Stone formation in the infected pediatric enterocystoplasty

Rejiv B. Mathoera

Stone Formation in the Infected Pediatric Enterocystoplasty

Steenvorming in de geïnfecteerde enterocystoplastiek van het kind

Stone formation in the infected pediatric enterocystoplasty
Rejiv B. Mathoera

Thesis, Erasmus University- with references and a summary in Dutch

ISBN 90-6734-276-9

Printed by Optima Grafische Communicatie, Rotterdam, The Netherlands
(www.ogc.nl)

Stone Formation in the Infected Pediatric Enterocystoplasty

Steenvorming in de geïnfecteerde enterocystoplastiek van het kind

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof.dr.ir. J.H. van Bemmel
en volgens het besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

Woensdag 21 mei 2003 om 11:45 uur

door

Rejiv Bikha Mathoera

geboren te Oranjestad (Aruba)

Promotiecommissie

Promotoren:	Prof.dr. F. H. Shröder Prof.dr. H. A. Verbrugh
Overige leden:	Prof.dr. R.J.M. Nijman Prof.dr. R. de Groot Dr. A.W.C. Einerhand
Copromotor:	Dr. D.J. Kok

Omslagillustratie van de voorkaft is een replica van een koperets van Aristaeus, de zoon van Cyrene en Apollo, die de veranderlijke en listige zeegod Proteus vastbindt en dwingt tot voorleggingen over de toekomst.

uit: "Tempel der Zanggodinnen" door Bernard Picart Le Romain en andere braeve meesters.
Gedrukt te Amsterdam door Zacharias Chatelain (1783)

Omslagillustratie van de achterkaft is een globale voorstelling van interacties die een rol spelen tussen de vier belangrijkste categorieën die bestudeerd worden in dit proefschrift: de bacterie, de gastheer, de omgeving (of, naar zal blijken, MUC2 en MUC5AC) en het kristal. (Gebaseerd op de klassieke infectie driehoek van interacties tussen gastheer, micro-organisme en omgeving)

Dit proefschrift werd bewerkt binnen de Medische Microbiologie & Infectieziekten en de Kinderurologische divisie van de vakgroep Urologie, Faculteit Geneeskunde en Gezondheidswetenschappen, Erasmus MC Universitair Medisch Centrum Rotterdam

Dit proefschrift en het onderliggende onderzoek werden mede mogelijk gemaakt door de financiële steun van de Stichting Urologisch Wetenschappelijk Onderzoek (SUWO)

Voor mijn ouders

Contents

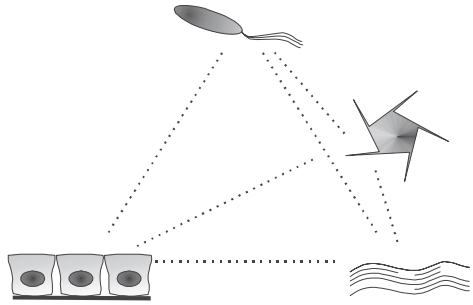
Abbreviations

AB-PAS	Alcian Blue- Periodic Acid Schiff
AEC	3-amino-9-ethylcarbazole
APES	aminopropyltriehoxysilane
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CaP	Calcium Phosphate
CCD	Charge Coupled Device
C.I.95%	95% Confidence Interval
CIC	Clean Intermittent Catheterization
CLSM	Confocal Laser Scanning Microscopy
CPM	Counts per minute
Cpn-60	Chaperonin – 60 kDa
CPS	Capsular (glyco)proteins
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic acid
DTT	dithiotreitol
EDTA	ethylene-diamine-tetraacetic acid
FBS	Fetal Bovine Serum
FITC	fluoro-isothiocyanate
GAG	glycosaminoglycans
GroEL	Historic name for Hsp 60 in *E.coli.* "Gro" stands for phage growth; "E" indicates that the growth defect can be overcome by a mutation in the phage head gene E; and "L" stands for "large subunit"
HCM	Human Colonic Mucin
HGM	Human Gastric Mucin
HMDS	hexamethyldisilazane
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
Hsp-60	Heat Shock Protein - 60 kDa
IL	Interleukine
IVP	Intravenous pyelogram
kDa	kilo Dalton
LC	Liquid Chromatography
LPS	Lipopolysaccharide
MASCOT® score	Matrixscience ® patented MOWSE based probability score for identification of peptide fragments
MDCK	Madin Darby Canine Kidney
MDH	Malate dehydrogenase
MMC	Meningo-myelocéle
MOWSE score	MOlecular Weight Search score
MR/K	Mannose Resistant- Klebsiella like fimbriae
MR/P	Mannose Resistant- Proteus like fimbriae
MS/MS	Tandem mass spectrometry
MTT	thiazolyl blue tetrazolium bromide = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAF	Non-agglutinating Fimbriae
NCCLS	National Committee for Clinical Laboratory Standards
NEAA	Non-essential Amino Acids
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phospate Buffer Solution
PBST	PBS + 0.1% Tween 20
PDGF	Platelet Derived Growth Factor
R.R.	Relative Risk= absolute risk of an index group/ absolute risk of the reference group
RASA	Relative Antibiotic Susceptibility Assay
RIPA	Radio-Immuno Precipitation Buffer
RNA	ribonucleic acid
ROS	Radical Oxygen Species
SDS	sodium-dodecylsulphate
THP	Tamm Horsfal Protein
TNF-α	Tumor Necrosis Factor-α
TRITC	thio-rhodamine-isothiocyanate
TXSP	Triton X-100 soluble membrane proteins
UCA	Urothelial Cell Adhesin
VACTERL	vertebra-anorectal-cardial-tracheal-esophageal-renal-limbs
XRMA	X-ray microanalysis

