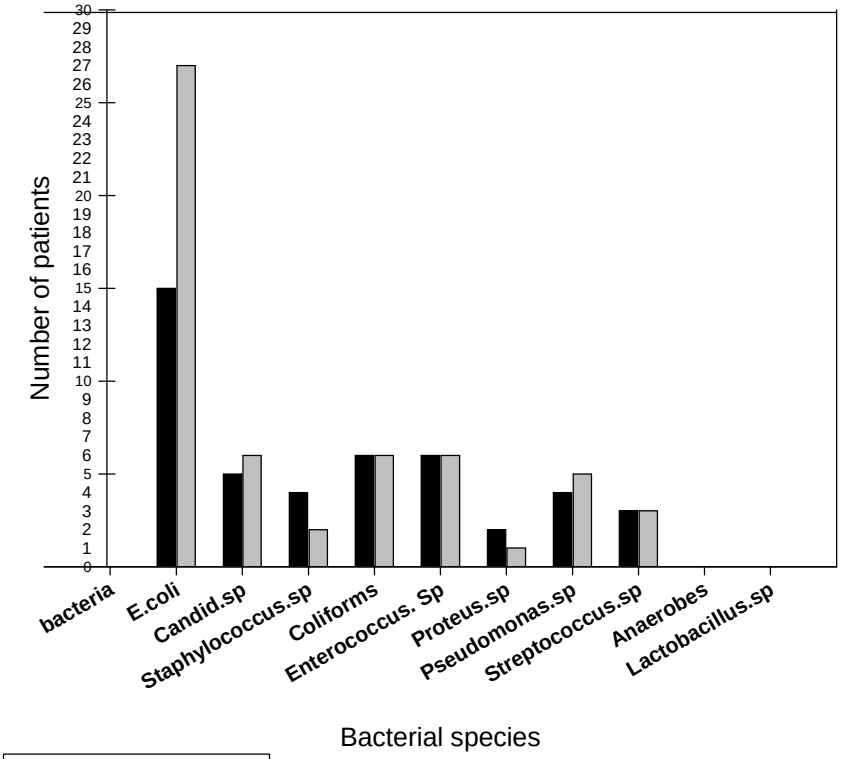
Figure 3.2 The Prominent bacteria found in CSU cultures



3.2.4 Discussion

These data demonstrate a more than doubling of the bacterial isolation rate when a more meticulous culture method is deployed. By using CSU sample, I could be confident that the isolates originate from the bladder. Figure 3.1 illustrates the fact that the differences between the methods occurred over the whole spectrum of the inflammatory response and was not limited to the most symptomatic. These data imply that the misgivings about the validity of routine culture methods apply to patients with symptoms of OAB. The routine methods for culturing urine to detect $\geq 10^5$ cfu ml^{-1}, when applied to patients with symptoms of OAB, may be failing to identify a significant proportion of patients with genuine urine infection. These data are supported by the study described in chapter 4 in which, in a similar cohort of patients, a positive culture result using the MSU (10^5cfu/ml^{-1}) method was 15%, using a CSU specimen (10^5cfu/ml^{-1}) the positive result was 20% and using a CSU (10^2cfu/ml^{-1}) the positive result was 29%. Thus, by using a MSU, we are potentially missing 5% of UTI's and a further 9% of patients by using a threshold of 10^5cfu/ml^{-1}. These data suggest that the laboratory diagnosis of UTI should be reviewed especially in the context of patients with chronic lower urinary tract symptoms such as OAB.

The findings of this study prompted me to develop a method of culturing urine to maximise the growth of any pathogenic bacteria and the second hypothesis was studied.

3.3 Second Hypothesis

3.3.1 Background

This study examined the association between OAB and intracellular bacterial colonisation of uroepithelial cells by pathogenic bacteria.

If the hypothesis is correct then culture of the urinary sediment, containing the shed uroepithelium, and bladder biopsies taken from OAB patients and normal controls would yield a greater number and, possibly, species, of bacteria than the routine urine culture currently conducted in the NHS which seeks a single dominant species of bacteria at 1 $x10^5$ cfu/ml^{-1} as a positive result.

Currently, there are no human studies in the literature to suggest methods of culturing the urinary sediment and so the study described below uses two pilot methods, the purpose of which was to allow the detection of bacteria in a urine sample and bladder biopsy by collecting the uroepithelial cells, hence culturing bacteria attached to and invading these cells. *Gatoria et al.* (185) describe a method of culturing the urinary sediment and bladder wall biopsies in samples taken from dogs. They centrifuged 5mls of collected urine and discarded the supernatant. They then used a loopful of the sediment collected for culture. Bladder biopsy tissue was first macerated and then cultured. The methods below are adapted from this study. In addition to the culture methods, a duplicate bladder biopsy taken from patients was sent for

histological examination by pathologists at the Whittington Hospital NHS Trust. This was only to confirm the presence of inflammation in the bladder and to exclude any malignancy as a cause of any of the changes seen in the bladder tissue.

3.3.2 Methods

89 women were recruited. The urine sediment was collected and plated on Columbia blood agar (CBA) and Fastidious anaerobic agar (FAA) and incubated both aerobically and anaerobically (2.9.2). A duplicate aliquot of urine was sent for routine culture at the Whittington Hospital microbiology laboratory, with an infection threshold of 10^5 cfu/ml (2.6). Bacteria were identified using biochemical tests and 16S rRNA gene typing (2.10-2.11).

An aliquot of unspun urine was examined immediately by microscopy using a haemocytometer and the urinary white cells evaluated (2.4.1.)

3.3.2.1 Bladder biopsy

Patients with Overactive bladder symptoms were provided with an information leaflet summarising the cystoscopy procedure and bladder biopsy (See appendix 3). The biopsy was taken as per section 2.7.

3.3.2.2 Bacterial Retrieval from bladder biopsies

Patients with OAB symptoms undergoing cystoscopy for investigation of their condition or for treatment of their condition with intravesical Botulinum toxin A (Botox A) consented to take part. The patients were characterised by using a validated questionnaire (appendix 1). The biopsies were obtained as described in section 2.7.

Each biopsy measured up to 1mm in length. The biopsy was placed in 400µl of normal saline (neat solution) and gently macerated with a sterile plastic pestle to break up the biopsy tissue. Serial dilutions tenfold, one hundred fold and one thousand fold were then prepared and 100µL of each dilution (50µL on each half) spread onto a CBA plate (E&O laboratories) and FAA plate (E&O laboratories). 10µL of the neat suspension was placed on a double frosted slide for Gram stain. The CBA plates were incubated at 37°C in 5% CO_2 for 48hrs. The FAA plates were placed in an anaerobic incubator at 37°C for 7 days. Colony counts and sub-culturing was carried out as per 2.9.3-2.9.4). A second identical biopsy was taken from the same site for histological

analysis at the Whittington hospital NHS Trust histopathology department. There were no biopsies taken from control volunteers.

3.3.3 Statistics

There were no data from previous studies to assist in a sample size calculation. The experiment was therefore conducted to capacity. The analysis performed explored the difference in total bacterial count (cfu/ml^{-1}) and largest bacterial count (cfu/ml^{-1}) of the most abundant bacteria between patients and volunteers.

The power to test the null hypothesis that the population means were equal was calculated. The criterion for statistical significance (alpha) was set at 0.050. The test was 2-tailed, meaning that an effect in either direction would be interpreted.

The statistical analysis of the data was conducted using one-way analyses of variance (ANOVA). The statistical program SPSS was used for analysing data and calculating (ANOVA) and 95% confidence intervals. Where the data were the average of means the standard errors of the means were used to construct distribution bars. In all other cases the distribution bars were the 95% CI.

3.3.4 Results

89 women provided specimens, 63 had OAB symptoms and provided a CSU mean age 56 (sd=17); 26 asymptomatic female controls, average age 29 (sd=12), of which 11 provided a MSU and 15 a CSU. The symptom profiles are described in table 2.1 Both MSU and CSU was used in this study despite previous reports of higher contamination rates to compare and validate the new culture method.

3.3.4.1 Urine culture results

The routine cultures at 10^5cfu ml^{-1} were positive in 7 of the 63 patients (11%); all of these (100%) had pyuria (>10 white blood cells per ml). The enhanced culture at 10^2cfu ml^{-1} was positive in 12 of the 63 patients (19%); 11 of these (92%) had pyuria. 7 patients proved positive on both cultures and all of these had pyuria.

2 out of 26 controls (8%) grew *E. coli* in both cultures, the others were found to be negative. These were both MSU specimens. One of the control volunteers had pyuria. Again, this was in an MSU specimen. In contrast, the spun sediment culture grew bacteria in all samples, with differences in colony counts related to OAB and to pyuria (F= 9.4, P=0.003): (fig 3.3 -3.5). Figure 3.3 compares the mean total colony counts cfu/ ml^{-1} between the patient groups and normal volunteer groups, showing the SEM. This graph shows a significant difference between the two groups in mean total bacterial count. Figure 3.4 below shows the total bacterial counts in cfu/ml^{-1} in the different

patient and normal volunteer groups. It shows a higher total bacterial count in both the patient groups (OAB patients with and without pyuria) when compared to the normal volunteer groups in samples taken by both CSU and MSU methods. OAB patients also grew a significantly higher count of the single most abundant colony type compared to the control volunteers (F= 4.664, df= 2, P= 0.012), (fig 3.5). The single most abundant colony type was also different in the different groups.

465 bacterial isolates were sub-cultured and purified from the sediment culture and subjected to biochemical tests and sequence analysis as described in section 2.10-2.11. 12 isolates were not identified by sequence analysis because of no growth on sub-culture.

The normal volunteers (N=26) grew between 1-4 $x10^3$ cfu ml^{-1}; there was no significant difference between MSU and CSU collected samples. OAB patients without pyuria (N= 40) grew $1x10^6$ cfu ml^{-1}. OAB patients with pyuria (N=23) grew $8x10^6$ cfu ml^{-1}.

In the OAB patients the dominant bacterial isolates were *Streptococcus spp* (18% of bacteria found), *Lactobacillus spp* (15% of bacteria found) and *Enterococcus spp* (10% of bacteria found) (fig.3.4).

The dominant bacteria found in the catheter control group (30% of bacteria found) were *Lactobacillus spp.* (fig 3.7) and the dominant isolates in the MSU volunteers were *Staphylococcus spp* (30% of bacteria found) (fig 3.8). Other prominent control isolates were *Enterococcus spp.* (13% of bacteria found)

and *Streptococcus spp* (11% of bacteria found) and *Corynebacterium spp.* (8% of bacteria found) in catheter control volunteers and *Corynebacterium spp. (*20% of bacteria found*), Streptococcus spp* (16% of bacteria found) and *E.coli* (11% of bacteria found) in MSU volunteers.

Figure 3.3 Log mean total bacterial colony count and SEM comparing OAB patients (N=63) with asymptomatic controls (N=26)culturing the urinary sediment.

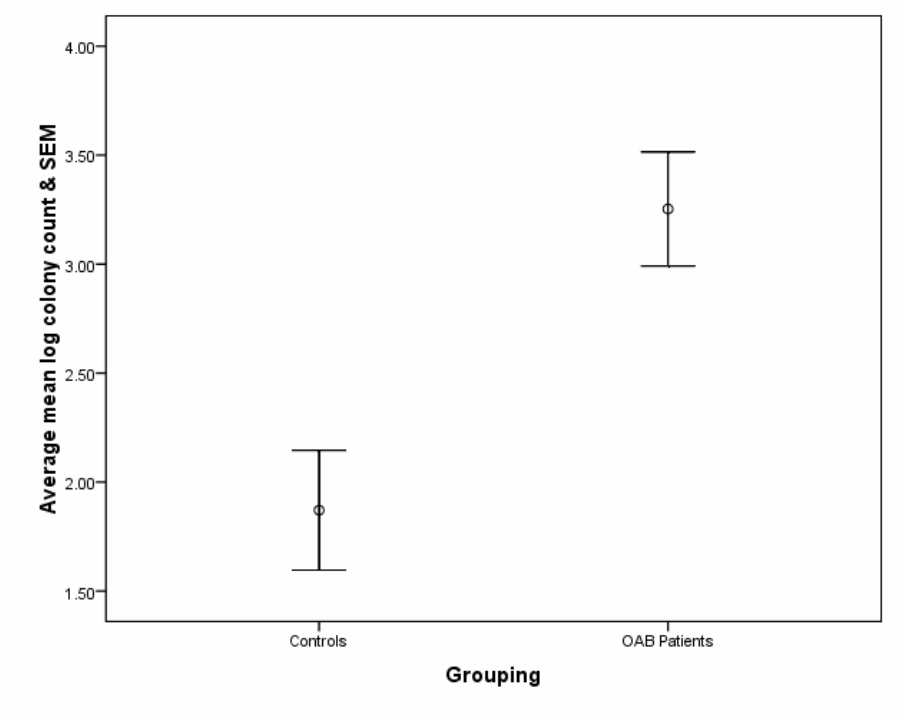


Figure 3.4 Log mean total bacterial colony count and SEM comparing OAB patients with and without pyuria and asymptomatic controls providing catheter specimens of urine and Mid-Stream specimens of urine subjected to culture of the urinary sediment.

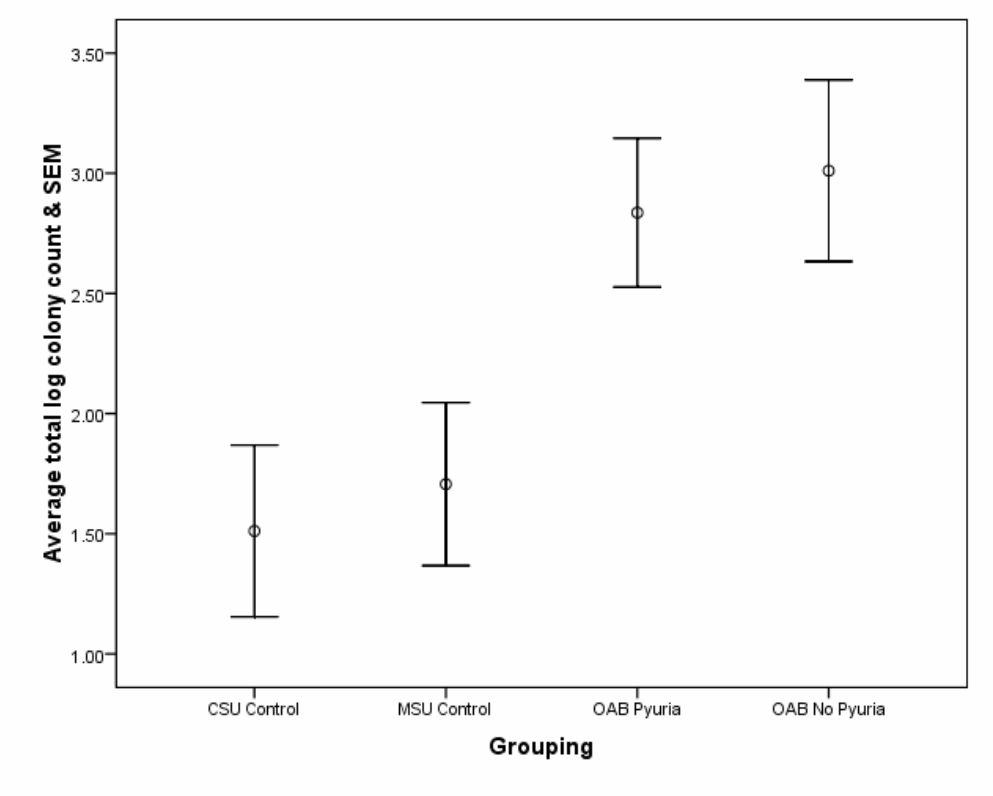


Figure 3.5 Log mean largest bacterial colony count and SE mean comparing OAB patients with asymptomatic controls providing catheter specimens and mid- stream urines subjected to culture of the urinary sediment .(Cont=control volunteers, OAB=Overactive bladder patient group)

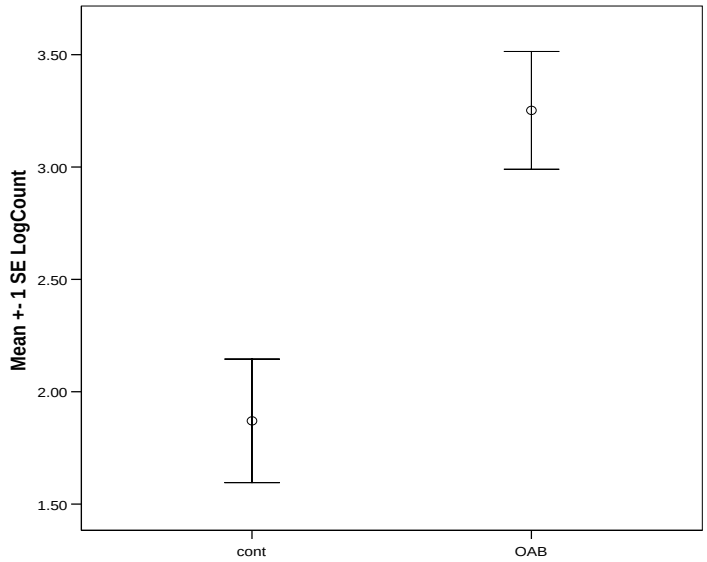


Figure 3.6 Bacterial species isolated in patients with overactive bladder

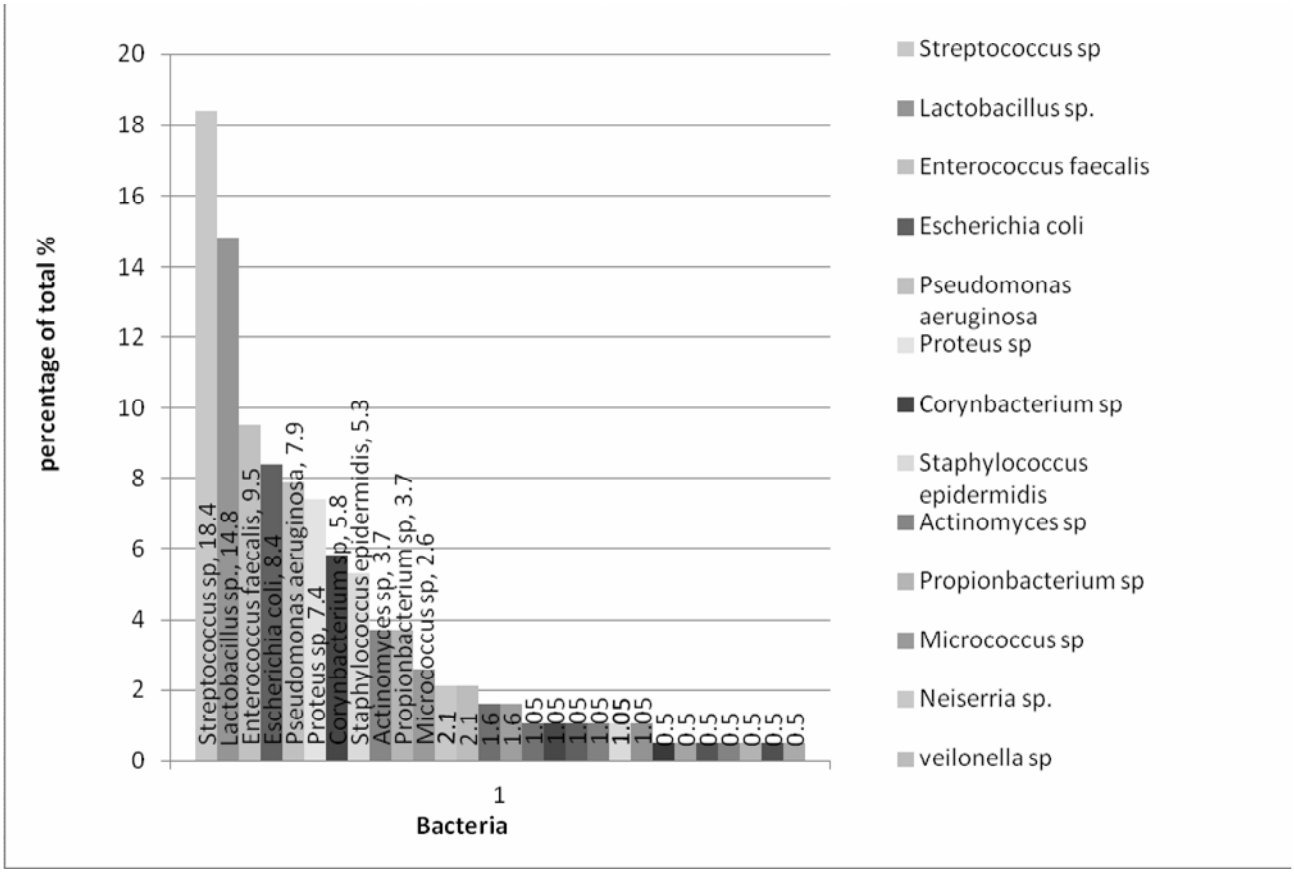


Figure 3.7 Bacterial species isolated from asymptomatic volunteers providing a catheter specimen of urine as a percentage of the total number of bacteria identified.

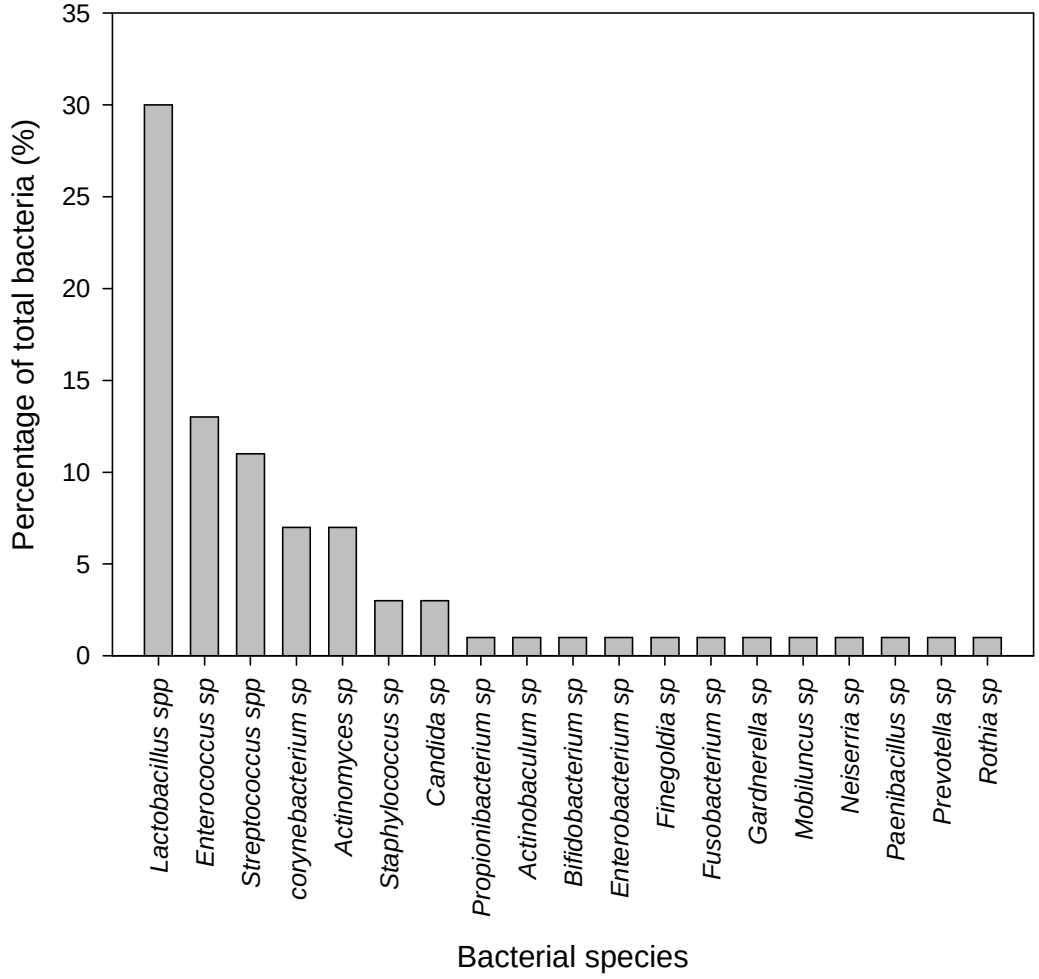
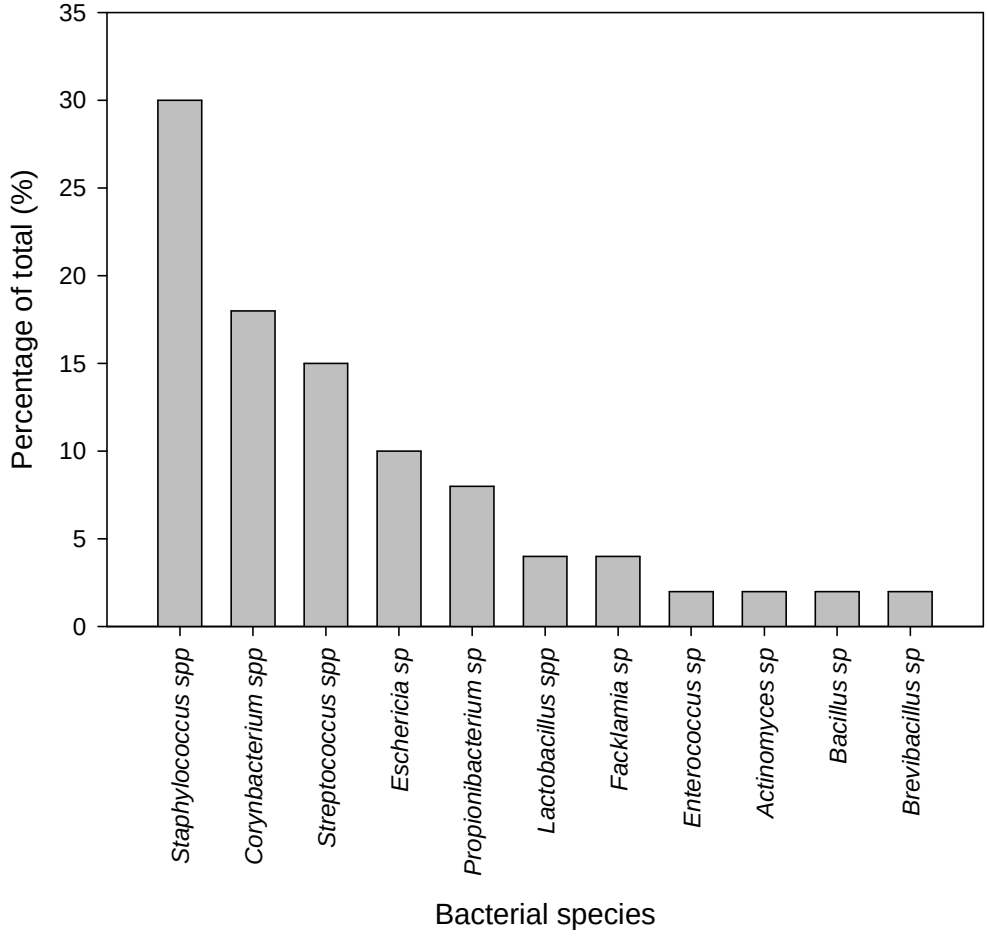


Figure 3.8 Bacterial species isolated in asymptomatic volunteers providing a mid-stream specimen of urine.



3.3.4.2 Biopsy culture

11 women, mean age 56 (sd=19) years with overactive bladder symptoms, provided bladder biopsy specimens for culture. The symptom profile is described in table 2.1

One woman had pyuria (>10 white blood cells per µL) at the time of the biopsy. 10/11 patients were on long-term antibiotic treatment (median time on antibiotics being 12 weeks) in preparation for the investigative procedure or to avoid infection during and after the procedure. 8/11 patients were taking Nitrofurantoin, 1/11 patient was taking Doxycycline and 1/11 patient was taking ciprofloxacin. 5/11 patients had negative sediment cultures with no bacterial growth. 6/11 patients grew bacteria from the biopsy culture despite taking antibiotics at the time. The average total bacterial count was $3.75x10^{3}$cfu ml^{-1} (sd=$8.1x10^{2}$cfu ml^{-1}). 2/5 culture positive patients grew anaerobes only. Table 3.1 illustrates the histology results of the bladder biopsy samples and the routine and sediment culture results. The table demonstrates an inflammatory reaction in all but two of the bladder biopsy samples according to the histology of the bladder biopsy. This confirms an inflammatory reaction in the bladders of these patients with OAB symptoms. However, the routine urine culture sent to the Whittington Hospital NHS Trust microbiology department (2.6) was negative in all but one patient.

Table 3.1 Routine culture results and histology results of bladder biopsies

Patient number	Routine urine culture result (>10^5cfu ml^{-1})	Sediment culture dominant bacteria	Pyuria count (Wbc mm^{-3})	Antibiotic status	Histology result
GW1	no growth	*Massilia.timonae*	0	Taking at time of biopsy	Normal urothelium
57	no growth	*Veillonella* spp	0	Taking at time of biopsy	Urothelium showing inflammation
58	no growth	*Veillonella* spp	0	Taking at time of biopsy	Urothelium showing inflammation
59	no growth	No growth	0	Taking at time of biopsy	Urothelium showing inflammation
67	no growth	*Streptococcus agalactiae*	0	Taking at time of biopsy	Urothelium showing inflammation
71	no growth	No growth	0	Taking at time of biopsy	Urothelium showing inflammation
74	no growth	*Neisseria sp*	0	Taking at time of biopsy	Urothelium showing inflammation
106	*Pseudomonas aeroginosa*	*Pseudomonas/ Bifidobacterium*	100	Taking at time of biopsy	Urothelium showing inflammation
108	no growth	No growth	0	Not taking	Urothelium showing inflammation
109	no growth	*Bifidobacterium*	0	Taking at time of biopsy	Urothelium showing inflammation
110	no growth	No growth	0	Taking at time of biopsy	Normal urothelium

3.3.5 Discussion

The symptom-free control group in the urine study provided some CSU samples as well as MSU's but these did not differ significantly in pyuria or total bacterial counts. They did differ in the prominent bacteria found however, fig 3.7 & 3.8. The CSU specimens contained fewer bacterial species and a larger number of samples grew *Lactobacillus* spp. This difference between MSU samples and CSU samples may be because of contamination of the MSU sample from the perineum. This confirms the findings of the study in chapter 2.5, suggesting that MSU specimens taken from women are contaminated from the perineum. However, the species that were isolated provide some discriminant power in judging the significance of the bacterium cultured. It is unclear as to why the CSU samples in the control group grew more *Lactobacillus spp* as compared to the MSU samples from the control group. Higher numbers are required to identify a meaningful difference.

Lactobacillus spp are vaginal commensals in women and are found in higher numbers in pre-menopausal menstruating women. The volunteers in the control group used in this study were significantly younger than the patient group (average age 29 years Vs 56 years) and this may account for the higher proportion of *Lactobacillus* spp in the control group. The *Lactobacillus* species identified are all commonly found in the vagina, including *Lactobacillus. crispatus, Lactobacillus. gasseri, Lactobacillus. johnsonni,* and *Lactobacillus. vaginalis*. It is also possible that the bacterial community is different between the patient group and control group, again, due to difference

in age and hormonal status. However, this does not account for the larger bacterial load found in the patient group, particularly of organisms such as *Streptococcus* spp. and *Enterococcus* spp. Although vaginal commensals, these are also found in high counts in disease states.

The largest *Streptococcus* groups amongst patients were *Strep. agalactiae and Strep. anginosus*. *Strep. agalactiae is a Group B Streptococcus (GBS)* and colonization of the urinary tract in women probably occurs by an ascending route from the vagina, where *GBS* can persist without causing symptoms. The literature is dominated by reference to *Group B Streptococcus* and its role in neonatal sepsis intrapartum, due to vaginal colonisation in the mother, however, several studies have also reported high rates of *GBS* UTI in non-pregnant adults (186) (187;188). One study by Trivalle et al. (189) reported *GBS* cultured from 39% of all cases of symptomatic UTI among nursing home residents >70 years of age. Several studies have reported the recovery of *GBS* from between 1 and 2% of all UTI cases (104;190). Interestingly, relatively high rates of treatment failure and poor clinical outcomes have been associated with *GBS* UTI and attributed to macrolide resistance (104).

Ulett et al. (191) investigated the early pathogenic mechanisms underlying *GBS* UTIs. Particularly, whether Uropathogenic *GBS* can bind to human bladder urothelium and trigger host responses as a means to colonize and cause inflammation in the bladder. They used an in vitro binding assay with 2 cell lines and a murine model of cystitis. They demonstrated that *GBS* binds

directly to human bladder urothelial cells, which facilitates colonization of the bladder in vivo. They also observed that the *GBS* isolate used in the study triggered a more potent induction of IL-1α than a virulent uropathogenic *E.coli* strain. These studies are very interesting in the context of this study as they give a possible explanation for the existence and persistence of these potential pathogens causing UTI in my patient group. *Strep. anginosus* bacteria are found as members of both the normal and pathogenic microbiota. They are most often found as part of the normal flora of the oral cavity and gastrointestinal tract of humans but these organisms have also been isolated from the normal microbiota of the respiratory and female genital tracts. The *S. anginosus* group has been associated with multiple types of infections. The first reported infections caused by these bacteria were oral infections; however, *S. anginosus* group bacteria are now known to be involved in infections in multiple body sites. *S. anginosus* is more commonly isolated from blood, urine, and soft tissue infections (192). There is very little in the literature in relation to this organism as a pathogen in UTIs and the mechanism of infection. *Strep. anginosus* is commonly associated with abscess formation and particularly with gastrointestinal infections. Several studies have found an association between *Strep. anginosus* and other pathogens in disease, particularly, the association with *E.coli* and *Pseudomonas spp*. in abscess formation (193).

Enterococcus.faecalis, again, although a vaginal commensal and found in the gastrointestinal tract, is not usually associated with UTI. The interaction between *Enterococcus spp* and urothelial tissue has been examined (194). In a study of *E. faecalis* isolates from patients with UTI and endocarditis,

Guzman et al showed that UTI isolates adhered efficiently to urothelial cells (195). The nature of the interaction of *Enterococcus spp* with urothelial tissue appears to be quite complex, with a role for bacterial cell surface carbohydrate and protein (196).

There were two obligate anaerobes isolated in the patient group: a *Peptostreptococcus* sp. and a *Veillonella* sp. commonly found in the vagina and implicated in bacterial vaginosis. Other commensal organisms were also found in smaller counts such as *Propionibacterium* sp*, Actinobaculum* sp*, Bifidobacterium* sp*, Enterobacterium* sp*, Finegoldia* sp*, Fusobacterium* sp*, Gardnerella* sp*, Mobiluncus* sp*, Neiserria* sp*, Paenibacillus* sp*, Prevotella* sp*, Rothia* sp.

This study compared the bacterial retrieval from uroepithelial cells compared to the standard culture used in the NHS which uses a loop of urine (both sediment and supernatant). This pilot study used a small number of patients and a control group which was not matched to the study group by age. This arose because of the difficulty in recruiting age matched control volunteers without any bladder symptoms such as nocturia, which can occur normally in later life for a variety of reasons. Despite this, the data demonstrate a very marked enhancement in the isolation of bacteria when the spun urinary cellular sediment is cultured. The data support the view that ordinary laboratory cultures, which focus on isolating *E. coli* at a threshold of 10^5 cfu ml^{-1}, are insensitive and inappropriate for screening out infection in patients with OAB. However, a much larger study would be required to confirm these findings and to implement any clinical change. Additionally, they support the

hypothesis that patients with OAB have a high probability of suffering from a bacterial UTI. The probability of bacterial growth, and the number of colonies, is increased if pyuria is detected. This is a coherent property because pyuria is a reflection of activation of the innate immune response. Pyuria has been found to affect about 35% of OAB patients when sampled by CSU, compared to 0% of asymptomatic controls (197).

A critical interaction between uropathogenic bacteria and the bladder epithelium appears to be adherence by bacteria through expression of surface adhesive organelles such as type 1 pili (152). It is possible that if the bacteria are attached to the urinary urothelial cells they may evade detection because current methods sample uncentrifuged urine. The sediment culture is directed at a concentrated suspension of the shed urothelial cells and may enhance detection because of selection of colonised cells. The literature supports this interaction in association with the most commonly found bacteria in the patient group (119;152;191;196;198;199).

It is an inevitable consequence of these data that we must implicate UTI in the aetiology of overactive bladder symptoms to a far greater extent than has previously been assumed. According to the accepted definition, most of the patients in this study, based on MSU culture data, would have received the diagnosis of OAB and not been offered treatment for urinary infection. This must be a cause for concern because of the considerable differences in bacterial colonisation between these patients and controls.

The sediment culture is evidently sensitive since bacteria were isolated from controls but in fewer orders of magnitude and the organisms were predominantly *Lactobacillus* spp.

Despite antibiotic treatment, bacteria were isolated from the urothelium taken at the time of bladder biopsy. In addition, the histology results of biopsies taken at the same time, confirm, that despite antibiotic treatment, an inflammatory process was present in the urothelium in most of the patient group. There are numerous causes for inflammation but the high bacterial counts and the species isolated are very supportive, but not proof, of infection being the cause of this inflammatory response. Again, only a small number of patients were recruited to the study and a biopsy at initial presentation, when the patient is not taking antibiotics, would be ideal. However, taking a biopsy without antibiotic prophylactic treatment may increase the risks to the patient and so this was not done.

These findings certainly cast doubt on current assumptions about the aetiology and the definition of OAB.

Chapter 4

Sensitivity and Specificity of Urinary Dipstick Test in the Detection of Urinary Tract Infections in Patients with Overactive Bladder

4.1. Hypothesis

The sensitivity and specificity of the popular leucocyte esterase and nitrite dipstick tests to exclude infection in patients with OAB are too low to be used to exclude infection in these patients.

4.2 Background

This study addressed the absence of validation data on the use of the leucocyte esterase and nitrite tests when applied to patients with non-dysuric, chronic lower urinary tract symptoms such as in OAB. This was approached by measuring the sensitivity and specificity of urinary dipsticks for UTI in patients with OAB, taking a 10^5 cfu ml^{-1} culture on MSU and both 10^2 cfu ml^{-1} and 10^5 cfu ml^{-1} on CSU as well as the results of microscopy of a fresh, unspun specimen of urine for pyuria using the same samples.

4.3 Methods

Researchers were blind to microbiological outcomes. Patients recorded their symptoms using a validated questionnaire and their reported urinary frequency and incontinence episodes were noted. From these data an urgency score (0 to 10) was calculated using a validated method as per section 2.2 (appendix 1) (170).

Data were also collected from a sample of asymptomatic controls.

Experiment 1 – A study, of MSU samples using the Gold standard 10^5 cfu ml^{-1}

MSU samples were obtained, an aliquot was tested by dipstick for leucocyte esterase (positive = trace and above) and nitrite; an aliquot was sent for routine laboratory culture; a fresh aliquot was examined immediately by microscopy.

Experiment 2 – A study of CSU samples using enhanced reference standard 10^2 cfu ml^{-1}

A sample of corresponding female patients with OAB was seen in order to obtain a CSU. The specimens were examined similarly but in addition aliquots were submitted to an enhanced culture (*vide infra*)

*MSU Collection***:** Samples were obtained by the midstream clean-catch method. (2.4.3.1)

CSU Collection: The procedure was performed on female patients only and by a doctor or specialist nurse (2.4.3.2)

Leukocyte Esterase and Nitrite Tests: The urine was dipped by a Multistix™ 8 SG. The leukocyte esterase pad sensitivity of the dipstick was stated to be

15 wbc μl^{-1} when “trace positive” and this level was considered to be positive for the study. The nitrite test pad sensitivity of the dipstick was stated to be 13-22 µmol L^{-1} (0.06-0.1 mg dL^{-1}) nitrite ions. These data were collected by clinic doctors and nurses (2.4.2).

Routine culture method (gold standard): The sampled urine was treated fresh, or after overnight storage at 4°C at the hospital laboratory. The samples were processed as in 2.4.6.

Enhanced culture method (enhanced reference standard): The sample obtained by CSU was processed immediately as described in 2.9.1. An aliquot of each CSU also underwent routine analysis at the threshold of 10^5 cfu ml^{-1} (2.6)

Microscopic white cell count: A fresh, unspun aliquot of urine was examined by microscopy as described in 2.4.1.

4.4 Statistics

The study followed the Standards for Reporting Diagnostic Accuracy (STARD) (200). The experiments were powered to detect a 75% sensitivity and 45% specificity so that 100 patients in each group culture positive / culture negative gave 85% power, alpha=0.05. The challenge was to obtain the 100 with positive cultures. The data were analysed by 2x2 contingency tables using Pearson Chi-Square (SPSS). Empty data points were excluded from analysis.

Patients with missing data were compared with those analysed using the variables of age, median 24-hour frequency, median 24-hour incontinence and urgency score by ANOVA. Differences in sex distribution were compared by Pearson Chi-Square.

4.5 Results

The STARD recruitment analysis is shown at the bottom of this section

Experiment 1 – A study, of MSU samples using the Gold standard 10^5 cfu ml^{-1}

This experiment analysed data from 508 patients (432 women and 76 men). These were newly presenting with OAB without acute frequency/dysuria between 2nd August 2004 and 6th January 2009. Their mean age was 51 (sd=19). No patients were taking antibiotics.

The median daily frequency was 8(IQ range 6-11), daily incontinence episodes 1 (IQ range 0-2) and the median urgency score was 2. (IQ range 0-5) reflecting the widespread overactive bladder symptoms. Of these 132 (35%) had pyuria on microscopy, 90 (24%) with sterile pyuria, and 79 (21%) had positive MSU cultures. Only 209 (55%) of patients with OAB symptoms had normal urine.

The data for this experiment are shown in table 4.1. The key findings were that the sensitivity for the gold standard, of leukocyte esterase was 56%,

Nitrite 10% and microscopic pyuria 56%; with specificities of 66%, 99% and 72% respectively. The sensitivity of leukocyte esterase for microscopic pyuria was 81% (specificity 83%)

Experiment 2 – A study of CSU samples using the Gold standard 10^5 cfu ml^{-1} and an enhanced reference standard 10^2 cfu ml^{-1}

470 women with OAB without acute frequency/dysuria agreed to provide a CSU between 1st October 2007 and 27th January 2009. Their mean age was 57 (sd=18). The median daily frequency was 8(IQ range 5-10), median daily incontinence episodes 1 (IQ range 0-2) and the median urgency score was 2 (IQ range 0-5).

The data for this experiment are shown in tables 4.2 and 4.3. The key finding was that on using CSU the gold standard culture method (10^5 cfu ml^{-1}) proved positive in 71 (15%) samples, in contrast to the MSU samples which were positive in 106 samples (21%).

In CSU samples the sensitivity for the gold standard, of leukocyte esterase was 59%, Nitrite 20% and microscopic pyuria 66%; with specificities of 84%, 97% and 73% respectively. The sensitivity of leukocyte esterase for microscopic pyuria was 64% (specificity 97%).

The enhanced method of CSU culture (10^2 cfu ml^{-1}) proved positive in 137 (29%); inevitably more than the gold standard 71 (15%). Commensurately, the surrogate markers were less sensitive for 10^2 cfu ml^{-1} (table 4.3).

Table 4.4 contains the data that compare the leukocyte esterase test results with the microscopic pyuria data.

Control subjects

42 volunteers with no lower urinary tract symptoms provided meticulously collected MSU specimens. There were 16 men and 26 women (mean age 34, sd-11). The volunteers recruited were hospital staff and students. Only two subjects (4.8%), both women, were leukocyte esterase positive and only one of these proved positive on urine culture, routine and enhanced. This woman provided the only nitrite positive specimen. Three controls (7%) showed pyuria on microscopy, one of these being leukocyte esterase positive.

Patients with positive cultures at different thresholds

There were no symptom differences in average daily frequency, incontinence or urge score between patients showing a positive culture only at the threshold of 10^5 cfu ml^{-1}, MSU or CSU, and those with a positive CSU at the 10^2 cfu ml^{-1}. The more sensitive culture did not identify a different symptomatic group (See table 4.5).

STARD participant recruitment analysis

Experiment 1

615 (526 female, 89 male) OAB without acute dysuria were identified: 20 (3%) patients could not provide a sample. Thus MSUs from 595 patients were provided. Of these 87 (15%) had missing data for one or all of the following: Leukocyte esterase 84 (14%); nitrite 80 (13%) microscopy 52 (9%). Thus the total analysed was 508 samples. Missing data occurred from equipment failures, equipment availability and omission of database entry during a busy clinic. The patients with missing data did not differ from those analysed in respect of age, sex, urinary frequency, urgency and incontinence (table 4.5).