Chapter 1

General Introduction

Bacterial tropism of enterocystoplasty infections and the influence of mucins and sugar moieties on Proteus mirabilis adhesion



R.B. Mathoera, D.J. Kok,
(Submitted)

Introduction

Proteus mirabilis is one of the most frequent bacterial agents that can induce infection stone formation[1] by urease production. In recent years the influence of *Proteus mirabilis* on stone formation in enterocystoplasties has been primarily related to the presence of urease. Usually the definition of a symptomatic infection: "The multiplication and maintenance of pathogenic bacteria, fungi, parasites or viruses after contamination, causing local inflammation or systemic disease of the host", was overlooked. Often the symptom "bacteriuria" was evaluated rather than the disease "symptomatic urinary tract infection" or "cystitis" which are correlated with stone formation in enterocystoplasties. Symptomatic infection assumes an initial interaction between bacteria and the bladder epithelium, followed by a cellular reaction that is associated with cellular, urinary and bacteriological factors. As an objective parameter for infection, bacteriuria or a nitrite test are usually regarded as a sufficient clinical indication of infection. The strong correlation between bacteriuria and cystitis in the normal bladder fuels misinterpretations of scientific data on the effect of treatment and of the definition of disease in enterocystoplasties by clinicians and scientists alike. Bacterial adherence to epithelial surfaces is important for starting the infection[2] and may be a target in prevention.

Swarming behavior is a typical characteristic of *Proteus* strains, although non-swarming wild types also occur. Swarming behaviour[3] and flagellation[4] have been regarded as motility enhancing traits that play a role in ascending urinary tract infections. Motility enhancing traits indeed facilitate ascending urinary tract infections but are not necessary for urinary tract infection with *P.mirabilis*[5]. *P.mirabilis* can adhere to different epithelial[6] surfaces and invade numerous cell types. Adhesion of *P.mirabilis* to epithelium depends upon bacterial characteristics and on epithelial adhesion molecules. It is not related to other adhesion molecules such as the glycosphingolipids ⇔ Type I fimbriae interaction in *Escherichia coli*, the most common urinary pathogen[7]. These interactions are most critical in cystitis in the early stage of infection[8].

In the normal bladder the epithelial lining responds with cytokines (interleukin (IL)-6, IL-8, platelet-derived growth factor (PDGF) and tumor necrosis factor-alpha, but not IL-1 beta, IL-2, IL-4 and IL-10)[9] . The epithelial lining acts as a barrier and exfoliation of epithelial cells will render adhering bacteria harmless[10]. Nevertheless in most cases exfoliation will not stop the bacterial infection. Bacterial invasion of epithelial cells may occur, providing a safe haven from antibiotic therapy and causing persistent infection[10, 11].

In the enterocystoplasty bacterial adhesion to the intestinal epithelial lining can furthermore persist due to absence of the normal bladder defense mechanisms and presence of factors that stimulate adhesion of bacteria and persistence of the bacterial infection. The intestinal epithelium in the bladder will change histologically to resemble urothelium[12] but to what extent this change influences the bacterial- host interaction is still unclear. Epithelial changes also occur in the catheterization channel due to catheterization although some of these abnormalities may have been there at the time of construction[14].

Consequently, antibiotic treatment is clinically less effective[13]. Bacterial infection in enterocystoplasties is thus often regarded as colonization of the intestinal segment[13] . In contrast, the infections in neurogenic bladders are often an indication for surgical treatment[15]. Whereas for the latter antimicrobiological therapy is preferred, colonization immunity can be achieved to

some extent by *Lactobaccilus* or *E.coli* strains [16] to prevent symptomatic urinary tract infection. The low level of *P.mirabilis* adherence may limit the effect of colonization immunity[17].
Effective anti-infection methods in addition to antibiotic therapy require overall insight into both the bacterial mechanisms involved in the adhesion of *P.mirabilis* to epithelial cells and the epithelial adhesion factors of enterocystoplasties and the normal bladder. We will describe the interactions of epithelial cells, *P.mirabilis* and crystals in relation to lower urinary tract infection and stone formation.

Enterocystoplasties as continent urinary diversions
Bowel segments have been used for more then a century in urologic surgery. Ileal reservoirs, ileocystoplasties, have become a more acceptable alternative for urinary diversion than the standard ileal conduit (**Fig**.1.) since their introduction and refinements in the 1950's and 1960's[18]. Patients usually evacuate their bladder by clean intermittent catheterization, usually prior to augmentation.

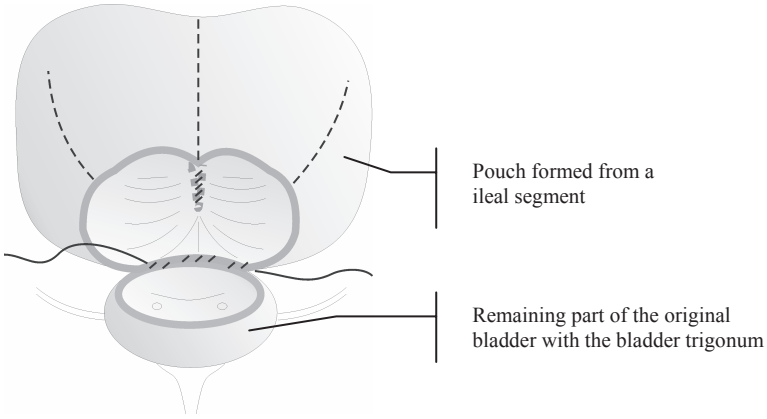


Fig.1.
Ileocystoplasty constructed according to the Goodwin Cup-Patch Technique [65]:
An ileal segment is isolated and opened along the anti-mesenteric border. A patch is formed to resemble a W, and both sides are connected to form a pouch. The pouch is anastomosed to the opened bladder. This was the first, but now historic suggestion of a continent reservoir.

The indications for bladder augmentations using intestinal segments differ for adults and children. In children most common bladder augmentations are ileocystoplasties and colocystoplasties, often to enlarge a small non-compliant bladder[15], usually neurogenic bladders in meningo-myelocoele patients, bladder extrophies or cloacal malformations. Sometimes auto-augmentations or gastrocystoplasties are constructed. These can be converted into ileocystoplasties if complications occur. In adults enterocystoplasties are often orthotopic bladder replacements constructed to completely replace the original bladder as part of the surgical treatment of bladder, prostatic and other tumors. They have their own indications and complications.
Alternative methods are under development such as epithelial ablation methods[19] that result in the removal of intestinal epithelium for urothelial overgrowth and methods that rely on in-vitro grown urothelium or smooth muscle regeneration in e.g. bladder acellular matrix graft[20]. Stone formation is still frequent in all cases. It becomes more relevant when the patient is young at the

age of augmentation and undergoes concomitant continence enhancing surgical procedures. Stone formation is often related to urease producing bacteria such as *P.mirabilis*[1] as presented in **Fig**.4. Bacteriuria has thus been regarded as one of the problems in enterocystoplasties although at present it is advised not to treat bacteriuria in enterocystoplasties unless symptomatic urinary tract infection occurs[14].

A number of considerations fuel this opinion that bacterial infection in enterocystoplasties should not always be treated as a complication, but as normal and a logical result of the use of intestinal tissue[14,15]. These are: the bacterial colonization of the intestinal segment before the construction of the enterocystoplasties, the absence of symptomatic urinary tract infection in many cases and the relatively small effect of antibiotic prevention or treatment on these infections[13,24,66]. In contrast, community acquired urinary tract infections in these patients have antibiotic resistance profiles resembling nosocomial infections which indicate frequent exposure to antibiotics and a possible explanation for the findings described above.

Regarding factors like bacterial adhesion, bacteriuria, electrolyte disturbances and stone formation, gastrocystoplasties are less likely to develop infection stones then ileocystoplasties[21]. The differences in epithelial surface and pH between ileocystoplasties, colocystoplasties, autoaugmentation, gastrocystoplasties and the normal bladder lead to specific bacterial interactions[24] and are expressed in the bacteriuria in these patients. A low pH level in gastrocystoplasties could be responsible for less stone formation. However, gastrocystoplasty patients have other complications such as the hematuria-dysuria syndrome[21]. Tumor formation is also more likely at epithelial junctions of gastrocystoplasties and ileocystoplasties[22]. Bacterial colonization or infection may contribute to this complication by the production of nitrosamines[23].

In non-infected enterocystoplasties, a relatively high average pH of 6.5 is present[25]. This rise in pH level may be attributed to urea uptake, which also decreases the available nitrogen for bacterial growth and correlates with the type and size of the intestinal segment in enterocystoplaties[25]. In infected enterocystoplasties urease activity further increases the pH. By concomitantly increasing the ammonium concentration it also increases the drive for formation of triple phosphate stones. In acidic solutions mucins precipitate according to turbity measurements[26], while formation and aggregation of crystals will occur at high pH levels that also insure the availability of free mucin molecules to enhance aggregation[27]. *E.coli* seems to be able to inhibit urease[28]. Apart from anti-adhesion effects, this feature of colonization by *E.coli* could give the augmented bladder protection from more pathogenic urease producing urinary pathogens such as *P.mirabilis*. Urinary acidification may also have a beneficial effect. Acidification and concentration of infected urine is more likely after inactivation of bacterial urease. Reduced fluids intake and lower clean intermittent catheterization frequency may be preferred by patients to prevent the increasing pain from the urinary tract infection, but they enhance urinary stasis, vesico-ureteral reflux and infection.
A diagram of the stone formation process with several patient related factors is presented in **Fig**.1.

In most cases the formation of a detectable stone will lead to a second surgical procedure to remove the stone. In children and adults with an enterocystoplasty this means either open surgery or stone extraction in most cases. Removing the stone as a whole prevents the formation of numerous smaller fragments that may form the nidus of the next stone.

Bacterial adhesion to epithelium

Symptomatic bacterial infection of enterocystoplasties requires the capacity to survive in urine and to adhere to the intestinal epithelium or the small amount of urothelium that may be left. Common uropathogens meet the requirements of survival in urine. Selection of the most prevalent infection must be determined by additional factors to explain the difference in bacteriological profile between normal bladders and enterocystoplasties. Possible additional factors are: the presence of intestinal-derived mucus and mucins, the abundant use of antibiotics, catheterization and urinary stasis and urine alkalinisation.

A number of bacterial fimbriae and pili may be responsible for bacterial adhesion to epithelial cells at a molecular level. Several of these adhesion molecules, such as UCA[32] (uroepithelial cell adhesin) specifically target the urothelium. NAF[33,34] (non-agglutinating fimbriae) and several MR/P[33] (Mannose Resistant fimbriae/ Proteus-like) are present on most Gram-negative bacteria found in urinary tract infections, such as *P.mirabilis*.