Experiment 2

607 women with OAB without acute frequency/dysuria attending outpatient clinic provided a CSU: 137 (23%) of these had incomplete data for one or all of the following: Leukocyte esterase 103 (17%); nitrite 101 (17%) microscopy 83 (14%); culture results 48 (8%). This arose from equipment failures but more commonly oversight in data entry and culture plate transport failures. The patients with missing data did not differ from those analysed in respect of age, sex, urinary frequency, urgency and incontinence (table 4.5).

Sensitivity and specificity applied to ordinary clinical practice

The receiver operator characteristic (ROC) curves for the leukocyte esterase and the nitrite tests as predictors of a routine MSU analysis with a threshold of

10^5 cfu ml^{-1}, reflecting normal clinical practice are shown in figures 4.1 and 4.2.

5.6 Discussion

This large study, examined of the reliability of the urinary dipstick test, as a surrogate for urinary infection, in patients without the classic symptoms of acute frequency /dysuria. Thus the experiment scrutinised a particular group for whom the accuracy of the test has greatest clinical relevance. These data show that the dipstick test in this context is insensitive, at best 59% sensitive (95% CI 47% to 70%) which is not appropriate for the exclusion of urinary tract infection when the symptoms are so noxious. It is remarkable that the Nitrite test which is commonly advocated had a sensitivity of 13% (95% CI 8% to 21%) at best. Nitrite stood out for its specificity of 99% (95% CI 98% to100%) but the data distribution would suggest that in such circumstances infection is likely to have been clinically overt. Recent NICE guidelines on female incontinence contain the statement “A urine dipstick test should be undertaken in all women presenting with urinary incontinence to detect the presence of blood, glucose, protein, leukocytes and nitrites in the urine” (201). The data reported here challenge the veracity of this. The International Consultation on Incontinence guidelines on lower urinary tract symptoms similarly recommends dipstick testing as a screening method (61). If this advice be followed it could be argued that significant number of patients with urinary infection become misdiagnosed with OAB. This is no small matter given the prevalence of lower urinary tract symptoms.

The inclusion of controls, albeit younger, permits comparisons that imply that the prevalence of pyuria (56% with MSU and 30% with CSU) and bacturia (20% with MSU at 10^5 cfu ml^{-1} and 30% with CSU at 10^2 cfu ml^{-1}) must be related to the disease process. This merits further scrutiny. The symptom analysis show that the patients, on average, had marked and disruptive symptoms of OAB. The youth of the controls arises from very exacting insistence on absent symptoms, particularly nocturia.

This study was conducted amidst normal clinical practice, giving it wide applicability. Nevertheless it resulted in more missing data than would be expected in non-pragmatic trials. A key quandary was the adoption of computer data entry to generate the hard copy in the notes. In pressure of the clinics it is very easy to overlook data entry cells. It was however reassuring that the demographic and symptomatic comparisons imply that a selection bias was not implicated.

The difference in sensitivities between MSU and CSU samples raises an additional concern. The tests proved less sensitive when CSU specimens were analysed whatever the method adopted. This implies a significant contamination of MSU samples. The differences in pyuria would indicate that the most plausible explanation would be vaginal contamination. The numbers were small but men did not show such dissociation. Again, the suitability of a MSU for screening out UTI in women must be questioned.

The use of a CSU submitted to enhanced culture with a diagnostic threshold of 10^2 cfu ml^{-1} did not reflect normal practice. It was affected in response to the doubts expressed by Stamm et al in 1982 (86) about the veracity of standard MSU culture for acute frequency/dysuria. This method did result in a higher yield with consequently reduced sensitivities for the tests. It would seem that it would be wise to scrutinise the gold standards that we use for identifying urinary infection in patients with OAB. It must also be acknowledged that the culture methods assume dominant pathogenicity from the *Enterobacteriaceae.* Whilst this might apply to acute infection there is no evidence that is appropriate to patients with non-dysuric OAB. Thus, there are no data to inform a selection of a suitable gold standard for diagnosing UTI in this context.

The performance of leukocyte esterase dipstick analysis for diagnosing acute UTI has been well studied but the literature is contradictory. The sensitivity and specificity vary considerably. The samples studied manifest differing prevalence's and that alone will affect PPV and NPV (202-205). Studies of populations with low prevalence of acute UTI show high NPV for leukocyte esterase, similar to our study (84% to 93%) (205;206) (205;207). However, these trials describe low sensitivity resulting in an objectionable under-treatment of genuine acute UTI.

In contrast to the findings of this study, some of the literature is confident about the efficacy of the nitrite test. The very high specificity may contribute to this since it means that the test is likely to be positive the more impressive the

urinary tract infection. Thus vivid recall may cause a cognitive bias perversely in favour of the test. In contrast, the test must excel where symptoms are unclear for it to be useful. This is transparently not the case.

The common nitrite producing organisms are *Proteus, E. coli, Klebsiella, Pseudomonas, Staphylococcus,* but there are other bacteria pathogenic to the bladder that do not reduce nitrate. Thus the nitrite test is inevitably going to miss a proportion of infections.

These data highlight a need to clarify the criteria for the exclusion of urinary tract infection in this group of patients. The limitations of the dipstick test have to be acknowledged when managing patients with non-dysuric OAB. It must be accepted that UTI may be erroneously excluded in the assessment of such symptoms, particularly OAB symptoms, and our understanding of this important condition may be very much at fault.

Table 4.1 Experiment 1: A study of MSU samples using the Gold standard 10^5cfu ml^{-1}.

MSU samples: Comparison of surrogate markers to gold standard 10^5 cfu ml^{-1}								
(1) Leukocyte esterase			**(2) Nitrite**			**(3) Microscopic pyuria**		
	Culture positive	Culture negative		Culture positive	Culture negative		Culture positive	Culture negative
Leukocyte positive	59 (12%)	138 (27%)	Nitrite Positive	11 (2%)	2 (0.4%)	Pyuria positive	59 (12%)	113 (22%)
Leukocyte negative	47 (9%)	264 (52%)	Nitrite Negative	95 (19%)	400 (79%)	Pyuria negative	46 (9%)	290 (57%)
Sensitivity = 56% (95% CI 46% to 66%)			**Sensitivity = 10%** (95% CI 6% to 18%)			**Sensitivity = 56%** (95% CI 46% to 66%)		
Specificity = 66% (95% CI 61% to 70%)			**Specificity = 99%** (95% CI 98% to 100%)			**Specificity= 72%** (95% CI 67% to 76%)		

Table 4.2 Experiment 2: A study of CSU samples using the Gold standard 10^5cfu ml^{-1}.

CSU samples: Comparison of surrogate markers to gold standard 10^5 cfu ml^{-1}								
(1) Leukocyte esterase			**(2) Nitrite**			**(3) Microscopic pyuria**		
	Culture positive	Culture negative		Culture positive	Culture negative		Culture positive	Culture negative
Leukocyte positive	42 (9%)	64 (14%)	Nitrite Positive	14 (3%)	10 (2%)	Pyuria positive	47 (10%)	106 (23%)
Leukocyte negative	29 (6%)	335 (71%)	Nitrite Negative	57 (12%)	389 (83%)	Pyuria negative	24 (5%)	293 (62%)
Sensitivity = 59% (95% CI 47% to 70%)			**Sensitivity= 20%** (95% CI 12% to 31%)			**Sensitivity = 66%** (95% CI 54% to 77%)		
Specificity = 84% (95% CI 80% to 87%)			**Specificity=97%** (95% CI 95% to 99%)			**Specificity = 73%** (95% CI 69% to 78%)		

Table 4.3 Experiment 2: A study of CSU samples using the Enhanced reference standard 10^5cfu ml^{-1}.

CSU samples: Comparison of surrogate markers to enhanced standard 10^2 cfu ml^{-1}								
(1) Leukocyte esterase			**(2) Nitrite**			**(3) Microscopic pyuria**		
	Culture positive	Culture negative		Culture positive	Culture negative		Culture positive	Culture negative
Leukocyte positive	61 (13%)	45 (10%)	Nitrite Positive	18 (4%)	6 (1%)	Pyuria positive	73 (16%)	80 (17%)
Leukocyte negative	76 (16%)	288 (61%)	Nitrite Negative	119 (25%)	327 (70%)	Pyuria negative	64 (14%)	253 (54%)
Sensitivity = 45% (95% CI 36% to 53%)			**Sensitivity = 13%** (95% CI 8% to 20%)			**Sensitivity = 53%** (95% CI 45% to 62%)		
Specificity = 86% (95% CI 82% to 90%)			**Specificity = 98%** (95% CI 96% to 99%)			**Specificity = 76%** (95% CI 71% to 80%)		

Table 4.4 Sensitivities and specificities of leukocyte esterase for detecting pyuria

MSU Samples - Leukocyte esterase compared with microscopic pyuria			**CSU Samples - Leukocyte esterase compared to microscopic pyuria**		
	Pyuria positive	Pyuria negative		Pyuria positive	Pyuria negative
Leukocyte esterase positive	140 (28%)	57 (11%)	Leukocyte esterase positive	98 (21%)	8 (2%)
Leukocyte esterase positive	32 (6%)	279 (55%)	Leukocyte esterase positive	55 (12%)	309 (66%)
Sensitivity = 81% (95% CI 75% to 87%)			**Sensitivity = 64%** (95% CI 56% to 71%)		
Specificity = 83% (95% CI 78% to 87%)			**Specificity = 97%** (95% CI 95% to 99%)		

Figure 4.1 ROC curve for urinary leukocyte esterase as a predictor of a positive routine MSU culture of 10^5 cfu ml^{-1}

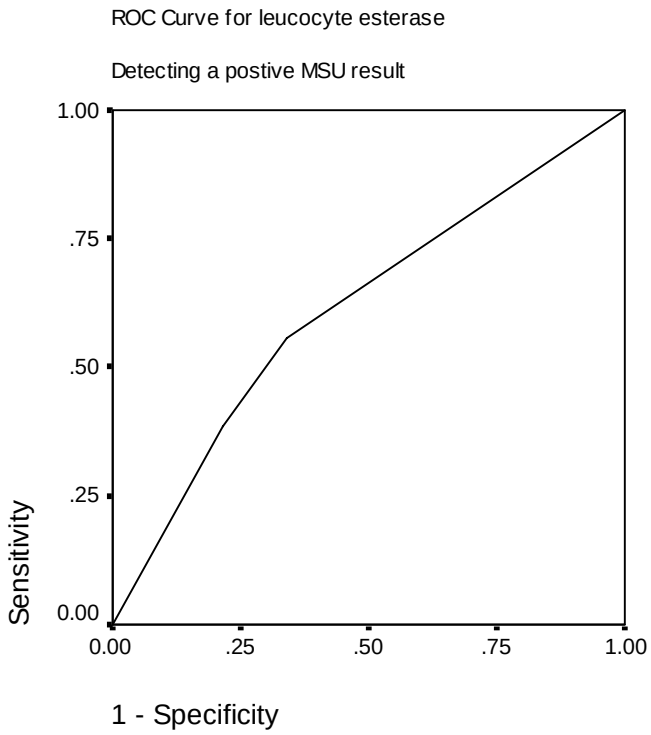


Figure 4.2 ROC curve for urinary nitrite as a predictor of a positive routine MSU culture of 10^5 cfu ml^{-1}

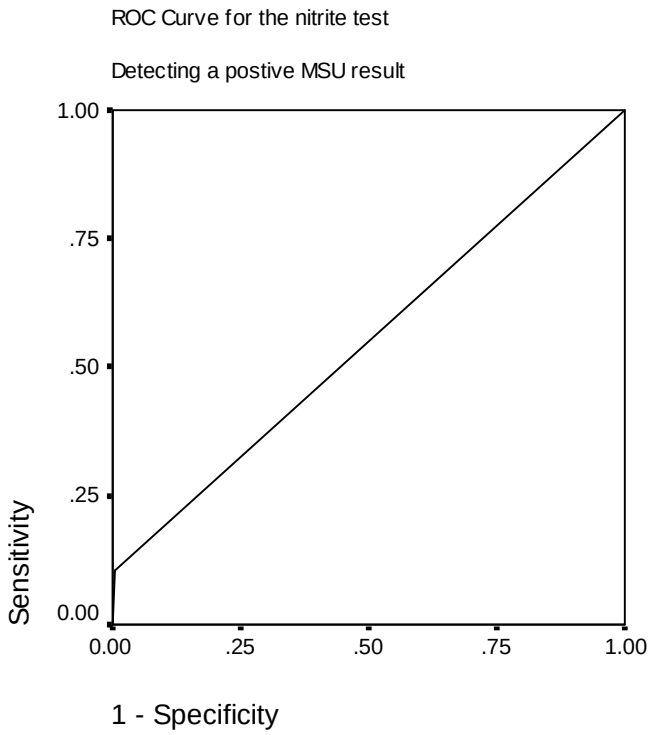


Table 4.5. The symptom profiles according to key patient groups. These comparative data demonstrate similar symptom profiles from patients with 10^5 cfu/ml and 10^2 cfu/ml. The 10^2 cfu/ml did not select a sub-set. Similarly the symptom profiles of patients with missing data imply that there was no selection bias.

Patient group	Mean Age	Median total Frequency episodes 24hrs	Median Urge Score	Median Total Incontinence episodes in 24hrs
MSU positive 10^5cfu/ml	50.84 (Sd=18.8)	8 (IQ range 6-11)	2 (IQ range 0-5))	1 (IQ range 0-2)
CSU positive 10^5cfu/ml	56.38 (Sd= 18.5)	8 (IQ range 5.5-11))	2 (IQ range 0-5)	1.5 (IQ range 0-2)
CSU positive 10^2cfu/ml	56.38 (Sd=18.5)	8.5 (IQ range 4-9.5)	2 (IQ range 0-5)	1 (IQ range 0-2.5)
Missing Data	50.81 (Sd=18.7)	8 (IQ range 5-12))	3 (IQ range 0-4)	1. (IQ range 0-2.5)

Chapter 5

A chemical Cytokine Response in the urine of Patients with Overactive Bladder

5.1 Hypothesis

The study tested the hypothesis that urinary IL-6 expression in the urine of patients with OAB would be increased in association with pyuria.

5.2 Background

There is ever increasing support in the literature for an inflammatory aetiology of many lower urinary tract syndromes, however, the inflammatory mediators that are involved in the pathophysiology of OAB are unclear. There are many studies describing the inflammatory signals in the urine in acute UTI and so these studies were used as a starting point to try and identify an additional inflammatory signal in the urine in patients with OAB which is also associated with urinary infection. The literature leans towards IL-6 in this respect, IL-6 being the most frequently identified cytokine in the urine in acute UTI (149;208-210);(126;150). Urothelial production of IL-6 occurs in response to bacterial challenge of the human urinary tract and at deliberate colonisation. Importantly, at disease recovery, IL-6 levels are normalised. Asymptomatic controls secrete very low, levels of IL-6 in urine and it has been proposed that IL-6 levels in urine could be used as a marker of UTI severity. This makes IL-6 an ideal cytokine to study between patients and normal control volunteers (211;211). Mouawad *et al. (212)* found the mean circulating IL-6 concentration in 40 healthy controls in blood serum to be 2.65 pg/ml. Healthy individuals also secrete IL-6 in their urine. Lamale *et al.* (130) reported the mean urinary

IL-6 level in 29 healthy individuals to be 0.63 pg/ml whilst Erickson *et al. (131)* reported healthy female IL-6 levels to be around 1.5 pg/ml. There seems to be no difference in urinary IL-6 between healthy males and females (213).

5.3 Methods

This was a blinded, observational cohort study. Women with symptoms of the OAB were asked to give consent to participate and provide catheter specimens of urine (CSU) for the study. Their symptoms were recorded using a validated questionnaire as per section 2.2. A CSU was obtained by inserting a Lofric (in and out) size 12 Fr catheter into the urinary bladder under aseptic conditions (2.4.3.2). Urine specimens were collected into a sterile container and a 4ml aliquot was taken immediately. A sample of 0.9µl was taken in order to affect a white blood cell count (2.4.1).

The samples were thawed and analysed in batches, blinded, once weekly. Human urinary IL-6 concentrations were determined using a commercial very high sensitivity sandwich ELISA with a limit of detection of 0.09pg/ml with a inter and intra assay coefficient of variation of less than 10% (R&D Systems, Oxon, UK).

5.3.1 ELISA Assay

4ml of urine from each sample was transferred to a sterile 10ml centrifuge tube. Tubes were placed in a Denley BR 401 centrifuge machine and centrifuged at 8000rcf for 5min. Subsequently, the supernatant was removed and placed in cryogenic tubes. Care was taken to ensure that the sediment pellet was not agitated at this stage. The tubes were frozen at -80 ºC in a Forma Scientific freezer. Freezing the samples was deemed more practical than immediate analysis, and it has been shown that IL-6 remains fairly stable even with up to 6 freeze-thaw cycles (214).

Urine IL-6 concentrations were measured by transferring the frozen samples to the Rayne Institute, UCL, and conducting an enzyme linked immunosorbent assay (ELISA). Samples were analysed in batches by defrosting a set number on the day of the ELISA and then refreezing them immediately afterwards, in case of a repeat being necessary. Repeated freeze-thaw cycles were avoided to ensure good conditions of urine were maintained. The samples were analysed blind with regard to the white cell content of the urine samples. The Quantakine high sensitivity IL-6 ELISA was used (© 2008 R and D Systems, MN, USA) and is based on a sandwich enzyme immunoassay system. IL-6 in samples added to the microplates, became bound to IL-6 immobilised monoclonal capture antibody coating the plates. After that, enzyme-linked polyclonal antibody was added to the plates, followed by substrate solution and amplifier solution. To end the colour producing reaction, stop acid solution was added to each well. Plates were washed with wash buffer in between

additions, to ensure removal of unbound material. In addition to the samples, standard IL-6 concentrations were added to the microplates in order to produce a calibration curve. In between additions and washes the microplate was incubated either on an orbital microplate shaker or in line with the protocol, on the benchtop. Finally, microplate colour optical densities were read with a microplate reader to determine IL- concentration. The reader produced standard curves and printed the results of each well automatically. The minimum detectable dose (MDD) of the ELISA kit according to the manufacturers ranges from 0.016-0.110 pg/ml, with an average MDD of 0.039 pg/ml. 40 of the samples were analysed twice to test the inter-assay precision and the mean value was taken, the other 64 samples were analysed only once. The intra-assay precision for urine assays suggested by the company is 5.5-9.8% and the inter-assay precision is suggested to 5.5-11.2%.

5.4 Statistics

The study was powered at 87% to detect a significant difference in IL-6 of 1 pg/ml with a standard deviation of 1.5. 1 pg/ml was chosen as the clinically significant difference to seek because low levels of IL-6 are detected in healthy individuals and there is a lack of data in the literature to define a cut-off level for chronic bladder conditions. The study was powered at 79%, with 35 patients in each group. In order to accommodate dropouts and missing data the target recruitment number of patients was rounded up to 40 in each group. In the event this goal was achieved without lost data so the ultimate power was 87%.

The between group differences in IL-6 were tested by use of the Mann–Whitney U test (Wilcoxon rank-sum test) for independent samples. Correlations between IL-6 and log pyuria were examined by means of Spearman's R.

5.5 Results

104 women with symptoms of the Overactive Bladder gave their consent to participate and provided catheter specimens of urine for the study. The patient demographics are described in table 2.1

Because of the exponential nature of increasing pyuria the log of the urinary white cell count was calculated. The median IL-6 level in the pyuria group was 2.36 pg/ml (IQ range 0.56-7.59) and the median IL-6 level in the non-pyuria group was 0.52 pg/ml (IQ range 0.16-1.91) see figure 5.1. The mean and 95% confidence interval has also been calculated because 95% CI plots are often better at illustrating the distributions to the human eye in figure 5.2. This would not be appropriate if non-parametic analysis had failed to identify a difference. There was a significant difference in IL-6 levels between those with pyuria and those without (95% CI of difference -2.9 to -0.11, p=0.34, t=-2.1) Figure 5.2.

It was found that there was a correlation between the log white cell count and IL-6 Spearman's R = 0.5, p=0.001 (Figure 5.3). There was no relationship between IL-6 and symptoms of urgency, frequency and incontinence.

Figure 5.1 Box plot showing IL-6 levels in pg/ml comparing patient samples with pyuria against no pyuria

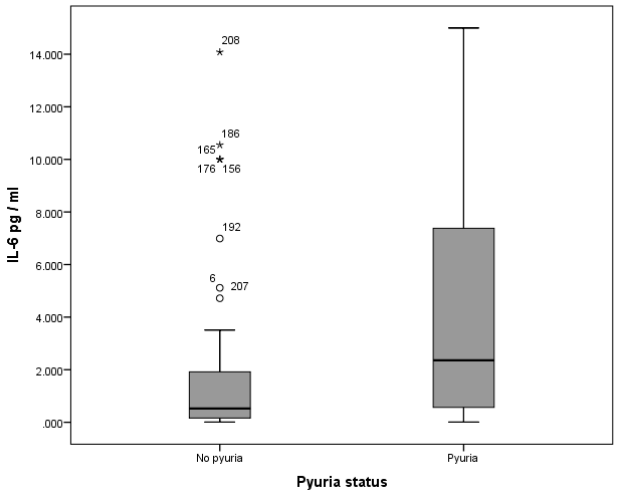


Figure 5.2 Mean Il-6 and 95% CI comparing pyuria against no pyuria

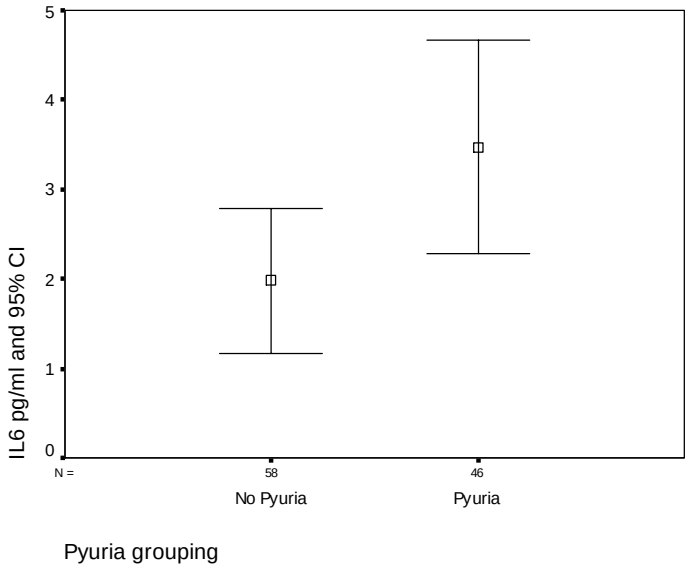
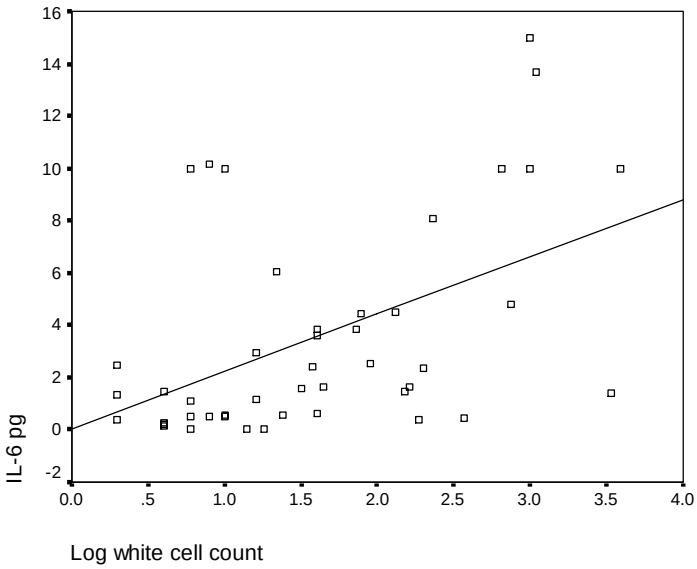


Figure 5.3 Scatter plot of Il-6 and log urinary white cell count showing correlation



5.6 Discussion

Several studies have identified various bladder epithelial cell derived mediators which are thought to be responsible for the vigorous neutrophil response in the bladder after acute bacterial infection (208;215). IL-6 is by far the most prominent cytokine detected in the urine of patients with acute UTI (198). There is also some evidence of multiple pathways in bladder epithelial cells for triggering the IL-6 response as an adaptation to avoid inactivation by uropathogenic bacteria (216). Identifying a cytokine response in patients with OAB supports the premise that some patients with OAB have an inflammatory aetiology to their condition.