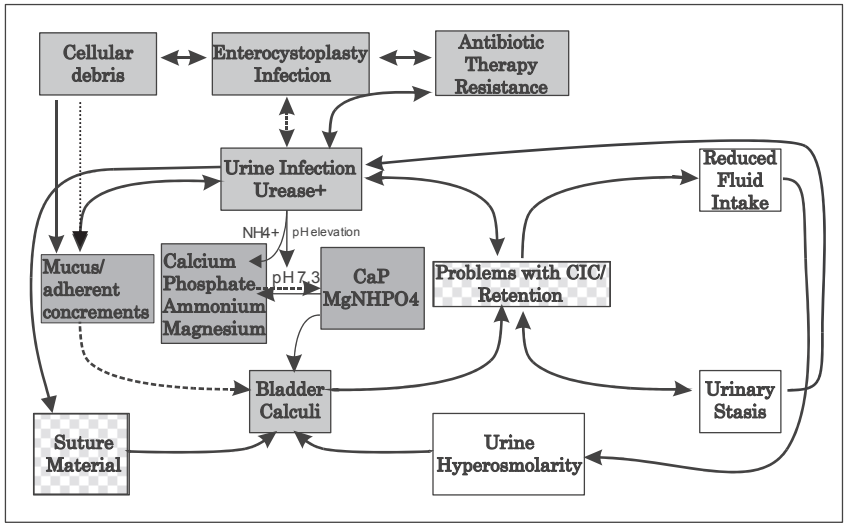


Fig2.

A proposed relationship between risk factors involved in stone formation. Broken lines indicate the involvement of mucins MUC2 and MUC5AC in the process. The checkered areas indicate hypothesized relations based on epidemiological data and literature. White areas indicate proposed relations based on literature and clinical experience. Suture materials and problems with Clean Intermittent Catheterization (CIC) are proposed as risk factors, based on correlations between respectively concomitant surgical procedures and consultation requiring catheterization problems with the incidence of bladder calculi. Bacterial colonization or infection with urease producing bacteria over longer periods of time due to bladder outlet procedures inducing urinary stasis[15] and the formation of concrements on suture material[29] and other foreign objects in the bladder also contribute to enterocystoplasty stone formation. Finally, both long-term histological and clean intermittent catheterisation problems [30,31] due to retention or pain contribute. The formation of a stone itself may in turn also lead to some of its own risk factors[31]. Therefore, the most common measures are to catheterize regularly, to use absorbable suture material in the bladder and be vigilant for stone formation and urease producing pathogens.

Host defense mechanisms

It has been reported that bacterial adhesion decreases when *P.mirabilis* is cultured in urine[34]. This may be the result of urinary proteins that block the adhesion of bacteria at the cellular surface, as is the case for Tamm-Horsfall protein and uroplakin Ia and Ib of *E.coli*[35]. Similarly an interaction between Tamm-Horsfall or mucins and the bacterial capsule of uropathogens may occur due to other bacterial-urine interactions such as crystallization on the bacterial capsule of *P.mirabilis*, **Fig**. 2. The effect of Tamm-Horsfall as an anti-adherence molecule is decreased by an increase in calcium, which induces precipitation of calcium salts[35]. Precipitation of calcium phosphate can occur on the bacterial capsule, as we visualized using scanning electron microscopy imaging[36].

It seems that bacterial calcification would only enhance the chance of infection stone formation after the bacteria have already adhered to the cellular surface or if they have formed a substantial mass by agglomeration. Non-adhered calcified bacteria are most likely washed out. Crystal precipitation on bacteria could thus be a host defense against infection with urease producing bacteria. On the other hand, it may also be a virulence factor, shielding the bacteria from antibiotics and is possibly involved in the increased resistance against antibacterial therapy in infection stones.

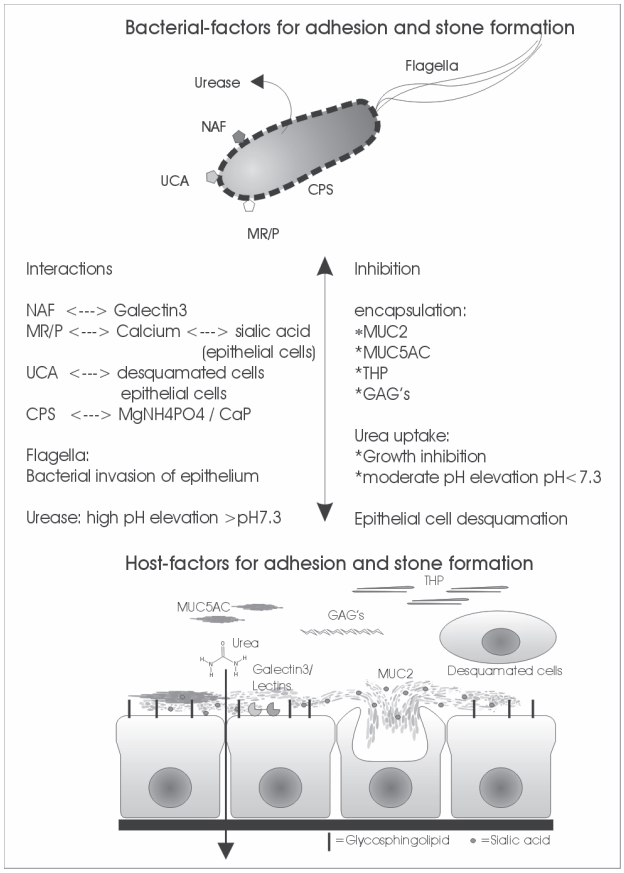


Fig.3.
Interacting bacterial and host factors in bacterial adhesion to the enterocystoplasty surface, relevant for the formation of an enterocystoplasty infection stone. The unknown factors are presented as part of the bacterial capsule or capsule associated proteins These will be discussed in chapter 4 and 7.