The role of bacterial infection in the aetiology of the overactive bladder

The pyuria found in these patients with OAB was associated with a marker of inflammation, namely IL-6. This provides some additional independent verification of the significance of pyuria detected by microscopy of fresh unspun specimens of urine. Whilst there was a correlation between the pyuria and IL-6 this was not sufficiently strong to encourage the view that IL-6 might prove to be a useful surrogate marker of pyuria.

The association between IL-6 and urine infection in the literature also leads us to hypothesise on an infective aetiology in these patients. In addition, there is growing evidence in the literature that the magnitude of the cytokine response and the type of cytokine found in the urine is influenced by the virulence of the pathogen. IL-6 has been particularly associated with fimbriae (208;217). One study by Wullt et al(218) showed a larger IL-6 response to bladder infection in mice due to P-fimbriated *E.coli* as compared to non-fimbriated *E.coli* The findings in this study support the view that OAB symptoms in some patients are associated with a urothelial inflammatory reaction which may be caused by urine infection.

Chapter 6

The presence of Intracellular bacteria in bladder epithelium of patients with OAB

6.1 Hypothesis

Uropathogenic bacteria invade bladder urothelial cells.

6.2 Background

The invasion assay used to determine intracellular invasion in this study was adapted and developed from Hultgren et al. 2001 (151), who used a method of determining intracellular invasion and growth of uropathogenic *E.coli* using a human bladder epithelial cell line. They infected the preparation with 100 *E.coli* per epithelial cell. After two hours they applied the antibiotic Gentamicin to kill the extant extracellular *E.coli*.

Urine specimens are likely to contain multiple bacterial species and not a pure culture of *E.coli*. Therefore, it was necessary to extend the Hultgren et al. 2001(151) method to address a wider bacterial community. The data in chapter 3 has also shown a dominance of *Group B Streptococcus*, *Enterococcus. sp* and *Lactobacillus spp.* in the patient group.

A recent study conducted by Bean et al (219) examined the antibiotic resistance of common uropathogens, particularly *E.Coli* from community and hospital based patients in East London. They published a table of resistance frequencies as below (Table 6.1). The table demonstrates low resistance in the community to Amoxicillin (12.4% resistance), Cefalexin (8.8% resistance),

Gentamicin (5.2% resistance), Nitrofurantoin (5.4% resistance) and Cefpodoxime (6.2% resistance).

Table 6.1Frequency of antibiotic susceptibility in relation to sex

Antibiotic	Female (*n* = 10157)		Male (*n* = 1656)	
	n	*n* (%) Resistant	*n*	*n* (%) Resistant
Ampicillin	10153	5460 (53.8)	1652	1051 (63.6)
Amoxicillin/clavulanate	9178	1139 (12.4)	1491	310 (20.8)
Cefalexin	10139	892 (8.8)	1643	321 (19.5)
Ciprofloxacin	10137	1038 (10.2)	1649	374 (22.7)
Gentamicin	10149	525 (5.2)	1655	214 (12.9)
Nitrofurantoin	10134	551 (5.4)	1647	142 (8.6)
Trimethoprim	10138	3989 (39.3)	1652	748 (45.3)
Cefpodoxime	8512	525 (6.2)	1418	215 (15.2)

Ann Clin Microbiol Antimicrob. 2008; 7: 13. Published online 2008 June 18. doi: 10.1186/1476-0711-7-13.

In deciding upon an appropriate antibiotic to use for selective destruction of extracellular microbes, it is important to consider the intracellular concentration of the antibiotics, which must be kept to a minimum so as to avoid elimination of intracellular bacteria. In 2006, Barcia-Macay et al (220) analyzed the pharmacodynamic factors influencing the activities of various antibiotics against intracellular forms of *S. aureus*. They measured the intracellular concentrations in relation to the time-lengths of exposure. Table 6.2 describes the intracellular and extracellular concentration of these antibiotics. The table demonstrates low intracellular to extracellular concentration for the antibiotics Gentamicin, Linezolid, Peniciliin V, Nafcillin, Amoxicillin and Ciprofloxacin.

Table 6.2. Cellular accumulation factor of antibiotics in THP-1 cells after 24 h of incubation at a fixed extracellular concentration

Antibiotic[a]	Cellular accumulation[b]	Extracellular concn (mg/liter)
Azithromycin	37.8 ± 1.3	5[c]
Telithromycin	27.9 ± 1.3	2[d]
Gentamicin	4.4 ± 0.1	250[c]
Linezolid	0.5 ± 0.0	250[c]
Penicillin V	1.2 ± 0.1	150[c]
Nafcillin	2.6 ± 0.1	400[c]
Amoxicillin	1.0 ± 0.1	150[c]
Oxacillin	4.0 ± 0.1	250[c]
Teicoplanin	7.4 ± 0.2	150[c]
Vancomycin	6.3 ± 0.1	100[c]
Oritavancin	148.0 ± 12.0	25[d]
Rifampin	17.6 ± 0.9	50[c]
Ciprofloxacin	5.1 ± 0.1	4.3[d]
Levofloxacin	7.0 ± 0.6	4[d]
Garenoxacin	9.1 ± 0.3	4[d]
Moxifloxacin	7.6 ± 0.3	4[d]

a The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular accumulation, b Apparent cellular concentration-to-extracellular concentration ratio, based on a cell volume of 5 µl per mg of cell protein. c A concentration larger than the Cmax was used because of a lack of sensitivity of the microbiological assay.
d Concentration corresponding to the Cmax
Taken from Pharmacodynamic Evaluation of the Intracellular Activities of Antibiotics against Staphylococcus aureus in a Model of THP-1 Macrophages
Maritza Barcia-Macay, Cristina Seral,† Marie-Paule Mingeot-Leclercq, Paul M. Tulkens, and Françoise Van Bambeke* Antimicrobial Agents and Chemotherapy, March 2006, p. 841-851, Vol. 50

These data therefore support the use of gentamicin and amoxicillin for invasion assays, as resistance to them in the community is infrequent and they achieve low intracellular concentrations. Gentamicin is an aminoglycoside antibiotic which works by binding to the bacterial 30S

ribosomal subunit, preventing the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, this leaves the bacteria unable to synthesize vital proteins. It is particularly useful against Gram negative rods (221). Amoxicillin is a β-Lactam antibiotic, which is bactericidal, and acts by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. It is used against Gram positive bacteria (222).

Whilst this project focused heavily on microbiological scrutiny of the phenomenon of interest I felt it was important to explore a complementary line of evidence so as not to rely wholly on one set of techniques. Therefore, microscopic evidence of uropathogenic bacterial invasion of the urothelium was sought.

A variety of methods have been used to visualise and discriminate intracellular and surface-adherent bacteria in cells. There are many direct microscopy methods with specific and non-specific staining techniques, including electron microscopy and immuno-fluorescent bacterium-specific antibodies and fluorescence techniques. Intracellular invasion assays such as described in Chapter 7 rely on the recovery of viable bacteria after their internalisation by urothelium cells, however, they do not discriminate between efficient bacterial invasion in combination with low intracellular survival or low invasiveness coupled to effective intracellular survival. Such assays can be complemented with microscopic evaluation of infected samples.

There are a number of fluorescent dyes that have been used to visualise intracellular bacteria.

Acridine orange is a nucleic acid selective fluorescent cationic dye. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When bound to DNA, it has an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When it associates with RNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red). Acridine orange has been used to enumerate intracellular organisms in bronchoalveolar lavage cells and HeLa cells, using crystal violet as a counter-stain as it is not cell permeable (223).

6.3 Method development and results

6.3.1 Microbiology methods

Patients with overactive bladder symptoms, attending the Incontinence clinic for the first time provided CSU specimens as described (2.4.3.2). An aliquot of urine was sent for routine culture and enhanced culture as described (2.6 and 2.9.1). Another aliquot underwent routine microscopy (2.4.1). The patients were not taking any antibiotics.

Urine was centrifuged at 8000rcf and the supernatant discarded. The sediment obtained was re-suspended in 400µL of sterile PBS and split into

two aliquots of 200µL. To re-concentrate these two halves each aliquot was centrifuged again as above and the sediment was collected. One aliquot was re-suspended in 1ml of Eagles minimum Growth medium supplemented with Non-Essential Amino Acids (NEAA) - 1% (Sigma), foetal bovine serum-5% (Sigma) and glutamine (Sigma) to 2mmol. This was to ensure a viable and supportive environment for the survival of the urothelial cells. To the medium, 200µg per ml of Gentamicin (Amdipharm plc) and 200µg per ml of Amoxicillin (Sigma) were added. This was to inhibit the growth and kill any extracellular bacteria attached to the urothelial cells. A viable epithelial cell count was conducted using 10µL of the suspension and 10µL of 1% Trypan blue (Sigma). 100µL of the suspension was plated on CBA and FAA in dilutions to $1x10^7$ (time zero count). This was in order to get an initial bacterial count from the urothelial cells which would represent both intracellular and extracellular bacteria. The rest of the suspension was seeded onto one well of a 12 well culture dish and incubated at 37°C, 5% CO_2 for 24 hrs. The second aliquot was plated as per 2.9.2. After 24 hours, the cells were viewed for adherence to the culture dish and for the presence of bacteria using a light microscope (Leica) at X 400 magnification. The supernatant was removed and the cells were washed three times with 400µL PBS. This was done to remove any remaining attached extracellular bacteria. The supernatant and every wash were plated in dilutions to $1x\ 10^3$ on CBA and FAA and incubated for 48hrs at 37°C, 5% Co_2 and 7 days in an anaerobic incubator at 37°C respectively. These bacterial counts allowed me to determine the effectiveness of the antibiotics and of washing the cells. Triton X 0.1% (Sigma) 200µl was added to the cells in the well to break down the cell membranes and release any

intracellular bacteria which could then be cultured. The cell suspension was cultured to 1x 10^3 dilutions on CBA and FAA as above.

All bacteria cultured were counted and sub-cultured as per section 2.9.3, biochemical identification tests were carried out as per section 2.10, 16srRNA gene typing was carried out as per section 2.11 and the samples were stored as per section 2.11.3.

6.3.2 Results of 1st Assay

First Sample (Study number 787). The patient was symptomatic and aged 54 years. Routine microscopy showed no pyuria. 195mls of urine was collected by CSU. Both the routine culture (10^5cfu/ml) and enhanced culture (10^2cfu/ ml) were negative. The reported frequency was 10 episodes per 24 hours, the urge score was 5 and the incontinence episodes reported were 1 per 24 hours. The total bacterial counts in both the aerobic and anaerobic cultures are described below in fig 6.1. There was a rise in total bacterial count after 24hrs of incubation in the culture media of 2.66x10^3cfu/ml (starting count 8.6x10^{-1} cfu/ml and count after 24 hours incubation 2.66x10^3 cfu/ml). This is labelled as 'supernatant 24hrs' on the graph. There was an increase of total bacterial count after the addition of Triton X of 6.18x10^1cfu/ml. This is labelled as 'Triton X' on the graph. The viable urothelial cell count was 1x10^5 cells per ml. Table 6.3 shows the morphologically different bacterial species identified by culture at each stage of the assay. It demonstrates the efficacy of the antibiotics and washes, since the colony count gradient is negative over

this phase. For the anaerobic culture it also demonstrates the presence of intracellular bacteria in the sample as the number of morphologically different bacteria increases after the addition of Triton X. Table 6.4 describes the identity of the bacterial species found in the assay. It is interesting to note that in the aerobic culture, a *Corynebacterium. sp* was identified after the addition of Triton X only, implying that it was growing intracellularly and in the anaerobic culture *Streptococcus* sp and *Lactobacillus* spp were found intracellularly.

Figure 6.1Total bacterial counts at different stages of the Intracellular bacterial isolation assay for sample 787 aerobic and anaerobic cultures. (Time zero=initial bacterial count at the start of the assay (cfu ml-1), Supernatant 24hrs= the bacterial count after 24hrs incubation of the urothelium with Gentamicin and Amoxicillin (cfu ml-1), Wash1= the bacterial count after washing the incubated cells in PBS for the first time (cfu ml-1), wash 2= the bacterial count after washing the incubated cells in PBS for the second time (cfu ml-1), wash 3= the bacterial count after washing the incubated cells in PBS for the third time(cfu ml-1), Triton x= the bacterial count after the addition of triton X. This corresponds to the intracellular bacterial count (cfu ml-1).

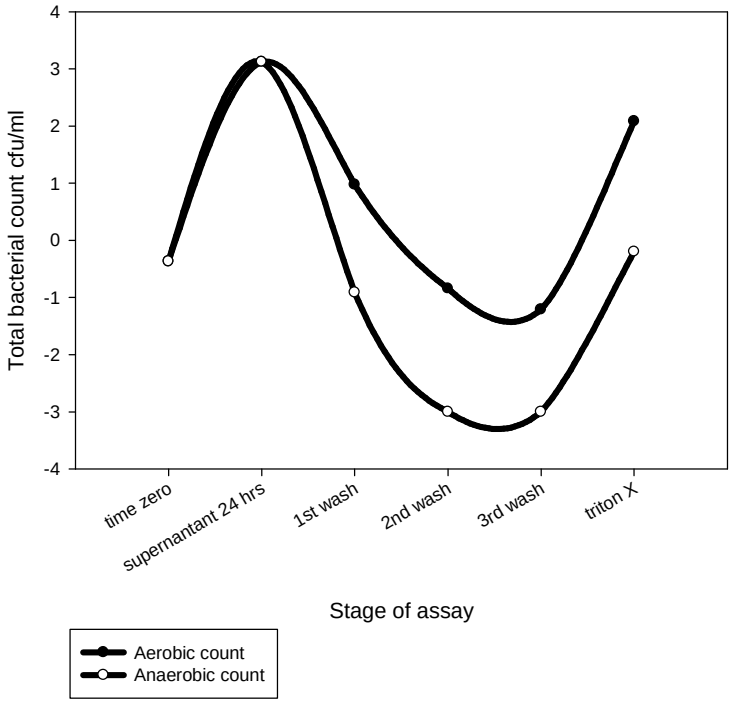


Table 6.3. The number of morphologically different colonies identified at each stage of the Intracellular bacterial isolation assay for sample 787 aerobic and anaerobic culture (Time zero=initial bacterial count at the start of the assay (cfu ml^{-1}), Supernatant 24hrs= the bacterial count after 24hrs incubation of the urothelium with gentamicin (cfu ml^{-1}), Wash1= the bacterial count after washing the incubated cells in PBS (cfu ml^{-1}), wash 2= the bacterial count after washing the incubated cells in PBS for the second time (cfu ml^{-1}), wash 3= the bacterial count after washing the incubated cells in PBS for the third time(cfu ml^{-1}), Triton x= the bacterial count after the addition of triton X. This corresponds to the intracellular bacterial count (cfu ml^{-1})

Wash	No. of different colonies aerobic	No of different colonies anaerobic
Time zero	3	3
Supernatant 24hrs	3	4
1^{st} wash	1	1
2^{nd} wash	1	0
3^{rd} wash	1	0
Triton x	1	2

Table 6.4. The identity of the different colonies at each stage of the Intracellular bacterial isolation assay for sample 787 aerobic and anaerobic culture.

Bacterial isolate (CO_2= identified in aerobic culture; 5% CO_2, Anaerobic = identified in anaerobic culture, Triton X =identified in culture after the addition of Triton X)	**Bacterial identification using 16srRNA gene typing**
787 A CO_2	***Strep.anginosus***
787 B CO_2	***Lactobacillus.* sp**
787 C CO_2	***Actinomyces* sp**
787 A Anaerobic	***Lactobacillus* sp**
787 B Anaerobic	***Lactobacillus* sp**
787 C Anaerobic	***Strep.anginosus***
787 D Anaerobic	***Strep. agalactiae***
787 A Triton X CO_2	***Corynebacterium* sp**
787 A Triton X Anaerobic	***Lactobacillus* sp**
787 B Triton X Anaerobic	***Strep. agalactiae***

6.3.3 Conclusion from 1st Assay

The experiment showed that despite addition of antibiotic to the growth medium, the supernatant after 24hrs. of incubation, sustained growth of bacteria. This implied resistance to the antibiotics. However, the three washes of the sediment reduced the total bacterial count, which increased after the addition of triton X. In addition, bacteria not cultured in the washes were present after the addition of Triton X. I concluded that the bacteria cultured after this step were therefore a combination of cell adherent bacteria and intracellular bacteria. Consequently, in order to identify the intracellular bacteria adequately, it was concluded that it was necessary to eliminate all extracellular bacteria and so the assay would have to be modified to achieve this.

6.3.4 Method development- washing urinary sediment cells before incubating them in growth media

Two specimens from new patients, not on antibiotic treatment were used. The assay was modified in an attempt to reduce the bacterial load in the urine sediment by washing the cells before incubation in growth medium. In addition, after incubation, all of the sediment was collected after gentle washing with PBS and centrifuge at 3000rcf. The centrifugation was reduced to 300rcf to protect the urothelial cells from damage. This process was

followed by washing as described above in 6.3.1. Bacteria cultured were treated as described above in section 6.3.1.

6.3.5 Results of method development- washing urinary sediment cells before incubating them in growth media

Two symptomatic patients provided CSU specimens as described previously. Patient 837 reported frequency of 9 episodes per 24 hours, an urgency score of 4 and 2 incontinence episodes per 24 hours. Patient 841 reported frequency of 11 episodes, an urgency score of 4 and 1 incontinence episode. One patient showed pyuria on microscopy. This patient grew a *Streptococcus* sp. on both routine (10^5cfu ml^{-1}) and enhanced cultures (10^2cfu ml^{-1}). The second patient, without pyuria, had a negative routine culture (10^5cfu ml^{-1}) and grew a *Streptococcus* sp. on the enhanced culture (10^2cfu ml^{-1}). The average viable epithelial cell count in the two specimens was $1x10^5$ cells ml^{-1}. The total bacterial counts at each step of the assay are illustrated below in figures 6.2 and 6.3. In each assay, the total bacterial count was reduced from the initial count through to the third washing step by an average of $1.09x10^3$cfu ml^{-1} (sd=$1.64x10^3$cfu ml^{-1}), there was an average rise in the total bacterial count by $5.37x10^2$cfu ml^{-1} (sd=$5.93x10^3$cfu ml^{-1}) after incubation in the culture medium for 24hrs, there was an average rise of total bacterial count after the addition of triton X of $3.02x10^2$cfu ml^{-1} (sd=$3.56x10^2$cfu ml^{-1}). Tables 6.5 and 6.7 show the number of morphologically different bacterial species identified by culture at each stage of the assay. There was no reduction in the number of different isolates seen after washing but a reduction in the total bacterial load. Table

6.6 shows the identity of the different bacterial species isolated. One of the colonies went unidentified because of failure to grow it in a pure culture. The table shows the presence of *Strep.pasterunis* and *Enterococcus. faecalis* intracellularly. Sample 841 initially cultured *Staphylococcus. aureus* and yet after the addition of Triton X only *Streptococcus. agalactiae*. Again, this suggests that the *Streptococcus*. *agalactiae* was an intracellular organism. These organisms were not grown as extracellular organisms in the initial culture. This could be explained by low extracellular numbers.

Figure 6.2 Sample 837: Total bacterial counts at different stages of the Intracellular bacterial isolation assay for sample 837 aerobic and anaerobic cultures with the addition of an extra washing stage. Viable cell count 2x 10^5 ml^{-1}. (Initial count=initial bacterial count at the start of the assay (cfu ml^{-1}), Wash1= the bacterial count after washing the cells in PBS (cfu ml^{-1}), wash 2= the bacterial count after washing the cells in PBS for the second time (cfu ml^{-1}), wash 3= the bacterial count after washing the cells in PBS for the third time(cfu ml^{-1})Time zero = bacterial count after addition of the urothelial cells to the growth media, Supernatant 24hrs= the bacterial count after 24hrs incubation of the urothelium with antibiotics (cfu ml^{-1}), , 1a= bacterial count after one wash of the cultured cells with PBS (cfu ml^{-1}), 2a= bacterial count after two washes of the cultured cells with PBS (cfu ml^{-1}), 3a= bacterial count after three washes of the cultured cells with PBS (cfu ml^{-1}), Triton x= the bacterial count after the addition of triton X. This corresponds to the intracellular bacterial count (cfu ml^{-1}).

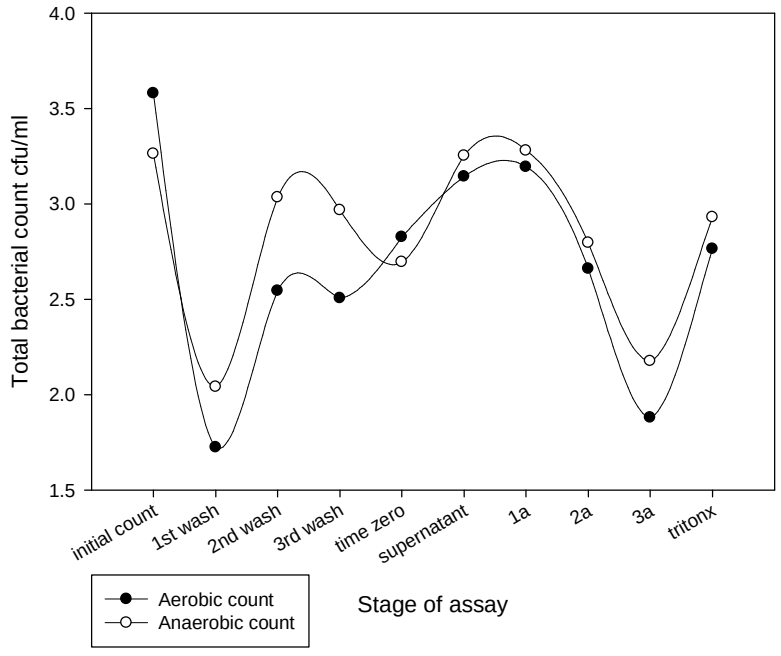


Table 6.5: The number of morphologically different colonies identified at each stage of the Intracellular bacterial isolation assay for sample 837 aerobic and anaerobic culture with the addition of an extra washing stage. (Initial count=bacterial count at the start of the assay (cfu ml^{-1}), Wash1= the bacterial count after washing the cells in PBS (cfu ml^{-1}), wash 2= the bacterial count after washing the cells in PBS for the second time (cfu ml^{-1}), wash 3= the bacterial count after washing the cells in PBS for the third time(cfu ml^{-1})Time zero = bacterial count after addition of the urothelial cells to the growth media, Supernatant 24hrs= the bacterial count after 24hrs incubation of the urothelium with antibiotics (cfu ml^{-1}), 1a= bacterial count after one wash of the cultured cells with PBS (cfu ml^{-1}), 2a= bacterial count after two washes of the cultured cells with PBS (cfu ml^{-1}), 3a= bacterial count after three washes of the cultured cells with PBS (cfu ml^{-1}), Triton x= the bacterial count after the addition of triton X. This corresponds to the intracellular bacterial count (cfu ml^{-1}).

Wash	No. of different colonies aerobic culture	No. of different colonies anaerobic culture
Initial Count	4	3
1st wash	4	4
2nd wash	4	4
3rd wash	4	4
Time zero	4	4
Supernatant 24hrs	1	3
1st wash	1	2
2nd wash	1	2
3rd wash	1	1
Triton x	1	1

Table 6.6. The identity of the colonies identified at each stage of the intracellular bacterial isolation assay for sample 837 aerobic and anaerobic culture.

Bacterial isolate (CO_2= identified in aerobic culture; 5% CO_2, Anaerobic = identified in anaerobic culture, Triton X =identified in culture after the addition of Triton X)	**Bacterial identification using 16srRNA gene typing**
837 A CO_2	***Unknown (unable to grown in pure culture)***
837 B CO_2	***Strep.pasterunis***
837 C CO_2	***Eschericia. coli***
837 D CO_2	***Enterococcus. faecalis***
837 A Anaerobic	***Enterococcus. faecalis***
837 B Anaerobic	***Alloscardovia. omnicolens***
837 C Anaerobic	***Strep.pasterunis***
837 D Anaerobic	***Strep. pasterunis***
837 A Triton X CO_2	***Strep.pasterunis***
837 A Triton X Anaerobic	***Enterococcus. faecalis***

Figure 6.3 Total bacterial counts at different stages of the Intracellular bacterial isolation assay for sample 841 aerobic and anaerobic culture with the addition of an extra washing stage. Viable cell count $1x10^3$ ml^{-1}

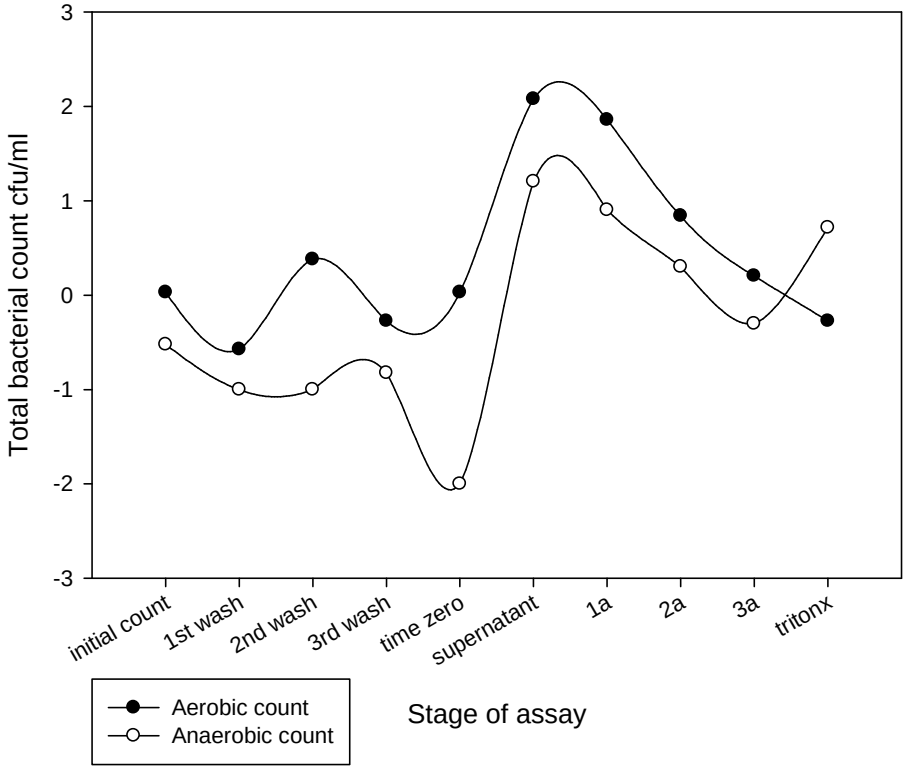


Table 6.7: The number of morphologically different colonies identified at each stage of the Intracellular bacterial isolation assay for sample 841 aerobic culture with the addition of an extra washing stage. (Initial count= bacterial count at the start of the assay (cfu ml^{-1}), Wash1= the bacterial count after washing the cells in PBS (cfu ml^{-1}), wash 2= the bacterial count after washing the cells in PBS for the second time (cfu ml^{-1}), wash 3= the bacterial count after washing the cells in PBS for the third time(cfu ml^{-1})Time zero = bacterial count after addition of the urothelial cells to the growth media, Supernatant 24hrs= the bacterial count after 24hrs incubation of the urothelium with antibiotics (cfu ml^{-1}), , 1a= bacterial count after one wash of the cultured cells with PBS (cfu ml^{-1}), 2a= bacterial count after two washes of the cultured cells with PBS (cfu ml^{-1}), 3a= bacterial count after three washes of the cultured cells with PBS (cfu ml^{-1}), Triton x= the bacterial count after the addition of triton X. This corresponds to the intracellular bacterial count (cfu ml^{-1}).

Wash	No. of different colonies aerobic cultures	No. of different colonies anaerobic cultures
Initial Count	1	1
1st wash	1	1
2nd wash	1	1
3rd wash	1	1
Time zero	1	1
Supernatant 24hrs	1	1
1st wash	1	1
2nd wash	1	1
3rd wash	1	1
Triton x	1	1

6.3.6 Conclusions from method development- washing urinary sediment cells before incubating them in growth media

The extra washing steps reduced the bacterial count, but there was still a rise of total bacterial count after incubation in the culture media, although this was not great ($<10^1$). There remained a possibility that resistant bacteria were propagating. The possible paths for development of this assay were to: 1. Change the antibiotics used, 2. Add another antibiotic, 3. Use another method of killing extracellular bacteria such as enzyme lysis.

6.3.7 Further development of the invasion assay-adding a further antibiotic or enzymes

The most common bacterial species isolated from the patients were *Enterococcus spp, Streptococcus spp* and *Lactobacillus spp*. *Enterococcus* antimicrobial susceptibility patterns vary among species of Enterococci, they are commonly resistant to high-level aminoglycosides and can also acquire resistance to vancomycin or the penicillins (224). *Streptococcus* strains display variable antibiotic resistance to azithromycin, clindamycin, erythromycin and tetracycline (225). *Lactobacillus spp* show antibiotic susceptibility patterns to β-lactam antibiotics (penicillin and ampicillin). Several strains are sensitive to tetracyline, however, most strains are resistant to erythromycin and the aminoglycoside group (226).

Linezolid has a very poor intracellular penetration and would be a good additional antibiotic. It is not commonly used, particularly for the treatment of

UTI and resistance would be expected to be low in the bacterial population. Linezolid is a synthetic antibiotic of the oxazolidinone class used for the treatment of infections caused by multi-resistant strains. Linezolid is effective against Gram-positive pathogens, notably *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus pneumoniae*, and *Streptococcus pyogenes.* It has almost no effect on Gram-negative bacteria and is only bacteriostatic against most *Enterococcus* species. Linezolid is also active against some anaerobic strains (227).

6.3.8 Methods of elimination of bacteria without the use of antibiotics; the use of enzymes.

Lysozyme, Lysostaphin and mutanalysin are three enzymes, which are active against bacteria. There are others but these are commonly used to eliminate bacteria from microbial assay experiments (228;229).

Lysozyme is found in high concentrations (>500 µg/ml) in mucosal surface fluids such as those lining the upper respiratory tract (230). Lysozyme is expressed by the epithelia and is also a major component of the granules of neutrophils, which may be recruited when the mucosa is acutely inflamed (231). Lysozyme has two distinct antibacterial activities (231). Its enzymatic muramidase activity hydrolyzes the conserved ß-1,4 glycosidic bond between *N*-acetyl glucosamine and *N*-acetyl muramic acid, the disaccharide residues of the bacterial peptidoglycan backbone. Hydrolysis of the glycan strands

leads to degradation of the cell wall and bacterial lysis. In addition, an antibacterial activity is observed with catalytically inactive lysozyme. This activity has been attributed to the disruption of bacterial membrane function by an inherent nine amino acid cationic antimicrobial peptide (CAMP). It has been used in invasion assays against Gram negative rods (232) (233);(234).

Lysostaphin is a zinc-containing metalloenzyme which has a specific lytic action against *Staphylococcus spp* by hydrolyzing glycylglycine bonds in the polyglycine bridges which form cross links between glycopeptide chains in the cell wall peptidoglycan of *S. aureus* cells. Its lytic activity was shown in tests with over 50 strains of *Staphylococcus.aureus*, and shown to be independent of phage type, resistance to other antibiotics, and the condition of the cell wall or degree of capsulation of the bacterium (235). It is widely used in cellular invasion assays (236) (237).

Mutanolysin is a N-Acetyl Muramidase. It is a muralytic enzyme that cleaves the N-acetylmuramyl-β(1-4)-N-acetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide. Mutanolysin provides cell lysis and is used for the isolation of biomolecules and RNA from the bacterial cell. Mutanolysin is particularly used for lysis of *Listeria. spp* and other Gram positive bacteria such as *Lactobacillus. spp* and *Lactococcus. Spp (238) (239)*.

The addition of these enzymes would potentially enhance the assay by achieving a greater extracellular kill.

6.3.9. Methods-Testing bacteria for sensitivity to proposed antibiotics and enzymes for the invasion assays

The six most commonly found bacteria in the patient population were selected for this study. *Streptococcus. anginosus, Enterococcus. faecalis, Escherichia.coli, Lactobacillus.gasseri*, *Corynebacteria.*sp *and Proteus.mirabilus. Pseudomonas* sp*.* was found in higher numbers as compared to *Proteus* sp but it is very difficult to work with, forming a film of slime like colony, difficult to enumerate and so *Proteus.mirabilis* was used instead. Colonies were obtained from the stored pure cultures isolated from patient samples. The strains were inoculated into a Columbia blood agar plate and incubated overnight at 37°C at 5% CO_2. The following day, a single colony of each bacterium was inoculated into 15mls of Brain Heart Infusion broth (Sigma) and incubated overnight at 37°C at 5% CO_2 in a shaking incubator (Progen scientific). As described by Hultgren et al. (151), Eagles Minimal Essential Medium (Sigma) was made up with 10% fetal bovine serum (Sigma), glutamine (Sigma) to 2mmol in 500mls and 1% non-essential amino acids (Sigma). The following additives were then added so that 14 different combinations of antibiotics and enzymes could be tested for their activity against the selected bacteria (Total volume 10mls):

1. 50µL Gentamicin from a stock solution of 40mg/ml (final concentration of 200µg/ml)

2. 1ml of Linezolid from a stock solution of 2mg/ml (final concentration of 200µg/ml)
3. 10 µL Amoxicillin from a stock solution of 200mg/ml (final concentration of 200µg/ml)
4. Gentamicin, Linezolid and Amoxicillin in the concentrations as above
5. Gentamicin and Linezolid in the concentrations as above
6. Gentamicin and Amoxicillin in the concentrations as above
7. Amoxicillin and Linezolid in the concentrations as above
8. Gentamicin in the concentration as above and Enzymes (Lysostaphin 50µg/ml, Mutanolysin 50µg/ml and Lysozyme 50µg/ml)
9. Linezolid and Enzymes in the concentrations as above
10. Amoxicilin and Enzymes in the concentrations as above
11. Gentamicin, Linezolid, Amoxicillin and Enzymes in the concentrations as above
12. Gentamicin, Linezolid and Enzymes in the concentrations as above
13. Gentamicin and Amoxicillin and Enzymes in the concentrations as above
14. Amoxicillin and Linezolid and Enzymes in the concentrations as above

The overnight bacterial cultures were centrifuged at 8000rcf for 5 minutes at room temperature using an Eppendorf 5804R centrifuge. The supernatant was gently removed and the bacterial pellet was re-suspended in 10ml of cell culture media as described above. The bacterial suspension was adjusted to an optical density at 600nm (OD_{600}) of 0.5 corresponding to $1x10^8$cfu/ml of bacteria. 250µL of this suspension was added to 10mls of Growth media as

above and the optical density of this bacterial suspension was adjusted to an optical density of 600nm (OD_{600}) of 0.05 corresponding to $1x10^6$cfu/ml of bacteria. This was further diluted to a final bacterial concentration of $1x10^3$cfu/ml. Three final concentrations of bacterial suspension were thus used. These concentrations were confirmed by plating to a dilution of $1X10^8$ with 200µL of each solution (100µL on each half) placed on a Columbia Blood Agar (CBA) plate (E&O laboratories).

To a 96 well plate (Sigma), 1ml of the Growth media was added to each well. For each bacterial isolate, 48 wells were inoculated with each bacterial concentration ($1X10^8$ shown in red, $1x10^6$ shown in green and $1x10^3$ shown in blue in table 6.8) and the antibiotic / enzyme mixtures described above were added as described in the following table (6.8), in triplicate, to test for bacterial resistance:

Table 6.8. 96 Well plate showing sequence of antibiotics and enzymes added to test bacterial strains for resistance. *G= Gentamicin, L= Linezolid, A= Amoxicillin, E= Enzymes (Lysostaphin, Lysozyme, mutanalysin)*

	1	2	3	4	5	6	7	8	9	10	11	12
A 1x10^{8}CFU/mL	Media only	Media only	Media only	Bacteria +media only	Bacteria +media only	Bacteria +media only	G	G	G	L	L	L
B 1x10^{8}CFU/mL	A	A	A	GLA	GLA	GLA	GL	GL	GL	GA	GA	GA
C 1x10^{8}CFU/mL	AL	AL	AL	GE	GE	GE	LE	LE	LE	AE	AE	AE
D 1x10^{8}CFU/mL	GLAE	GLAE	GLAE	GLE	GLE	GLE	GAE	GAE	GAE	ALE	ALE	ALE
E 1x10^{6}CFU/mL	Media only	Media only	Media only	Bacteria +media only	Bacteria +media only	Bacteria +media only	G	G	G	L	L	L
F 1x10^{6}CFU/mL	A	A	A	GLA	GLA	GLA	GL	GL	GL	GA	GA	GA
G 1x10^{6}CFU/mL	AL	AL	AL	GE	GE	GE	LE	LE	LE	AE	AE	AE
H 1x10^{6}CFU/mL	GLAE	GLAE	GLAE	GLE	GLE	GLE	GAE	GAE	GAE	ALE	ALE	ALE

	1	2	3	4	5	6	7	8	9	10	11	12
A 1x10^{3}CFU/mL	Media only	Media only	Media only	Bacteria +media only	Bacteria +media only	Bacteria +media only	G	G	G	L	L	L
B 1x10^{3}CFU/mL	A	A	A	GLA	GLA	GLA	GL	GL	GL	GA	GA	GA
C 1x10^{3}CFU/mL	AL	AL	AL	GE	GE	GE	LE	LE	LE	AE	AE	AE
D 1x10^{3}CFU/mL	GLAE	GLAE	GLAE	GLE	GLE	GLE	GAE	GAE	GAE	ALE	ALE	ALE

As can be seen in Table 6.8, triplicate wells were also inoculated with cell growth media alone and others with a bacterial suspension without any antibiotics or enzymes, these acting as controls. An absorbance reading was taken from each plate using a spectrophotometer and the plates were incubated overnight at 37°C in 5% CO_2. The following day, another absorbance reading was taken to confirm bacterial growth and the wells were inoculated onto Columbia blood agar using multipoint inoculators (Denley) delivering approximately 10uL from each well onto the plate. The plates were then incubated overnight at 37°C in 5% CO_2. Positive growth was noted the following day. In all wells that showed negative growth, the entire well volume (1ml) was inoculated onto a Columbia blood agar plate and incubated at 37°C in 5% CO_2 for 24 hours to confirm negative growth in that well.

All bacterial isolates were tested twice in the same manner to re-evaluate resistance.

6.3.10 Results of sensitivity tests

Tables 6.9, 6.10 and 6.11 show the sensitivity of the bacterial isolates to each combination of antibiotic and enzymes. There was no growth in the culture medium alone but there was growth in every well which contained growth medium with bacterial isolates alone. Culture confirmed the optical densities OD_{600} of 0.5 to correspond to a bacterial count of $1x10^8$cfu/ml, OD_{600} of 0.05 corresponding to $1x10^6$cfu/ml and the bacterial suspension diluted fourfold to $1x10^3$cfu/ml in each case.

The tables show that all bacterial isolates were sensitive to Gentamicin at a concentration of 200µg/ml except *Enterococcus.faecalis* at $1x10^8$cfu/ml, which was sensitive to Linezolid. *E coli* was resistant to Amoxicillin 200µg/ml at all bacterial concentrations. *Streptococcus.anginosus and Proteus. mirabilis* were resistant to Amoxicillin 200µg/ml at bacterial concentrations of $1x10^8$cfu/ml and $1x10^6$cfu/ml. *Lactobacillus. gasseri*, *Corynebacterium.*Sp were sensitive to all antibiotics. The addition of enzymes did not improve the sensitivity of the bacterial isolates to the antibiotics at any concentration of bacteria.

Table 6.9. Bacterial sensitivity at bacterial count of 1x108cfu/ml to combinations of gentamicin 200µg/ml, Linezolid 200µg/ml, amoxicillin 200µg/ml and mutanalysin 50µg/ml, Lysostaphin 50µg/ml and Lysozyme 50µg/ml. G= Gentamicin, L= Linezolid, A= Amoxicillin, E= Enzymes (Lysostaphin, Lysozyme, mutanalysin)

	Bacteria 1x10^{8}cfu/ml	***E.coli***	***E.faecalis***	***Strep.anginosus***	***Corynebacterium. Sp.***	***Lactobacillus. gasseri***	***Proteus. mirabilus***
Consistency of media							
Media+bacteria		Growth	Growth	Growth	Growth	Growth	Growth
G		No growth	Growth	No growth	No growth	No growth	No growth
L		Growth	faint growth	No growth	No growth	No growth	Growth
A		Growth	Growth	Growth	No growth	No growth	Growth
GLA		No growth	No growth	No growth	No growth	No growth	No growth
GL		No growth	No growth	No growth	No growth	No growth	No growth
GA		No growth	Growth	No growth	No growth	No growth	No growth
AL		Growth	faint Growth	No growth	No growth	No growth	Growth
GE		No growth	Growth	No growth	No growth	No growth	No growth
LE		Growth	faint growth	No growth	No growth	No growth	Growth
AE		Growth	Growth	Growth	No growth	No growth	Growth
GLAE		No growth	No growth	No growth	No growth	No growth	No growth
GLE		No growth	No growth	No growth	No growth	No growth	No growth
GAE		No growth	Growth	No growth	No growth	No growth	No growth
ALE		Growth	Growth	No growth	No growth	No growth	Growth

Table 6.10. Bacterial sensitivity at bacterial count of 1x106cfu/ml to combinations of gentamicin 200µg/ml, Linezolid 200µg/ml, amoxicillin 200µg/ml and mutanalysin 50µg/ml, Lysostaphin 50µg/ml and Lysozyme 50µg/ml. G= Gentamicin, L= Linezolid, A= Amoxicillin, E= Enzymes (Lysostaphin, Lysozyme, mutanalysin)

	Bacteria 1x10^{6}cfu/ml	***E.coli***	***E.faecalis***	***Strep.anginosus***	***Corynebacterium. Sp***	***Lactobacillus. gasseri***	***Proteus.mirabilus***
Consistency of media							
Media+bacteria		Growth	Growth	Growth	Growth	Growth	Growth
G		No growth	No growth	No growth	No growth	No growth	No growth
L		Growth	No growth	No growth	No growth	No growth	Growth
A		Growth	No growth	Growth	No growth	No growth	Growth
GLA		No growth	No growth	No growth	No growth	No growth	No growth
GL		No growth	No growth	No growth	No growth	No growth	No growth
GA		No growth	No growth	No growth	No growth	No growth	No growth
AL		Growth	No growth	No growth	No growth	No growth	Growth
GE		No growth	No growth	No growth	No growth	No growth	No growth
LE		Growth	No growth	No growth	No growth	No growth	Growth
AE		Growth	No growth	Growth	No growth	No growth	Growth
GLAE		No growth	No growth	No growth	No growth	No growth	No growth
GLE		No growth	No growth	No growth	No growth	No growth	No growth
GAE		No growth	No growth	No growth	No growth	No growth	No growth
ALE		Growth	No growth	No growth	No growth	No growth	Growth

Table 6.11. Bacterial sensitivity at bacterial count of 1x103cfu/ml to combinations of gentamicin 200µg/ml, Linezolid 200µg/ml, amoxicillin 200µg/ml and mutanalysin 50µg/ml, Lysostaphin 50µg/ml and Lysozyme 50µg/ml. G= Gentamicin, L= Linezolid, A= Amoxicillin, E= Enzymes (Lysostaphin, Lysozyme, mutanalysin)

Bacteria 1x10^{3}cfu/ml	***E.coli***	***E.faecalis***	***Strep.anginosus***	***Corynebacterium. Sp***	***Lactobacillus. gasseri***	***Proteus.mirabilus***
Consistency of media						
Media+bacteria	Growth	Growth	Growth	Growth	Growth	Growth
G	No growth	No growth	No growth	No growth	No growth	No growth
L	faint Growth	No growth	No growth	No growth	No growth	No growth
A	Growth	No growth	No growth	No growth	No growth	No growth
GLA	No growth	No growth	No growth	No growth	No growth	No growth
GL	No growth	No growth	No growth	No growth	No growth	No growth
GA	No growth	No growth	No growth	No growth	No growth	No growth
AL	Growth	No growth	No growth	No growth	No growth	No growth
GE	No growth	No growth	No growth	No growth	No growth	No growth
LE	Growth	No growth	No growth	No growth	No growth	No growth
AE	Growth	No growth	No growth	No growth	No growth	No growth
GLAE	No growth	No growth	No growth	No growth	No growth	No growth
GLE	No growth	No growth	No growth	No growth	No growth	No growth

6.3.11 Conclusion from sensitivity testing

All of the isolates were sensitive to Gentamicin except *Enterococcus faecalis* at $1x10^8$cfu/ml, which was sensitive to Linezolid. For this reason, Gentamicin, Amoxicillin and Linezolid 200µg/ml were used in the following experiment. The addition of enzymes did not seem to make a difference to the susceptibility of the bacteria to the antibiotics. It was therefore concluded that the addition of Linezoid alone to the existing assay would improve the elimination of extracellular bacteria and therefore enhance the retrieval of intracellular bacteria.