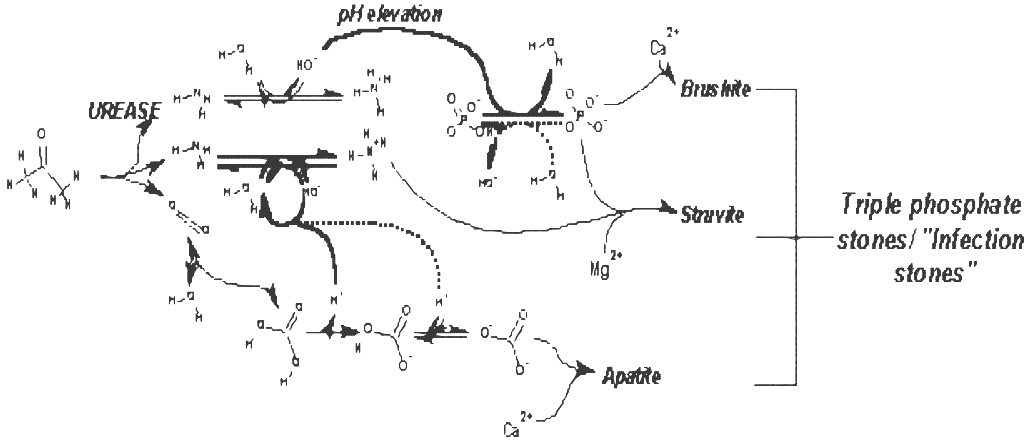


Fig.4. *The formation of infection stones according to experimental in-vitro data from Grenabo e.a., Hedelin e.a. and Petterson e.a. by the action of urease in urine. Urease in the bladder is generally produced by bacteria, usually gram-negative rods in a fashion described and reviewed in detail by H.M Mobley*

<u>*Bacterial adhesion factors*</u>

Adhesion may involve (glyco)protein ⇔ (glyco)protein (glyco)protein ⇔ polysaccharide or (glyco)lipid ⇔ (glyco)protein interactions and often includes cellular proteins described as lectins, such as galectins[37]. The bacterial adhesion molecules enhance bacterial infection and pathogenicity and are therefore classified as virulence factors. One of the cellular proteins involved in the interaction with *Proteus mirabilis*, NAF, was identified on MDCK cells as galectin 3 [38]. Galectin 3 has regions rich in tyrosine, glycine and proline which are very similar to the tandem repeats found in mucins in that they are the carbohydrate binding domains of the molecule[38] and that the bacterial capsule interacts with sugar moieties on the molecule[39]. Typical for Galectin 3 is also the possibility for multimerisation, transforming a 36 kDa monomer into a high molecular weight glycoprotein. Mucin interacting molecules also include GroEL (Hsp60), a heat shock protein which acts as a protein folding molecule. GroEL has been established as an intracellular molecule that is sometimes found at the bacterial surface of *Legionella pneumophilia*[40] *Haemophilus*[41] strains or *Clostridium difficile*[42]. GroEL seems to interact with intestinal mucins. It is not yet clear when an intracellular molecule would migrate to the bacterial surface, but increased expression of Hsp60 (Cpn60) in a hostile environment and altruistic lysis of single bacteria may benefit the survival of a colony.

<u>*Epithelial adhesion factors*</u>

Bacteria-cell interactions take place at the surface of the cell, which is often covered by high molecular weight substances (glycocalyx and mucus layer) rich in sugar residues that protect the cellular surface. These surface molecules may act as a barrier to external chemical or biochemical or microbiological processes, isolate the cell to preserve cellular function or act as receptors or signal molecules. All surface molecules are possible targets for bacterial adhesion. The most prevalent molecule for a certain tissue type is a likely microbiological target for adhesion and infection, even when they were produced by the cell to resist bacterial adhesion. A surface glycoprotein target in enterocystoplasties is mucin, the most important structural component of the mucus layer.

The large amount of intestinal tissue incorporated in enterocystoplasties produces and secretes either MUC2 or MUC5AC. It seems from interaction studies that addition of sialic acid and reduction of mucin sugar-O-rings decrease bacterial interaction with MUC2 and MUC5AC[43]. Therefore the interaction probably involves the sugar residues common in both mucins and galectin 3 although other interactions including three-dimensional recognition cannot be excluded.

Mucins

The many different mucins differ in size and three-dimensional shape but share sequence and glycosylation similarities. Before achieving a MUC designation, mucins are usually described by their function, mucin-like or mucoproteins. To date there are approximately 16 mucin types (MUC1-13, and MUC16-18) of which 14 have been approved by the Human Genome Organization Gene Nomenclature Committee (http:/www.hugo-international.org/hugo/). MUC1 carries a number of other names such as episialin, or epithelial membrane antigen. The gene sequences of MUC1-7 have been determined and partial gene sequences are available for all mucin types including endomucin (MUC14). MUC1- MUC7 are the best studied so far.

Mucins are high molecular weight o-glycosylated glycoproteins with galactose, fucose, Gal-Nac, Glc-Nac, sulfated sugars or sialic acid and unique carboxylated ends and tandem repeats often called mucin domains or PTS regions, high in Proline, Threonine, and Serine (20-50% of amino-acids, 50-80% of molecular weight). They contain at least 50 weight% O-glycans in their mucin domains and often show additional N-glycosylation. They are found in epithelial mucous membranes of the gastro-intestinal, respiratory and urogenital tract, on endothelial cells and on leucocytes.[44] They share several domains including a Von Willibrand factor domain which is expressed in both MUC2 and MUC5AC.
However, most of these domains are not restricted to the mucin family. MUC4 and MUC1 are the most dominant mucin types in the genitourinary tract. In one third MUC3 is also expressed[45]. MUC4 is also present next to MUC2 and MUC5AC in the normal epithelium of the respiratory tract[46]. MUC2 is the most common mucin type in ileum and colon[47], whereas MUC5AC is typical for epithelial stomach tissue[48] and otherwise only markedly present in the duodenum, bladder trigonum, or in case of a bladder tumor. MUC6 can also be found in the antrum of the stomach, but is produced by the glandular tissue[48]. The secretory MUC2, MUC5AC, MUC5B and MUC6 seem to have similar and unique cysteine rich domains and are encoded on the 11p15 chromosomal locus. Since a similar cluster of mucin genes can be found on the 7q22 chromosomal gene locus, restructuring of the mucin family results in tentative subgroups of 11p15 mucins and 7q22 mucins. Restructuring of the mucin family tree could clarify the relationship within the mucin family between the different mucins and explain how mucins and mucin candidates with varying characteristics can be classified. This subject was eloquently addressed elsewhere[49]. Many of these glycoproteins are highly sulfated and contain sialic acid to produce a relatively large rigid structure by absorbing water to form a viscous solution in their hydrated form.

Mucins in enterocystoplasties

After incorporating the intestinal segment in the bladder, mucin expression changes. Up-regulation of MUC1 and MUC4 expression occurs in transposed ileal segments resembling the mucin excretion pattern of normal bladder epithelium, whereas ileal segments in clam enterocystoplasty showed an up-regulation of MUC2, 3, 4 and 5AC expression towards the site

of anastomosis with the ileal segment[46]. These changes, some of which may be due to the exposure to urine, coincide with a change from ileal sialomucins to colonic sulfomucins by a change in glycosylation[50]. The mucins bind calcium and may form a template resembling the crystal structure on which crystals are formed and grow, as has been described for gallbladder stones[51] .

On the surface of the epithelial cells lining the bladder and intestine different mucins are expressed. MUC2 and MUC5AC are characteristic mucins along the gastro-intestinal tract. They form a barrier against the microbiologically colonized intestinal lumen but also supply the intestinal flora with a growth surface. When transposed to the bladder these mucins are still produced. In the lung and bladder mucins have a protecting function and the mucus blanket is discarded with any adhering bacteria. *P.mirabilis* overcomes this barrier with UCA, NAF and MRP fimbriae to persist in the bladder and lung.

In the bladder exfoliation of the bladder epithelium is a method to remove bacteria when the mucous blanket fails[52]. In addition desquamated epithelial cells may have an antibacterial effect[53], attributed by some to bladder mucin and glycosaminoglycans[54]. It is likely that the intestinal mucins which act as a barrier and tolerate bacterial colonization function in the same manner when transposed to the bladder. In enterocystoplasties, adhesion of *P.mirabilis* to the augmented bladder with its sequela of urease production and pH rise will then make the bladder susceptible to stone formation. We have previously shown that these strains thereby bind to the most commonly available targets in the enterocystoplasty (MUC2 and MUC5AC) *(Chapter 6 and 7).*

Infection prevention by interaction inhibition

As adherence of bacteria is the pivotal step in the infection process[5] and the infection and damaging of epithelial surfaces in the bladder with urease producing bacteria is key to the stone formation, this has to be prevented**.** This may be accomplished with relative easy, because most of the patients are already on CIC and some experimental success has already been achieved by others as discussed in this section From interaction experiments we have concluded that sialic acid and MUC3 may prevent bacterial- epithelial interaction. Furthermore we found that intact sugar-O-rings were important for the bacterial mucin interaction. This opens some leads to prevention of adherence.

Several groups have tested rinsing solution that aim at preventing infection. Several specific carbohydrate-bacteria interactions were discovered. One interaction inhibition method using N-acetylneuraminyl (α2-3) lactose was developed against *H. pylori*[39]. Also moderately effective rinsing solutions were developed using competing bacteria, such as *Lactobacillus*, to counteract colonization by *P.mirabilis*[16]. Sugar solutions containing galactose and fucose were effectively used in the treatment of resistant infections with *Pseudomonas aeruginosa.*[55] *Pseudomonas* strains also depend on the presence of sialic acid and specific sugars commonly found in mucins for binding to the epithelial cells of the host[56] . Other interactions have been described with specific mucin types, such as MUC1[57] . *Pseudomonas aeruginosa* and *E.coli* were able to up regulate MUC2 and MUC5AC in infected cells by the lipopolysaccharides in their bacterial capsule[58]. In this case bacteria exploit the mucin secretion defense mechanism by the host as a cellular adhesion molecule. Asialic derivatives of mucins could act as interactions inhibitors for *Pseudomonas aeruginosa* to host cells[59]. However, the commercial antibody detection methods used to show this seem unreliable and asialic derivatives appear involved in the bacterial