6.3.12 Methods- Addition of Linezolid for the elimination of extracellular bacteria

The assays were conducted as described in 6.3 with the addition of Linezolid 200µg/ml into the antibiotic Growth medium. This method was adapted from (232-234) Herbert, Kristian and Ellison.

6.3.13 Results of assay development- Addition of Linezolid for elimination of extracellular bacteria

23 women were studied, mean age 56 (sd=17); 16 had OAB symptoms and provided a CSU; 7 asymptomatic female controls, average age 29 (sd=12), provided a CSU. The patient demographics are described in table 2.1 The routine culture at 10^5cfu/ml were reported as positive in 3 OAB patients, none of whom had pyuria. 15/16 of the OAB samples showed evidence of intracellular bacterial colonisation of the bladder epithelium and only 2/7 of the control specimens showed the same. The intracellular bacteria retrieved from the last stage of the assay were subjected to biochemical tests and sequence analysis (section 2.10 and 2.11) and are shown in table 6.12. The average total intracellular bacterial count retrieved in the patient group was $1.5x10^6$ cfu/ml. By contrast, the average total intracellular bacterial count in the control group was $3.63x10^1$ cfu/ml so that the difference was 5 orders of magnitude, ie $\approx 10^5$ ($F=5.668$, $df=1$, $P=0.027$) (Fig 6.6). The bacterial cell counts at different stages of the assay in both patient and control groups are shown in figures 6.4 and 6.5. By the 3rd wash, after the addition of Gentamicin, Amoxicillin and Linezolid, counts approaching zero were obtained and so cultures obtained after the addition of Triton X are presumed to have intracellular origins.

Table 6.12. Bacterial isolates retrieved from patient and control samples using the intracellular protection assay.

Identity	Patient/Control	Bacteria Retrieved Intracellularily	Intracellular bacterial count cfu/ml^{-1}
1	Patient	*Corynebacterium/ Lactobacillus sp.*	1.22 x10^{2}
2	Patient	*Enterococus faecalis*	1.43 x10^{3}
3	Patient	*Streptococcus agalactiae*	5.73
4	Patient	*Enterococus faecalis/ E.coli*	2.00 x10^{7}
5	Patient	*Enterococus faecalis/ Pseudomonas sp*	2.5 x10^{2}
6	Patient	*Streptococcus anginosus*	5.19x10
7	Patient	*Proteus sp*	3.51x10
8	Patient	*Proteus sp*	7.43 x10^{2}
9	Patient	*Enterococus faecalis*	1.39 x10
10	Patient	*Streptococcus anginosus*	1x10^{6}
11	Patient	*No growth*	0.00
12	Patient	*Streptococcus agalactiae*	1.00 x10^{6}
13	Patient	*No growth*	0
14	Patient	*No growth*	0
15	Patient	*Streptococcus agalactiae*	0.1
16	Patient	*E.coli*	2.00 x10^{2}
1	Control	*No growth*	0.00
2	Control	*Enterococus faecalis*	2.43x10^{2}
3	Control	*No growth*	0.00
4	Control	*Streptococcus agalactiae*	1.08x10
5	Control	*No growth*	0.00
6	Control	*No growth*	0.00
7	Control	*No growth*	0.00

Figure 6.4 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial isolation assay for patients with Overactive bladder, using the antibiotics Gentamicin, Amoxicillin and Linezolid at concentrations of 200µg/ml. 1=Initial bacterial count before incubation with the antibiotics, 2= Bacterial count after 24hrs incubation with the antibiotics, 3=Bacterial count after 3rd wash with PBS, 4=Bacterial count after addition of Triton X. Average epithelial cell count $1x10^5$cells per ml.

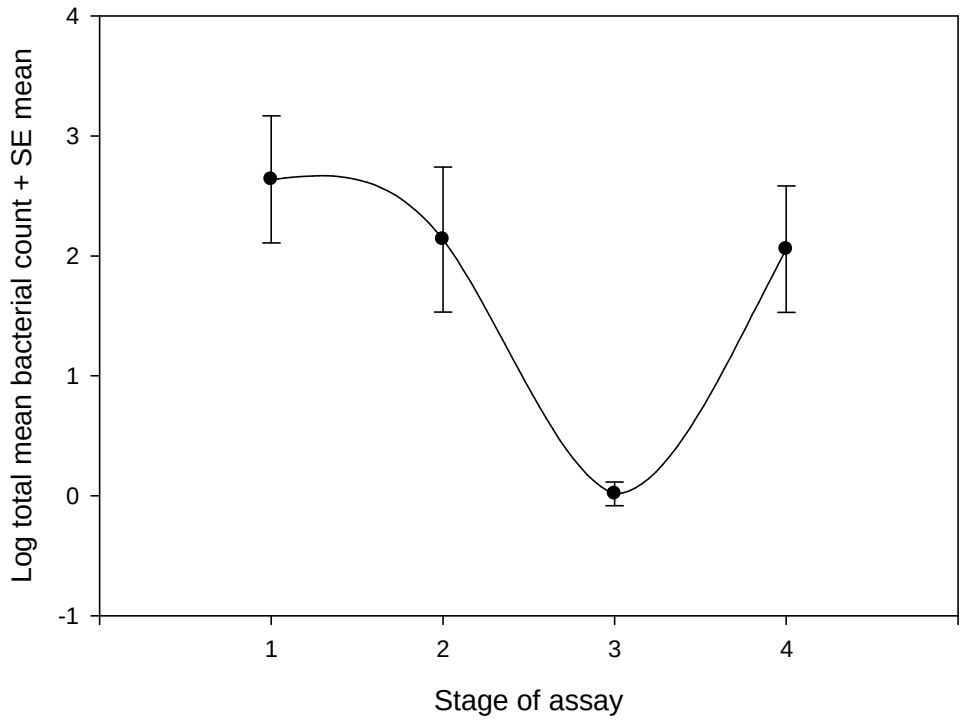


Figure 6.5 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial isolation assay for control volunteers, using the antibiotics Gentamicin, Amoxicillin and Linezolid at concentrations of 200µg/ml. 1=Initial bacterial count before incubation with the antibiotics, 2= Bacterial count after 24hrs incubation with the antibiotics, 3=Bacterial count after 3rd wash with PBS, 4=Bacterial count after addition of Triton X.. Average epithelial cell count 2.3x10^{4}cells per ml.

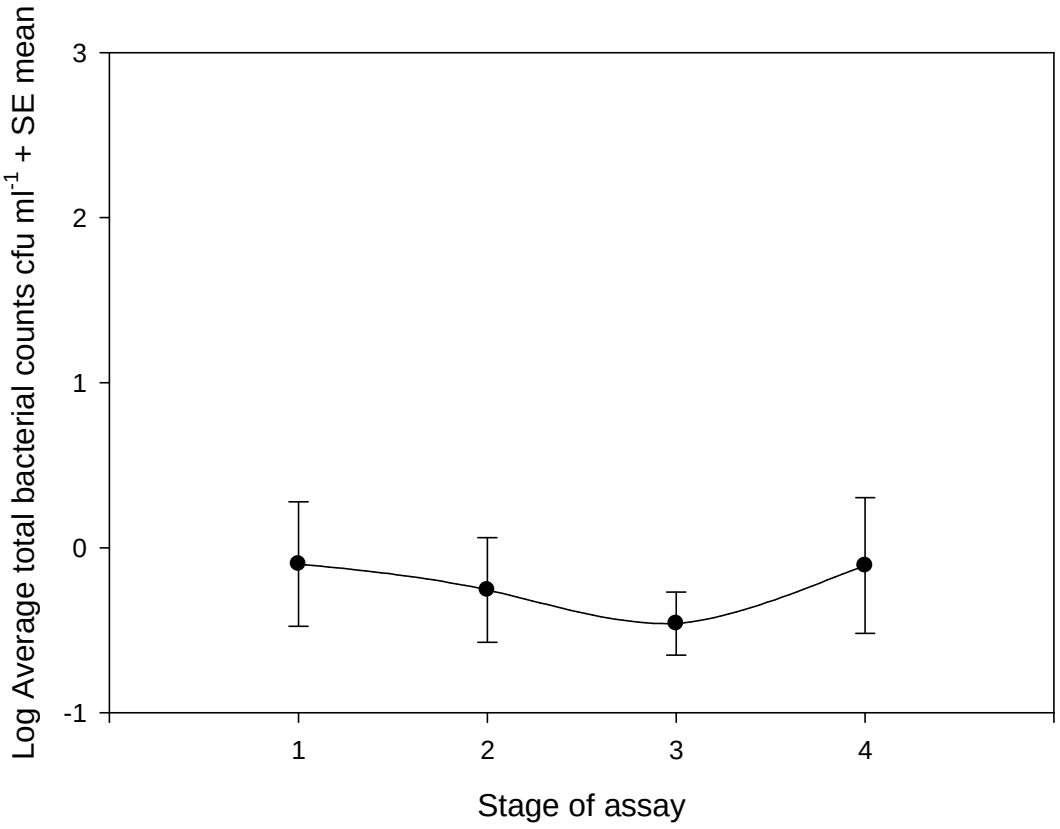
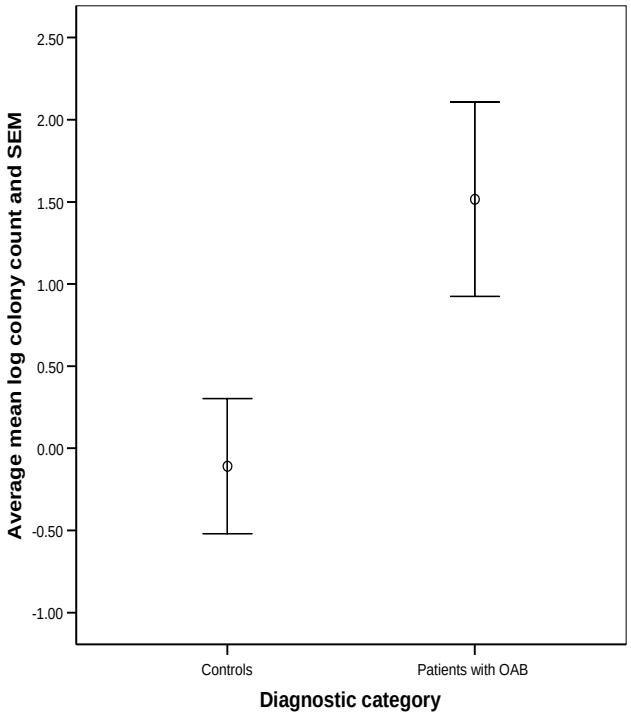


Figure 6.6 Log total intracellular bacterial counts cfu/ml-1 after the addition of Triton X and SE of the mean comparing the Overactive bladder patient group and the control volunteers.



6.3.14 Methods of microscopic examination of intracellular invasion

This method was adapted and developed from Miliotis et al. (223).

Thirty four patients attending the incontinence clinic provided urine specimens by the CSU method (2.4.3.2). Eight asymptomatic controls, attending the Day Surgery unit for an unrelated condition, agreed to provide a catheter specimen of urine. An aliquot of urine was used for routine microscopy (2.4) and another aliquot was used for routine culture and enhanced culture (2.6 and 2.9.1). A further aliquot was used for sediment culture (2.9.2).

An aliquot of urine was centrifuged at 800rcf for 5 minutes at room temperature using an Eppendorf 5804R centrifuge. The supernatant was removed gently and the sediment was kept. The sediment was washed in 200µl of Hank's balanced salt solution (SIGMA). Hank's balanced solution contains glucose and it provides an energy source for cell metabolism. It also provides cells with water and certain bulk inorganic ions and it provides a buffering system in order to maintain the solution within the physiological pH range of 7.2 to 7.6 (from the product information sheet by SIGMA).The cells were then stained with 100µl of 0.01% acridine orange(PARIS ANTICORPS) in Gey's solution (SIGMA) for 45 seconds. The sediment was then rinsed with 200µl of Hanks balanced salt solution, and counterstained with 100µl of 0.05% crystal violet (Fluka Biochemika) in for 45 seconds. The sediment was then washed again with Hanks balanced salt solution. The sediment was placed onto a double frosted glass slide and a cover slip was added which was sealed with clear nail-varnish. Slides were then viewed immediately under a Olympus BX-40 fluorescence microscope by using incident light at x200 magnification for screening and x400 magnification - for quantification. The number of green (viable) fluorescent bacteria in the first 30 cells was counted, and the mean number of bacteria per cell was determined.

The initial protocol was taken from experiments conducted by Miliotis et al. (223). However, when viewing the samples under the fluorescent light microscope, there was no fluorescence detected from the sample. The protocol was therefore modified by increasing the exposure time of both the acridine orange solution and the crystal violet counterstain. The time the acridine orange solution was left on the cells was increased to 30 minutes

while the crystal violet counter staining was left on for 5 minutes. The green fluorescence from the acridine orange solution showed that the stain was binding to viable cells. Nevertheless, all of the extracellular bacteria were not being quenched by the crystal violet solution. Further experiments focused on increasing the exposure time of the crystal violet solution to the cells. The exposure time was increased from 5 minutes to 10, 15, 20 and 25 minutes. Investigations were carried out using the Olympus BX-40 fluorescent light microscope. It was found out that leaving the crystal violet counter stain for 20 minutes was sufficient time for the extracellular bacteria to be quenched. The intensity of the fluorescence from intracellular bacteria was discernible from the fluorescence from extracellular bacteria.

6.3.15 Statistics-Acridine Orange staining to visualise intracellular bacteria

A non-parametric test for Independent samples, the Mann Whitney U test was used to compare the median number of bacteria visualised per cell between the patient and control groups.

6.3.16 Results-Acridine Orange staining to visualise intracellular bacteria

Of the thirty four patient samples, after modifications to the assay, twenty four samples were used for the final experiment and all eight of the control samples. The assay was disappointing; the fluorescence emitted under the BX-40 microscope was poor. This meant that some cells fluoresced well and a neighbouring cell would fluoresce poorly. This may have occurred because of the variability of urine pH and the variability of urea and various ions in the urine. However, Hanks balanced salt solution should have corrected for this irregularity.

No patients were taking antibiotics. 16 had pyuria on microscopy. The sediment culture in the patient group showed a mean total bacterial count of $7.28x10^4$cfu/ml. The asymptomatic control group had a mean total bacterial count of $8.67x10^3$cfu/ml. The mean number of bacteria counted per cell for the patient group was 2.94 bacteria per cell and for the control group 0.22 bacteria per cell. This was significantly different (p= .01). The mean number of bacteria counted per sample for the patient group was 49.9 and 6.6 for the control group. This was significantly different (p= .01). Figure 6.7 shows the mean number of bacteria per cell between patients and controls. Although significant, the graph demonstrates the wide variance in the mean in the patient group. This may have occurred because of the larger number of shed cells in the patient group seen on microscopy. Figures 6.8-6.9 are

photographs taken of the fluorescent microscopy showing intracellular bacteria in patient samples.

Figure 6.7 Log mean number of bacteria counted in the first 30 cells and the 95% confidence interval of the mean using acridine orange staining comparing the Overactive bladder patient group and the control volunteers.

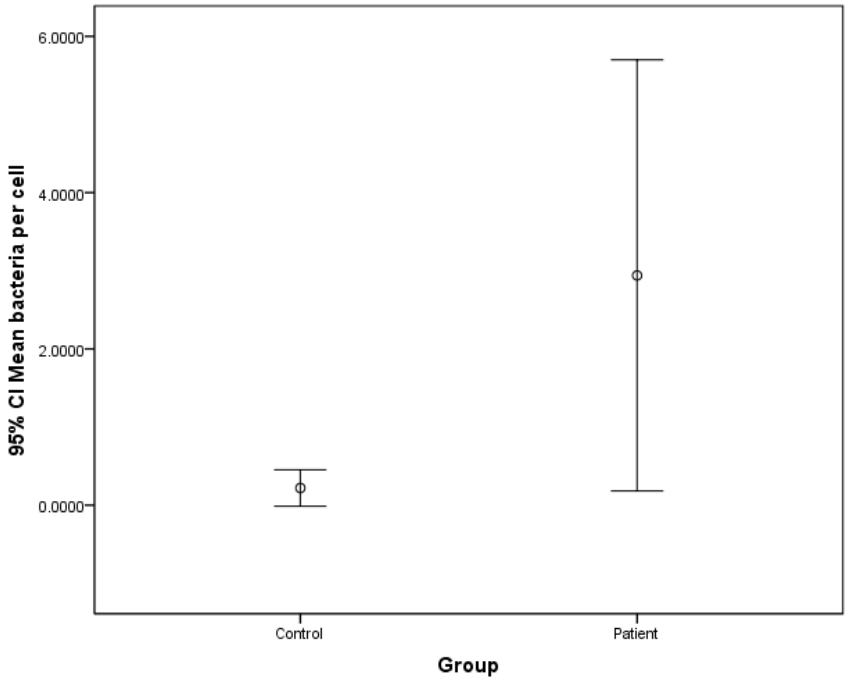


Figure 6.8 Fluorescent microscopy x400 magnification using the Olympus BX-40 showing intracellular rods in a bladder epithelial cell.

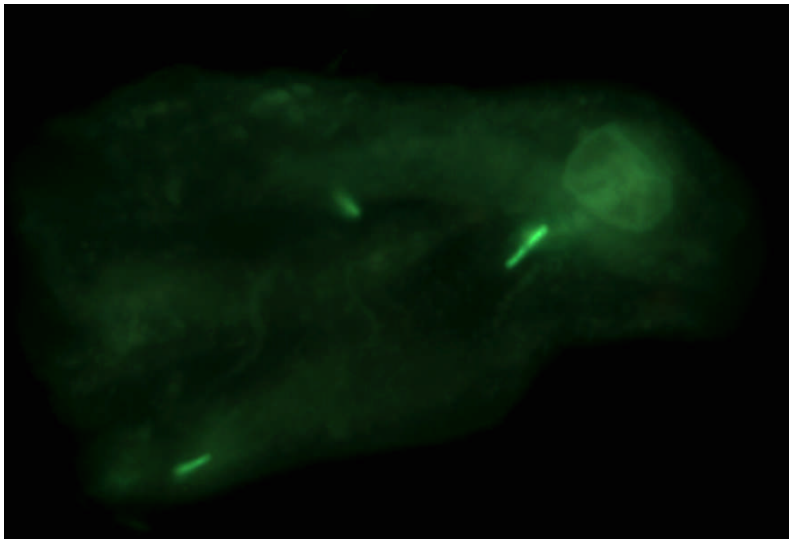
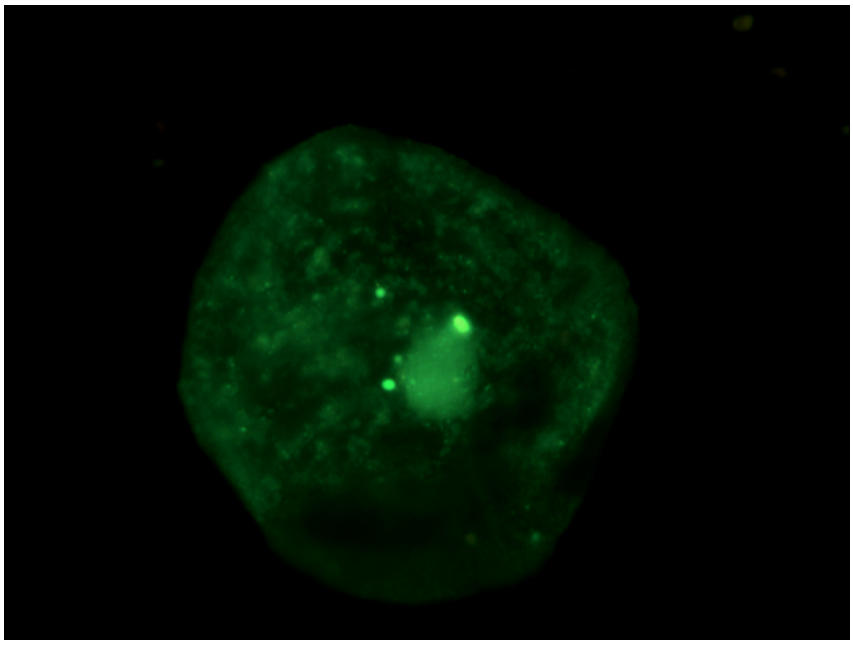


Figure 6.9 Fluorescent microscopy x 400 magnification using the Olympus BX-40 showing intracellular organisms in a bladder epithelial cell.



6.4 Discussion

These experiments resulted in a very marked discrimination between patients with OAB and normal controls. Whilst imaging through confocal microscopy would be the most direct means of locating intracellular bacteria, the data presented here constitute a microbiological test of this hypothesis which stands in the face of the results.

The dominant bacteria in this sample of patients was *Enterococcus faecalis*, *Streptococcus agalactiae*, *Streptococcus anginosus, Escherichia coli and Proteus mirabilis.* The findings affirm the results reported in chapter 3, where similar species dominated isolates obtained from other OAB patients. Additionally, *A. omnicolens* was isolated from one of the patients. *A. omnicolens* has been rarely isolated from human clinical specimens. This organisms is a member of the normal microbiota of the human gastrointestinal tract and oral cavity, and some strains have been used as probiotics to protect against gut pathogens (240), (241).

Mahlen et al. (242), found *A. omnicolens* and *Bifidobacterium* isolates as causative agents of UTI in symptomatic patients with pyuria. They concluded that these organisms should not be ignored when isolated from clinical specimens. Clinical laboratories may consider these organisms to be normal microbiota or not recover these organisms since they are slow growing and are difficult to identify (243).

This is the first time that this evidence for intracellular bacterial invasion of bladder epithelium in patients with OAB has been shown. Intracellular invasion is a known mechanism of bacterial evasion of the host immune response leading to persistence and treatment failure.

The failure of the enzymes to influence the demise of the extracellular bacteria was disappointing and, at the time, perplexing. On reflection there may well be good reason for this observation. A finding from these studies and those of other groups such as Hultgren et al. is that the bacteria of interest seem to be very well adapted to life inside a host cell. The implication is that there may be an extensive evolutionary story leading to these behaviours. Recent attention, albeit limited, to innate immune function of epithelia, particularly of the intestine, provide some signposts.

In response to the pathogens, the urothelium mounts a, bipartite, innate immune response (149). Recognition of pathogen lipopolysaccharides by TLR4 on the urothelial cells initiates an intracellular cascade which leads to the transcription and release of antimicrobial peptides, inflammatory cytokines and chemokines, and co-stimulatory molecules that promote the many elements of the innate immune response to common bacteria, including the recruitment of polymorphonuclear neutrophils (PMN). Because the bacteria are located inside the cells they can increase in number while evading the action of PMNs as well as antibiotics and other defence mechanisms. There are intracellular innate immune mechanisms, notably the fusion of the bacteria

containing endosomes with lysososmes. It has come to light that some microbes have evolved methods of escaping such attacks. Others seem to depend on the fusion of the endosome with the lysosome because the bacteria concerned have evolved mechanisms that permit them to thrive in an environment that contains lysosome enzymes which the microbes seem to be controlling (244) (245) (246). These findings certainly imply lysosyme resistance in the bacteria that were cultured from the patients, both from the intracellular and extracellular spaces.

The use of the acridine orange stain was disappointing as it was a difficult stain to use with variance in its staining from cell to cell. Confocal microscopy techniques have been used by Hultgren et al. (149), allowing visualisation of the cell membrane and the bacteria inside the cell. However, that group were using a known bacterium, *E.coli* and so they were able to use immunological staining specific for *E.coli* bacterial components. In this study the problem was that staining was that a variety of bacteria was present in the urine and so no specific stains could be used.

Chapter 7

Intracellular Invasion of a Bladder Epithelial Cell Line

7.1 Hypothesis

Bacteria isolated from patients with the overactive bladder syndrome will invade a human bladder epithelial cell line in contrast to similar bacteria isolated from the urine of asymptomatic controls.

7.2 Background

This study examined four of the isolates retrieved from OAB patients to investigate whether they would invade a human bladder epithelial cell line. This was to test the hypothesis that bacteria from patients would exhibit the capability of cell invasion in contrast to microbes isolated from controls. This provided a different path to scrutinising the veracity of the results reported in chapter 6.

The method was adapted from Martinez et al., 2000 (247), who used *E.coli* to infect a human bladder cell line, Marouni et al., 2004 (248), who used a *Group A Streptococcus* to infect Hep-2 epithelial cells and Fabretti et al., 2009 who used *Enterococcus faecalis* to infect Hep-2 epithelial cells (249).

7.3 Methods

Four bacterial isolates were studied, previously isolated from patients presenting with symptoms of OAB. These were *Eschericia. coli, Enterococcus. faecalis*, *Streptococcus. anginosus* and *Proteus. mirabilis.* One isolate from a control volunteer *Lactobacillus. gaseri* was also used. These isolates had been previously cultured during the intracellular growth assays described in chapter 6. The isolates were identified as per section 2.10-2.11

A bladder epithelial cell line from a transitional cell carcinoma (EJ138) was grown to confluency in Eagles Minimal Essential Medium supplemented with Glutamine to 2mmol (Sigma), fetal bovine serum 10% (Sigma) and Non-essential amino acids 1% (Sigma) in a 5% Co_2 atmosphere. Cells were passaged at a ratio of 1:5 twice weekly. All experiments were performed on the cells between the 3rd and 15th passage. All experiments were conducted in triplicate. The experiments were conducted as follows:

Day 1

The bacteria required (*Eschericia. coli, Enterococcus. faecalis*, *Streptococcus. anginosus, Proteus. mirabilis and Lactobacillus.gaseri*) were retrieved from frozen storage (2.11.3) and a loop of the isolate was streaked onto a CBA plate and cultured overnight in 5% CO_2 at 37ºC.

Day 2

A single colony of the bacterium was added to 15ml of Brain Heart infusion broth (Oxoid Ltd) and incubated overnight at 37ºC in 5% CO_2. This was in order to get a bacterial suspension in which the bacterial count could be adjusted for the experiment.

The human bladder cell line EJ138, was split and seeded into 5 wells of a 24 well plate (Sigma) at $1x10^5$ cells per well and grown to confluency overnight at 37ºC in 5% CO_2.

Day 3

The bacterial suspension was centrifuged at 600g for 5 minutes. The supernatant was removed and the sediment was re-suspend in 10mls of Eagles Minimal Essential Medium and adjusted to an optical density at 600nm (OD_{600}) of 0.5, so that a final multiplicity of infection (M.O.I) of 100 bacteria per bladder cell could be achieved. CBA plates were inoculated with the bacterial suspension at dilutions to x1000. This was in order to check the adjusted bacterial suspension and confirm the colony count.

The 24 well plate was removed from the incubator and microscopy was performed at x400 magnification with an inverted microscope (Olympus) to check for confluency. The cell media were removed from the wells and each well was washed with 1ml PBS. The PBS was plated onto CBA and incubated overnight at 37ºC, 5% CO_2 to check for bacterial growth prior to inoculation of the bladder cells with bacteria.

The bacterial suspension was added at the correct volume to give a final MOI of 100 bacteria per cell and additional Eagles Minimal Essential Medium supplemented with Glutamine to 2mmol (Sigma), fetal bovine serum 10% (Sigma) and Non-essential amino acids 1% (Sigma) for a final volume of 1ml.

The triplicates were as follows:

1. Media only, no cells, no bacteria, (control)
2. Bacteria, no cells (control)
3. Cells only (control)
4. Cells and bacteria (To give the total bacterial counts, both intracellular and extracellular)
5. Cells and bacteria (To give the counts of the adhesive and invasive bacteria)
6. Cells and bacteria (To give the counts of the intracellular bacteria only)
7. Cells and bacteria (for viable epithelial cell line counts at the end of the assay)

The wells were incubated at 37° C, 5% CO_2 for 2 hours.

After incubation, the media were removed and replaced with media containing gentamicin at 100µg/mL to triplicates 3, 6, 7 only (to kill any extracellular bacteria) and wells 1,2,3,6,7 were incubated for 2 hours.

To triplicate 4, triton X was added for a final concentration of 0.1% for 5 minutes. The solution in triplicate 4 was plated on CBA at dilutions to x1000 and incubated at 37° C, 5% CO_2. This gave the total bacterial count both intracellular and extracellular.

To triplicate 5, the media were removed and the wells were washed with 1ml PBS 5 times. The total volume of the last wash was kept and plated onto CBA

and incubated overnight at 37° C, 5% CO_2. The cells in the well were then lysed with Triton X at a final concentration of 0.1% for 5 minutes. The solution in triplicate 5 was plated on CBA at dilutions to x1000 and incubated at 37° C, 5% CO_2. This gave the bacterial counts for the adherent and invasive bacteria.

After a further 2 hour incubation period for triplicates 1, 3, 6, 7 the media were removed and the total volumes were plated on CBA and incubated at 37° C, 5% CO_2 overnight for the initial bacterial counts after incubation with gentamicin.

Triplicates 1,3,6,7 were then washed with PBS 1ml 10 times and the last wash was plated on CBA and incubated at 37° C, 5% CO_2 overnight. This step was to remove any cell adherent bacteria after incubation. Triton X was then added for a final concentration of 0.1% for 5 minutes to lyse the epithelial cells and release any intracellular bacteria.

Triplicates 1,2,3,6,7 were then treated as follows:

Triplicate 1: The entire volume was plated on CBA and incubated at 37° C, 5% CO_2 overnight. This well was to insure that the culture media were not contaminated.

Triplicate 2: The total volume of the wells was plated on CBA and incubated at 37° C, 5% CO_2 overnight and then the wells were washed with 1ml PBS 5 times and every wash was plated on CBA and incubated at 37° C, 5% CO_2 overnight . Triton X was then added for a final concentration of 0.1% for 5 minutes and the entire well volume was plated on CBA and incubated at 37°

C, 5% CO_2 overnight. This was to make sure the bacteria grew in the media and was not altered by the addition of Triton X.

Triplicate 3: The entire volume of the wells was plated on CBA and incubated at 37° C, 5% CO_2 overnight. This well contained bladder cells only, bathed in culture media + gentamicin. This again was to make sure that no bacteria grew in these wells as a control.

Triplicate 6: Serial dilutions of the solution in the well were performed to a dilution of x1000 and plated on CBA and incubated at 37° C, 5% CO_2 overnight. This would give the intracellular bacterial count (invasive bacteria).

Triplicate 7: 100µL trypsin was added to the well and incubated for 2-3 minutes. 20µL of trypan blue was added to 20µL of the well suspension. This gave a viable bladder epithelial cell count.

7.4 Results from invasion assay incubating with Gentamicin 100µg/ml for 2 hours

The viable urothelial cell counts obtained at the end of the assay were between 1.6-2x10^5 cells.

All control wells were negative for bacterial growth; wells 1 and wells 3. The bacterial wells (2) grew a lawn of bacteria, even at x 1000 dilution and after washing 10 times and adding Triton X.

After incubation with antibiotics, the media removed from wells 6 showed bacterial growth, for all bacterial isolates. After washing the attached cells in the well 10 times, again, with each bacterium, the wells still showed bacterial growth. After the addition of Triton X, bacteria were retrieved from all wells for each bacteria tested.

7.5 Conclusions from invasion assay incubating with Gentamicin 100µg/ml for 2 hours

The results of the experiment showed bacterial growth even after the addition of gentamicin. This meant that the assay could not differentiate intracellular from extracellular bacteria. This was a little surprising as various authors in the literature have consistently used a 2 hour incubation period for bacterial kills of similar numbers of bacteria. This meant that the gentamicin was possibly only bacteriostatic at 2 hours or not active against the bacterial isolates used. The same isolates had been used in chapter 6 and those experiments had shown these isolates to be sensitive to gentamicin at 24 hours.

Therefore, I tested the isolates by inoculating them with gentamicin 100µg/mL for 2 hours, 12 hours and 24 hours to test the ability of the gentamicin to kill the bacteria at variable time intervals.

7.6 Method development inoculating with gentamicin for variable lengths of time

The experiment was conducted as above but wells 3,6,7 were inoculated with gentamicin 100µg/mL for 2 hours, 12 hours and 24 hours. *Enterococcus. faecalis* was used in this experiment.

7.7 Results of variable antibiotic inoculation times

The viable urothelial cell counts obtained at the end of the assay were between 1.4-1.6x10^5 cells.

All control wells were negative for bacterial growth; wells 1 and wells 3. The bacterial wells (2) grew a lawn of bacteria, even at x 1000 dilution and after washing 5 times and adding Triton X.

Incubation at 2 hrs. with gentamicin confirmed bacterial growth. At 12 hours and 24 hours incubation with gentamicin, again there was bacterial growth. At 12 hours there was an average of 65 bacteria per ml and at 24 hours there was an average of 6 bacteria per ml.

7.8 Conclusion from variable antibiotic inoculation times

The experiment showed that the greater incubation time gave a greater kill. However, at 24 hours there was still not a total kill achieved. This would make it difficult to feel confident that growth post cell lysis implied that invasion was occurring. I decided to use different antibiotic concentrations. In my previous experiments in chapter 6, I had used 200µg per ml of gentamicin. This was because there was an indeterminate amount of bacteria in the human samples but in this experiment, I had control over the number of bacteria that were added to the assay (100 bacteria per epithelial cell). I decided to repeat the experiment, using an incubation period of 24 hours but with 100µg, 150µg, 200µg per ml of gentamicin to test the different concentrations to achieve a complete kill after incubation.

7.9 Method improvement. Incubating with different concentrations of gentamicin

The above experiment was repeated, incubating for 24 hours in gentamicin 100µg/ml, 150µg/ml and 200µg/ml in wells 3,6,7. *Enterococcus. faecalis* was used in the experiment.

7.10 Results of incubating with different concentrations of gentamicin

The viable urothelial cell counts obtained at the end of the assay were between 1.4-2x10^5 cells.

All control wells were negative for bacterial growth; wells 1 and wells 3. The bacterial wells (2) grew a lawn of bacteria, even at x 1000 dilution and after washing 5 times and adding Triton X.

At 100µg/ml the average growth was 60 bacterial per ml, at 150µg/ml the average growth was 43 bacteria per ml and at 200µg/ml there was no bacterial growth.

7.11 Conclusion from invasion assay improvement; using different concentrations of gentamicin.

This experiment showed incomplete kills at 100µg/ml and 150µg/ml. There was not much difference between the two concentrations. At 200µg/ml, however, there was complete kill, confirmed in all triplicate wells. For this reason, I conducted all further experiments using gentamicin 200µg/ml with an incubation period of 24 hours.

7.12 Methods with improved invasion assay using gentamicin 200µg/ml

The experiment was repeated as per section 7.3 using *Eschericia. coli, Enterococcus. faecalis*, *Streptococcus. anginosus, Proteus. mirabilis or Lactobacillus. gaseri.*Wells 3,6,7 were incubated with gentamicin 200µg/ml for 24 hours.

7.13 Results from improved assay using gentamicin 200µg/ml.

The viable urothelial cell counts obtained at the end of the assay were between 1.4-2x10^5 cells.

All control wells were negative for bacterial growth; wells 1 and wells 3. The bacterial wells (2) grew a lawn of bacteria, even at x 1000 dilution and after washing 10 times and adding Triton X.

Figures 7.1-7.5 below show the bacterial counts at different stages of the assay for each bacterium. The graphs demonstrate a complete kill of extracellular bacteria after the addition of Gentamicin 200µg/ml for 24 hours. They also demonstrate the release of intracellular bacteria after cell lysis with the addition of triton X in all cases except for *Lactobacillus.gaseri*, in which no bacterial growth is shown after cell lysis. The average bacterial counts post cell lysis (intracellular bacterial count) were: *E.coli* 1.8x10 cfu ml^{-1}, *E.faecalis* $1.02x10^{4}$cfu ml^{-1}, *Strep.anginosus* $2.69x10^{3}$cfu ml^{-1}, *Proteus.mirabilus* 3.20 $x10^{3}$cfu ml^{-1}, *Lactobacillus. Gaserri* 0 cfu ml^{-1}.

Figure 7.1 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial invasion assay for Eshericia.coli. Start=Initial bacterial count before incubation with the antibiotics, 2hrs= Bacterial count after 2hrs incubation without antibiotics, mixed=Adhesion and invasion count, Gent=Bacterial count after addition of Gentamicin and incubation for 24hrs, Wash= Bacterial count after washing with PBS 10 times to remove any adherent bacteria, Invasion=Bacterial count after cell lysis with Triton X.

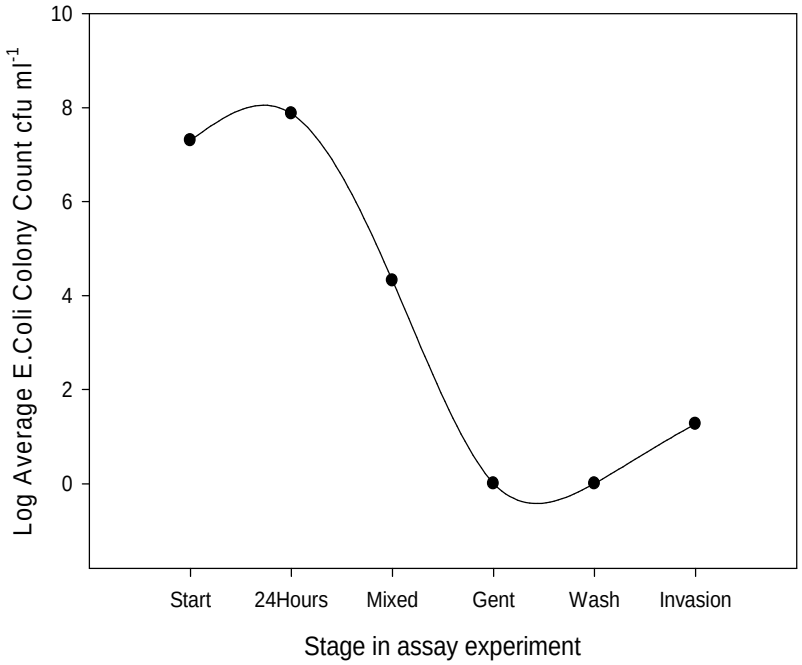


Figure 7.2 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial invasion assay for Enterococcus.faecalis. Start=Initial bacterial count before incubation with the antibiotics, 2hrs= Bacterial count after 2hrs incubation without antibiotics, mixed=Adhesion and invasion count, Gent=Bacterial count after addition of Gentamicin and incubation for 24hrs, Wash= Bacterial count after washing with PBS 10 times to remove any adherent bacteria, Invasion=Bacterial count after cell lysis with Triton X.

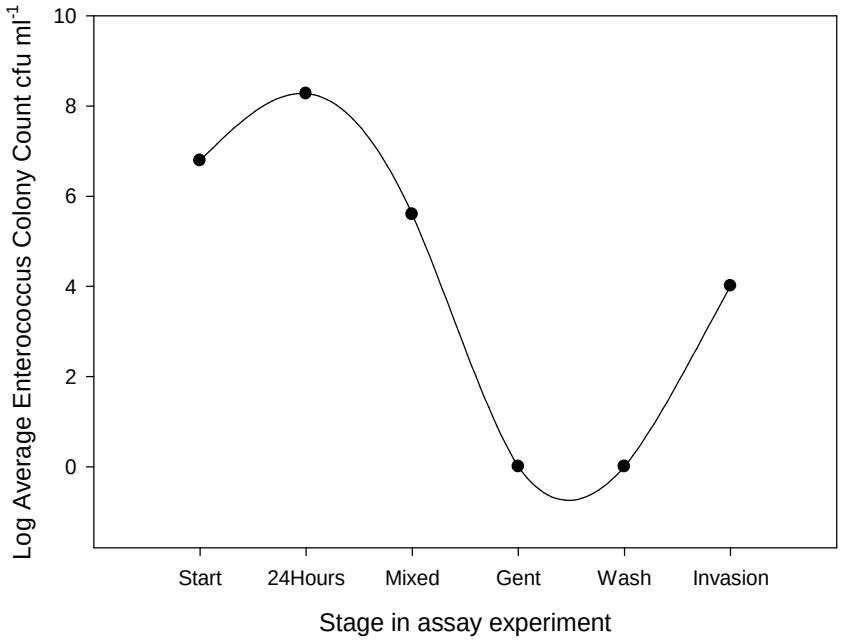


Figure 7.3 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial invasion assay for Streptococcus.anginosus. Start=Initial bacterial count before incubation with the antibiotics, 2hrs= Bacterial count after 2hrs incubation without antibiotics, mixed=Adhesion and invasion count, Gent=Bacterial count after addition of Gentamicin and incubation for 24hrs, Wash= Bacterial count after washing with PBS 10 times to remove any adherent bacteria, Invasion=Bacterial count after cell lysis with Triton X.

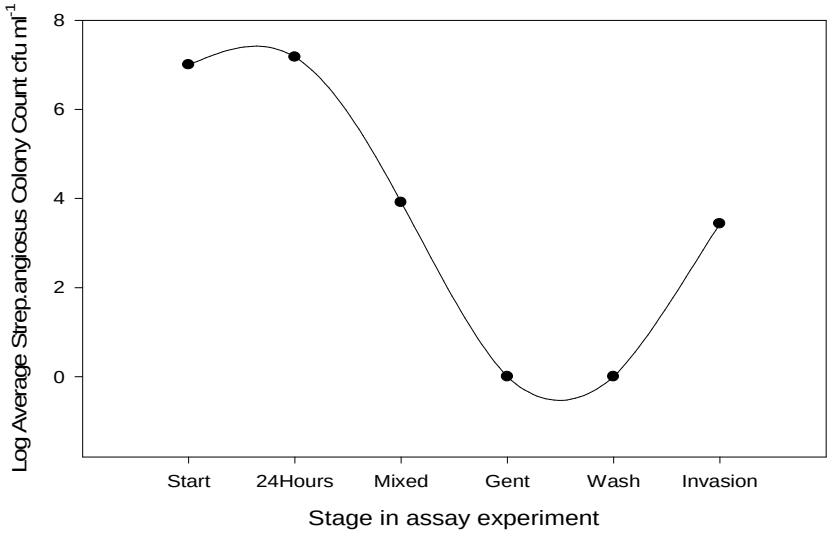


Figure 7.4 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial invasion assay for Proteus.mirabilus. Start=Initial bacterial count before incubation with the antibiotics, 2hrs= Bacterial count after 2hrs incubation without antibiotics, mixed=Adhesion and invasion count, Gent=Bacterial count after addition of Gentamicin and incubation for 24hrs, Wash= Bacterial count after washing with PBS 10 times to remove any adherent bacteria, Invasion=Bacterial count after cell lysis with Triton X.