interaction in biofilms rather then the bacteria-cell interaction. Mannose and N-acetyl-D-galactosamine inhibited adhesion of *E. coli* and *P. aeruginosa* to epithelial cells, whereas only mannose inhibited adhesion of *S. zooepidemicus* to endometrial epithelium. N-acetyl-D-glucosamine, D-(+)-glucose, galactose, and N-acetyl-neuraminic acid had no effect on the bacterial adherence of these strains[60]. Overall, bacterial interactions with host epithelial cells depend to a large degree on specific sugar interactions. This makes treatment using specific sugars possible. For research purposes the use of pure sugars or glycoproteins is inevitable. Unfortunately, treatments using a mixture of these purified sugars could be very expensive unless such mixtures are obtained from a natural source. To select the best mixtures, it is necessary to study the possible interactions of uropathogens with the epithelial cells in combination with interaction studies, to find the best combinations or requirements of interaction inhibitors. From the interaction characteristics of bacterial adhesion molecules, the interaction inhibiting molecules and their epithelial counterparts a database may be compiled. This database and the expression of adhesion molecules on uropathogens could be used to determine the correct additives for bladder rinsing solutions, tailored to the patients needs.

The anti-infective effect of glycosaminoglycans support that sugars may be effective in the prevention of urinary tract infections. Furthermore anti-adhesion treatment with N-Acetylcysteine for *Streptococcus pneumoniae* and *Haemophilus influenzae* strains was effective[61] in vitro on oropharyngeal epithelial cells. Mucoprotective substances such as ecabet sodium and rebamipide also seem to have a positive therapeutic effect on bacterial adhesion of *H.pylori* to the gastric mucosa[62] by creating a protective layer on the gastric epithelial surface, shielding the host from the bacterial agent . The haemagglutinins of *Bacteroides forsythus* strains seem to be preferentially inhibited by amino-acids such as L-arginine and L-histidine which also inhibit their interaction with polymorphonuclear leucocytes, but this interaction can be altered by trypsinisation of the leucocytes[63]. So other options are available besides sugar compounds which may be therapeutically helpful on their own, in synergistic combinations or in combination with antibiotics.

Nevertheless, at present sugars seem the best option for effective prevention of urinary tract infections and of infections in enterocystoplasties since the sugar binding properties of uropathogenic bacteria are well conserved among strains and comparable between several gram-negative uropathogens. Until now most improvements on bladder irrigation fluids are achieved using antiseptics and anti-fungal agents. As a prophylactic, the prerequisite for success is the ability of a solution to inhibit the occurrence of bacterial infections and infection stones for a lifetime without complications or loss of effect. In light of the bladder rinsing solutions in use in urologic practice at present there certainly is a lot of improvement possible using sugar solutions which have proven successful in vitro[64]. Bladder irrigation fluids that would act on the most important urease producing bacteria and the most common uropathogens in the augmented bladder would greatly improve the urologic success of the enterocystoplasty as a continent diversion and should be our aim for the future.

References

1. Takeuchi H, Yamamoto S, Terai A, Kurazono H, Takeda Y, Okada Y, Yoshida O., Detection of Proteus mirabilis urease gene in urinary calculi by polymerase chain reaction. Int J Urol. 3(3):202-6; 1996
2. Roberts DD., Interactions of respiratory pathogens with host cell surface and extracellular matrix components. Am J Respir Cell Mol Biol 3(3):181-6; 1990
3. Allison C, Emody L, Coleman N, Hughes C., The role of swarm cell differentiation and multicellular migration in the uropathogenicity of Proteus mirabilis. J Infect Dis. 169(5):1155-8; 1994
4. Mobley HL, Belas R, Lockatell V, Chippendale G, Trifillis AL, Johnson DE, Warren JW. Construction of a flagellum-negative mutant of Proteus mirabilis: effect on internalization by human renal epithelial cells and virulence in a mouse model of ascending urinary tract infection. Infect Immun. 64(12):5332-40; 1996
5. Roberts JA, Fussell EN, Kaack MB. Bacterial adherence to urethral catheters. J Urol. 144(2 Pt 1):264-9; 1990
6. Savoia D, Martinetto P, Achino A, Pugliese A. Adhesion of Proteus species to various cell types. Eur J Clin Microbiol. 2(6):571-6; 1983
7. Lomberg H, Larsson P, Leffler H, Svanborg-Eden C., Different binding specificities of P. mirabilis compared to E. coli. Scand J Infect Dis Suppl. 33:37-42; 1982
8. Mardh PA, Colleen S, Hovelius B., Attachment of bacteria to exfoliated cells from the urogenital tract. Invest Urol 16(5):322-6; 1979
9. Funfstuck R, Franke S, Hellberg M, Ott U, Knofel B, Straube E, Sommer M, Hacker J. Secretion of cytokines by uroepithelial cells stimulated by Escherichia coli and Citrobacter spp. nt J Antimicrob Agents 17(4):253-8; 2001
10. Schilling JD, Mulvey MA, Hultgren SJ. Dynamic interactions between host and pathogen during acute urinary tract infections. Urology 57(6 Suppl 1):56-61; 2001
11. Mulvey MA, Schilling JD, Hultgren SJ., Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. Infect Immun. 69(7):4572-9; 2001
12. Little JS Jr, Klee LW, Hoover DM, Rink RC., Long-term histopathological changes observed in rats subjected to augmentation cystoplasty. J Urol. 152(2 Pt 2):720-4;1994
13. Akerlund S, Campanello M, Kaijser B, Jonsson O. Bacteriuria in patients with a continent ileal reservoir for urinary diversion does not regularly require antibiotic treatment. Br J Urol. 74(2):177-81; 1994
14. Singh S, Sharma S, Sen R, Garg P, Airon R., Comparative evaluation of commonly used catheters through histopathological changes induced in bladder urothelium. Urol Int. 53(3):155-7; 1994
15. Mathoera RB, Kok DJ, Nijman RJM., Bladder Calculi in augmentation cystoplasty in children. Urology 56(3):482-7; 2000
16. Asahara T, Nomoto K, Watanuki M, Yokokura T., Antimicrobial activity of intraurethrally administered probiotic Lactobacillus casei in a murine model of Escherichia coli urinary tract infection. Antimicrob Agents Chemother 45(6):1751-60; 2001
17. Osset J, Bartolome RM, Garcia E, Andreu A., Assessment of the capacity of Lactobacillus to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. J Infect Dis. 183(3):485-491; 2001
18. Perez L.M., Webster G.D., History of urinary diversion techniques. In: Webster G.D., Goldwasser B. (eds.), *Urinary diversion, Scientific Foundations and Clinical Practice.*, 1st ed. pp. 2-22. Isis Medical Media Ltd., Oxford,UK.
19. Demirbilek S, Aydin G, Ozardali HI, Baykara S., Chemically induced intestinal de-epithelialization using silver nitrate for bladder augmentation. Urol Res 29(1):29-33; 2001
20. Wefer J, Sievert KD, Schlote N, Wefer AE, Nunes L, Dahiya R, Gleason CA, Tanagho EA., Time dependent smooth muscle regeneration and maturation in a bladder acellular matrix graft: histological studies and in vivo functional evaluation. J Urol 165(5):1755-9; 2001
21. Di Benedetto V, Monfort G., Stomach versus sigmoid colon in children undergoing major reconstruction of the lower urinary tract. Pediatr Surg Int 12(5-6):393-6; 1997