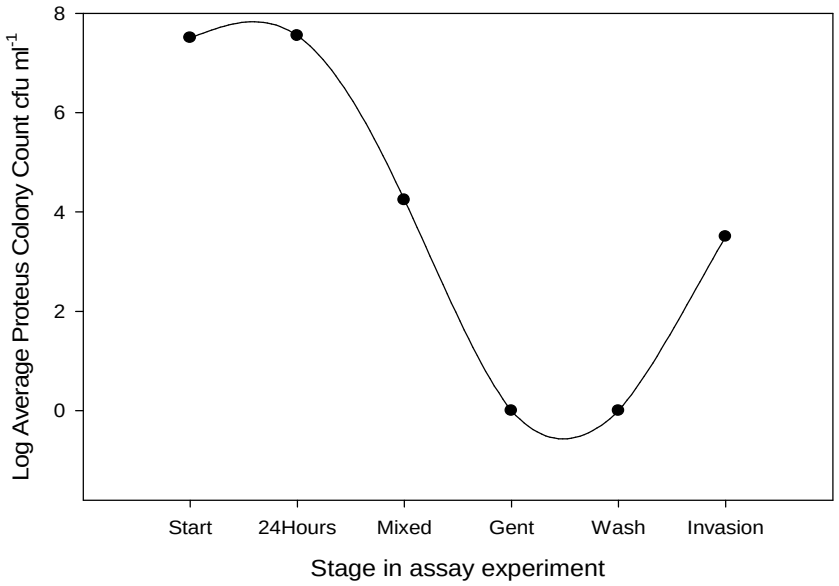
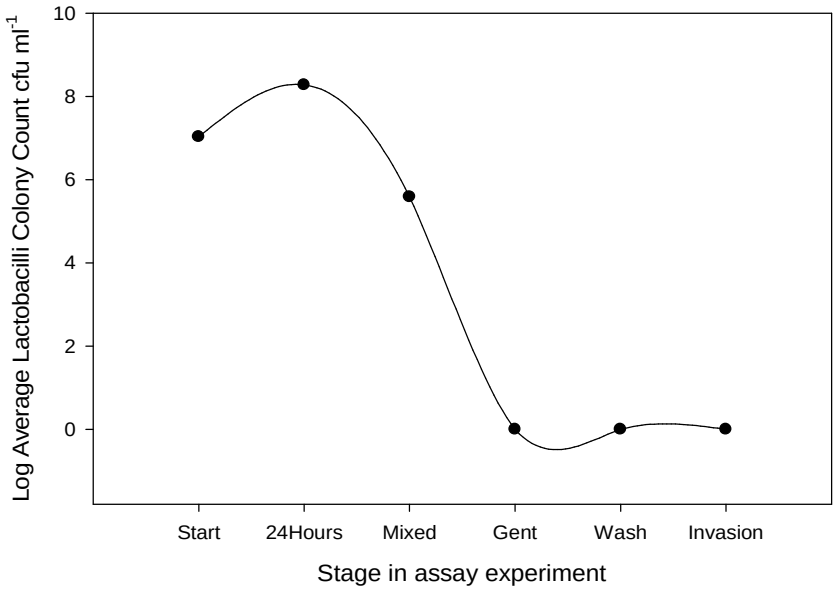


Figure 7.5 Total bacterial counts (cfu/ml-1) at different stages of the Intracellular bacterial invasion assay for Lactobacillus.gasseri. Start=Initial bacterial count before incubation with the antibiotics, 2hrs= Bacterial count after 2hrs incubation without antibiotics, mixed=Adhesion and invasion count, Gent=Bacterial count after addition of Gentamicin and incubation for 24hrs, Wash= Bacterial count after washing with PBS 10 times to remove any adherent bacteria, Invasion=Bacterial count after cell lysis with Triton X.



7.14 Discussion

These experiments confirm intracellular invasion of a cell line by prominent bacteria isolated from patients suffering with overactive bladder symptoms. It is interesting that this did not occur in the *Lactobacillus. gasseri* species isolated from a control volunteer. However, all species from both patients and controls should be scrutinised in this manner to fully understand the differences in invasion ability and invasion frequency manifest by the various bacteria isolated. It is too premature to assume that all isolates from patients will invade bladder epithelia *in vitro* and all isolates from control volunteers will not.

This experiment features some additional interesting findings. The inability of gentamicin at 100µg/ml for 2 hours to achieve a complete bacterial kill of each isolate suggests an adaptation of these bacteria for gentamicin resistance at low concentrations. *Enterococci* are well known for their ability to adapt to an antibiotic exposure, mainly due to their natural resistant to low-level concentrations of ß-lactams and aminoglycosides (250) Kobayashi et al (251); Bujdáková et al (252). Resistance to aminoglycosides is mostly thought to be caused by the production of many aminoglycoside-modifying enzymes with the prevalence of the enzyme 6'-acetyltransferase–2''-phosphotransferase (253)

A recent study by Mulvey et al. 2010 (254), showed the persistence of uropathogenic *E.coli in vitro* and *in vivo* in a mouse model, despite the use of

multiple antibiotics including gentamicin. *E.coli* resistance to gentamicin, although acknowledged, is poorly understood. One study by Kühberger et al., 1979 (255), showed that gentamicin resistant *E.coli* strains contained mutations correlating to an altered L6 ribosomal protein, requiring an increased minimal inhibitory concentration of the drug by about 5 to 10-fold.

High level resistance to aminoglycosides has been reported in various *Streptococcal spp*, including *Group B Streptococcus*. This also occurs in *Proteus. mirabilis*, the resistance genes aph(3 ')-III gene and aminoglycoside-modifying enzymes being identified as the cause. It is well established that a transposable sequence of DNA encoding resistance to various antibiotics is capable of transposing to other plasmids that exist within the same cell (256) (257;258) and this may account for aminoglycoside resistance in urinary pathogens and in the isolates used in this study, particularly, where there is a large commensal community of bacteria, providing opportunistic pathogens.

Urothelial invasion by *E.coli and Proteus. mirabilis* has been studied as a model for UTI by Allison et al. (259). Urothelial invasion by *Streptococcal Spp* and *Enterococcus Spp* has not been widely studied. Biofilm formation as a mechanism of pathogenesis has been widely documented in the *Enterococcal spp* and *Enterococcus* UTI is often associated with the use of a urinary catheter (260) and so it is difficult to speculate on a mechanism for intracellular invasion for these bacteria. However, there are various mechanisms reported in the literature responsible for bacterial adhesion and invasion of various human cells. *Streptococci* are known to colonise different

sites in the human body because they express multiple adhesin proteins that recognize specific receptors, often sugars or oligosaccharides, expressed at various body sites. Following initial adherence processes, bacteria grow and survive depending upon the physical and chemical environment. The initial adherence of a single cell gives rise to micro-colonies. This results in the formation of what has been termed a linking film. Further cell division leads to the generation of a small society with the incorporation and accumulation of other bacterial cells to establish a community. In a dental plaque community, for example, there may be over 100 different kinds of bacteria present. Multiple adhesive and metabolic interactions occur between bacterial cells in these communities. Fimbriae are present on the surfaces of some *streptococcal* cells which allow long range adhesion to occur (261).

Many urinary pathogens, including *E.coli*, use quorum sensing genes in order to regulate virulence genes during bacterial invasion. Three systems of quorum sensing exist in bacteria. The first uses acyl homoserine lactone (acyl HSL) molecules for signalling. This system is used by gram-negative bacteria. The second system is found in gram-positive bacteria that uses short cyclic peptides and a receptor kinase signalling pathway. The third system, used by both gram negative and gram positive bacteria uses elements of both the first and second systems (262).

These communications systems control various host–pathogen interactions. Of the entire complement of genes that contribute to virulence in bacterial pathogens, quorum sensing molecules are a major regulator. Up to 15% of

the open reading frames of bacteria are controlled by quorum sensing molecules. Quorum sensing can promote the growth of related strains of bacteria and simultaneously inhibit the growth of other bacterial or even fungi competing for the same ecologic niche. Critical virulence determinants such as toxin production, sporulation, plasmid transfer, invasion gene synthesis, and various immune evasion mechanisms of bacteria are controlled by quorum sensing genes. This is certainly a possible mechanism of bacterial survival and disease in the bladder, considering the vast commensal community of the perineum.

Chapter 8

General Discussions and Conclusion

8.1 Introduction

I set out to explore a key problem that manifests clinically as an attenuated response of patients with the Overactive Bladder syndrome to the widely used antimuscarinic agents developed for this condition. This resistant response could be attributed to the lack of drug efficacy other than the fact there have been many cases of symptom recrudescence whilst on medication in patients who previously had responded very well (263-266). If we accept that the antimuscarinics are not associated with tachyphylaxis then another explanation must be sought.

A reasonable proposition would be that the problems are being caused by undiagnosed infection although many would claim that this has been ruled out by urinalysis and culture. This position is predicated on the premise that the tests being used are capable of ruling out infection. I asked the questions are the tests sufficiently sensitive? If not how can we search for bacteria as aetiological agents in the Overactive Bladder if they exist?

8.2 Limitations and criticisms of the study

I have been able to present several pilot studies, identifying and questioning inconsistencies in the literature with regards to the identification and exclusion of infection in patients with the Overactive bladder. Although the total number of subjects studied was large, the subgroups in each of the studies was small, making it difficult to draw final conclusions from these studies. In addition, because of the reliance on catheter specimens of urine and the exclusion in the studies of volunteers with any bladder symptoms, the asymptomatic volunteer groups were small and not age matched. Although, in the literature, *Lactobacillus spp* are reported to reduce after the menopause (267;268), there is no consensus on predominance of other species and no studies on the presence of *Streptococcus spp* in the post-menopausal state. Nevertheless, the ideal standard would be to compare the patient group with a similar age matched group.

Only a few men were investigated in this study. At the time of the study, women made up the majority of patients presenting as new referrals to the clinic and therefore this was the most practical approach, although the prevalence of Overactive bladder is similar in both men and women and it is difficult to generalise these findings to men.

8. 3 The reliability of existing tests to exclude urine infection in patients with OAB

I produced evidence of the serious limitations in the sensitivity of current dipstick testing to exclude infection in patients with OAB which are very similar to other data on patients with acute frequency/dysuria (68;202-205).

Suspicion has been raised not just over the surrogate markers but the culture systems used to detect infection (179;206). I assimilated the criticisms that have been levelled at the routine MSU, that it produces a contaminated sample for microbiological analysis, particularly in women (76;80;180;181). I attempted a number of new methods in the hope of obviating the problems:

An enhanced threshold (10^2 cfu ml^{-1}) applied to CSU sent to the NHS laboratory demonstrated that there was territory to explore but with an understanding that any threshold reduction would always mean that there would be increased identification of apparent pathology. Thus controls and the differentiation of the patient groups became very important to the interpretation of a pathological signal.

I next used CSU, the enhanced threshold and non-selective media and that produced an increased isolation of microbes. The proportion of patients identified by these means was not as great as I might have expected, given

the clinical narratives and the experience with the use of antibiotics in the clinical service. It seemed that despite the very best circumstances for detecting microbes the results were still insufficiently reassuring.

8.4 Evidence of an inflammatory response in the bladder

If bacterial infection was to be implicated in the aetiology of the Overactive bladder, as well as examining the existing tests that are used to exclude urine infection in these patients, it was important to show some evidence of inflammation in the bladder, which is unlikely to exist if the existing 'theories' of the Overactive bladder are to be upheld. I studied the acute phase cytokine IL-6 signal in the urine of patients with Overactive bladder syndrome as compared to normal volunteers as a marker of inflammation. Pyuria in patients with OAB symptoms was associated with urinary expression of IL-6. This finding was supportive of the view that OAB symptoms in some patients was associated with a urothelial inflammatory reaction and further basis to the premise that bacterial infection, as a cause of inflammation, should be excluded.

8.5 The urine cellular sediment

Whilst studying the urine sediment as part of the exploration of the surrogate markers, I was struck by the common occurrence of clue cells and the apparent clustering of microbes around urothelial cells, possibly adhering to

them. The Hultgren groups had also published on their murine model and thereby raised the question of bacteria avoiding detection through intracellular colonisation (151;152;247).

The first step in this new road was to extract the urothelial cells and culture those. If the bacteria were adherent to the cells then their numbers should be increased considerably in cell concentrates much as bound antibodies are used to extract proteins and cells of particular interest. Perhaps sampling of the ordinary urine failed to trap cells in significant numbers and the urine represented a dilute suspension of planktonic bacteria.

The very first non-selective cultures of spun sediment started to produce extraordinary results with very clear quantitative and qualitative discrimination between the patient groups and the controls. It was interesting to note that the bacteria isolated, although implicated in urinary tract infection, were not the most abundant bacteria reported in cases of acute urine infection, notably, *E.coli.* The largest group of bacteria isolated were the *Streptococcus spp*. and *Enterococcus spp.* These are known vaginal commensals but also implicated in disease states, particularly urine infection and in the case of *Streptococcus spp*. in vaginal infection and neonatal sepsis in pregnant women (104;186;195;248;261;269). These were not the largest groups identified in the control volunteers.

It would be tempting to stop here and promote a new culture method with the associated clinical data justifying the case. This however would not be

satisfactory as a pathophysiological mechanism and explanation would be lacking. If bacterial adhesion were part of the process of cell invasion then the identification of viable intracellular colonisation would present a suitable target. Whilst the Hultgren group have used Electron microscopy to identify intracellular colonisation their case was far more convincing in the murine model than were the data that they presented in acute cystitis in humans (151;152). Commensal organisms are a ubiquitous manifestation of the lower urinary tract, and given the evolutionary history of the eukaryocyte, the harmless colonisation of cells would not be a preposterous proposition. It would be far more informative if the study of colonisation were rooted in the careful identification of the microbes concerned so that species differences could be studied between controls and patients and the findings analysed in the light of what we know about the pathological properties of the isolated microbes.

In addition, we are less sure that the imaging studies to date have shown intracellular colonisation of human urothelial cells beyond reasonable doubt. There remains the question of whether the bacteria visualised are only attached to the cells. I therefore proposed a culture based analysis of the colonisation of urothelial cells from patients and controls. This was then to be followed by an investigation of the invasion properties of the bacteria isolated from patients and controls. Imaging studies would have been very welcome adjuncts to these experiments but a very great deal of work needed to be conducted on differential staining methods before suitable confocal

microscopic methods could be deployed. Given the time constraints I elected to focus on the microbiological approach.

I hence had to invent a whole new set of methods to achieve my goals and much of my work has focused on method development and validation, nevertheless, despite the many difficulties the data present a very convincing case of a pathological colonisation of urothelial cells and a possible cause of OAB. The data reflected other studies of other, non-bladder, epithelial cells, showing intracellular colonisation of different body surfaces by these pathogens in the disease state (191;232;248;261;270). Another important finding, whilst developing the intracellular invasion assays, was the resistance of the bacteria retrieved from patients to lytic enzymes, again reflecting a possible immune adaptation for intracellular survival.

8.6 Future Work

This study raises many questions. Future work should include looking more closely at a larger cohort of patients, including men, with age matched control volunteers and be directed particularly at the imaging of intracellular bacterial pathogens in patients with the overactive bladder, longitudinal cohort studies of the microbiological ecology in patients with the Overactive bladder and any changes in the ecology over time; the symptoms in relation to colonisation patterns over time, the effect of antibiotic intervention and a proper

widespread analysis of the innate immune interactions so as to explain the pathophysiology and the microbial adaption. The innate immune system might well prove to be a rich source of new surrogate markers and measures of disease progress or regression under treatment.

8.7 Conclusion

The hypothesis that this thesis set out to test were as follows:

The presence of pyuria ≥ 10 wbc μl^{-1} in patients with OAB is associated with other evidence of an inflammatory response.

The traditional diagnostic methods deployed to exclude urinary infection in OAB patients are missing genuine infection.

OAB is associated with intracellular bacterial colonisation of uroepithelial cells by pathogenic bacteria

All three hypotheses are supported by the work in this thesis. The role of bacterial infection in the overactive bladder syndrome cannot be reliably excluded by the current tests available in routine practice. Patients with overactive bladder syndrome display an IL-6 inflammatory signal in the urine, not shown previously; suggesting that core to the aetiology of the condition is an inflammatory mechanism from the urothelium. This finding is supported by the urothelial biopsies taken from these patients, invariably showing

histopathological inflammatory changes. Intracellular bacterial colonisation, particularly by the *Streptococcus spp* and *Enterococcus spp.* is predominantly found in patients with overactive bladder as compared with control volunteers. These bacteria not only show the ability to invade the bladder epithelium but possibly an ability to evade immune defences as evidenced by their resistance to lytic enzymes.

Appendices

Appendix 1

A 10-Item Scale to Measure Urinary Urgency

Question	Yes Score	No Score
Do you suffer from urgency	1	0
Do you suffer from urge incontinence	1	0
Do you have urgency on rising after waking in the morning	1	0
Do you have urge incontinence on rising after waking in the morning	1	0
Do you have urgency on putting a key in the front door when arriving home (latchkey urgency)	1	0
Do you have urge incontinence on putting a key in the front door when arriving home (latchkey urgency)	1	0
Do you have urgency aggravated by the sound of running water	1	0
Do you have urge incontinence aggravated by the sound of running water	1	0
Are your urge symptoms aggravated by cold	1	0
Are your urge symptoms aggravated by fatigue or anxiety	1	0
Possible Maximum and Minimum Scores	10	0

Appendix 2

Midstream Urine Specimen

For correct test results, follow instructions carefully.

A bowl, a plastic container, and hand wipe and a wet gauze will be given to you

Cleansing before collecting the urine specimen

1. Wipe your hands with the hand towel that we give you and discard it

2. Thoroughly cleanse the entire genital area using the special gauze that we give you

    - Females – Hold the outer edges of labia apart and cleanse from front to back with the gauze

    - Males – retract foreskin if not circumcised and wipe the end of the penis with the gauze

Do not throw the gauze into the toilet bowl throw it into the yellow bin available in the toilet

Collecting the urine specimen

- Females – continue to hold labia apart while urinating

- Males – continue to retract foreskin while urinating

    1. Urinate (pee) a small amount of urine into the toilet.
    2. Then without stopping, catch some urine into the bowl by passing it into the urine stream.
    3. As the stream comes to the end move the bowl away and urinate (pee) the rest into the toilet
    4. Pour the urine in the bowl into the plastic container and screw the lid down

Wash hands after collecting the urine specimen

Appendix 3

The Whittington Hospital NHS
NHS Trust

PATIENT INFORMATION SHEET

10/08/06 (Cystoscopy) Ver 2

A study of bladder and patient symptoms

You are being invited to take part in a research study. Before you decide whether participation is right for you, please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

You may also obtain information from the hospital Patient Advice and Liaison Service (PALS), Whittington Hospital, Tel 020 7288 5956 or 020 7288 5957

Thank you for taking the time to read this information and for considering helping us with our research project.

What is the purpose of the project?

Many people suffer with urinary incontinence (leaking from the bladder). It is a very unpleasant problem, which affects people's lives at home, at work and their general well-being. The aim of this study is to examine samples of the bladder to better understand how the bladder works and relate these findings to patient symptoms. This involves studying both patients who have urinary symptoms and those that do not, in order to make a comparison.

The Whittington Hospital NHS
NHS Trust

Why have I been chosen?

The urologist who is currently treating you is carrying out a procedure which involves an examination of the inside of the bladder via cystoscopy (key hole procedure).

This provides an opportunity for the surgeon to take a sample (biopsy) of the bladder lining. We should like to do this so as to use the sample for research purposes.

Do I have to take part?

It is up to you if you decide whether or not to take part. If you do decide to take part you will be given a copy of this information sheet to keep. You will be asked to sign a consent form a copy of which will be given to you. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

If you decide to take part in the project the research clinician will arrange to see you before your operation takes place. You will be asked you some questions about your bladder and how well it works for you. Your answers will be recorded on a form stored on a computer. This will take about 30-45 minutes. The computer record is closely guarded by the NHS security system so there is no unauthorised access to you record. Blood samples may be taken as part of your normal clinical care. We should like to record the results on these in you research record.

When you have the cystoscopy, the doctor will take a very small sample of bladder tissue (about 2-3mm or a quarter inch in size) in addition to any that may be needed in order to check your condition or make a diagnosis.

What will be done with the biopsy tissue that is taken for this study?

The bladder sample taken at biopsy will be studied and the tissue sample will be destroyed once the research is complete. The use of your specimen, where it goes and the results of any experiments that are conducted on it will be kept on the record and could be made available to you should you so wish. In effect there will be the full story of your specimen.

The Whittington Hospital NHS
NHS Trust

What are the alternatives to participating in the study

It is entirely your decision if you wish to take part in the study and it will not affect your future care if you do not wish to do so. If you do not wish to take part you will have your operation in the usual way, a biopsy may be taken (as a routine), but no extra research sample will be taken.

What are the possible disadvantages and risks of taking part?

Taking an extra sample of bladder tissue means that the procedure will take a bit longer than usual. With each biopsy is a risk of a small amount of bleeding after the surgery, this will require no further treatment and stops after a day or so.

What are the possible benefits of taking part?

The sample of bladder tissue taken for this research will be considered as a 'gift' to the university. There are no benefits directly to you for taking part. But your participation will help us to have a better understanding of how the bladder works and may lead to better treatments being available to people with bladder problems in the future.

What happens if something goes wrong?

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

What if I want to withdraw from the study?

You are free to withdraw from the study at any time before and after signing the consent form without needing to give any explanations. If you wish to withdraw after the bladder sample has been taken then you may contact us and we will not use the sample for any experiments.

The Whittington Hospital NHS
NHS Trust

Will my taking part in this study be kept confidential?

All information, which is collected about you, during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital / surgery will have your name and address removed so that you cannot be recognised from it.

Your GP will be notified of your involvement in the study unless you do not wish us to do so.

What will happen to the results of the research?

The results of the experiments made on the samples of bladder muscle will be published in scientific journals so that other researchers working on improving treatments for people with incontinence will benefit from our knowledge. You will not be identified on any publications.

Who is organising and funding the research?

This study is organised by the Institute of Urology and Nephrology and the Department of Medicine at the Whittington Hospital.

Who has reviewed the study?

The Moorfields and Whittington Research Ethics Committee has reviewed this study.

Contact for further information

If you have any questions please don't hesitate to contact us.

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