22. Gitlin JS, Wu XR, Sun TT, Ritchey ML, Shapiro E., New concepts of histological changes in experimental augmentation cystoplasty: insights into the development of neoplastic transformation at the enterovesical and gastrovesical anastomosis. J Urol 162(3 Pt 2):1096-100; 1999
23. Woodhams SD, Greenwell TJ, Smalley T, Mundy AR., Factors causing variation in urinary N-nitrosamine levels in enterocystoplasties. BJU Int 88(3):187-91; 2001
24. Sakai Y, Fujisawa M, Nakano Y, Miyazaki S, Arakawa S, Kamidono S., Bacterial adherence in a rat bladder augmentation model: ileocystoplasty versus colocystoplasty. J Urol 164(6):2104-7; 2000
25. Lockhart JL, Davies R, Persky L, Figueroa TE, Ramirez G., Acid-base changes following urinary tract reconstruction for continent diversion and orthotopic bladder replacement. J Urol 152(2 Pt 1):338-42; 1994
26. Bhaskar R.K., Gang D., Bansil R., Pajevic S., Hamilton J.A., Turner B.S., LaMont T.J., Profound increase in viscosity and aggregation of pig gastric mucin at low pH. Am. J. Physiol. 261: G827-G832; 1991
27. Mathoera, R.B., D.J. Kok, W.J. Visser, C.M. Verduin, R.J.M. Nijman. Cellular membrane associated mucins in artificial urine as mediators of crystal adhesion: an in vitro enterocystoplasty model. J Urol. 166: 2329-2336 ; 2001
28. Edin-Liljegren A, Grenabo L, Hedelin H, Larsson P, Pettersson S., Influence of Escherichia coli on urease-induced crystallisation in human urine., Scand J Urol Nephrol 27(2):163-7; 1993
29. Edlich RF, Rodeheaver GT, Thacker JG., Considerations in the choice of sutures for wound closure of the genitourinary tract. J Urol 137(3):373-9; 1987
30. Barroso U, Jednak R, Fleming P, Barthold JS, Gonzalez R., Bladder calculi in children who perform clean intermittent catheterization. BJU Int 85(7):879-84; 2000
31. Solomon MH, Koff SA, Diokno AC., Bladder calculi complicating intermittent catheterization. J Urol. 1980 Jul;124(1):140-1.
32. Wray SK, Hull SI, Cook RG, Barrish J, Hull RA.,Identification and characterization of a uroepithelial cell adhesin from a uropathogenic isolate of Proteus mirabilis. Infect Immun. 54(1):43-9;1986
33. R K.Latta, A Grondin, H. C Jarrell, RG. Nicholls, L R. Bérubé1. Differential expression of nonagglutinating fimbriae and MR/P pili in swarming colonies of Proteus mirabilis. J Bacteriol 181(10):3220-3225; 1999
34. Tolson DL, Harrison BA, Latta RK, Lee KK, Altman E. The expression of nonagglutinating fimbriae and its role in Proteus mirabilis adherence to epithelial cells. Can J Microbiol. 43(8):709-17; 1997
35. Sobota AE, Apicella LL., Reduction in the anti-adherence activity of Tamm-Horsfall protein with increasing concentration of calcium. Urol Res. 19(3):177-80; 1991
36. Mathoera R.B. Kok D.J., Verduin C.M., Nijman R.J.M., Pathological and therapeutical significance of cellular invasion by Proteus mirabilis in an enterocystoplasty infection stone model. Infect. Immun. 70(12): 7022-7032; 2002
37. Barondes S.H., D.N.W. Cooper, M.A. Gitt, H. Leffler., Galectins, Structure and function of a large family of animal lectins. J. Biol. Chem. 269: 20807-20810;1994
38. Altmann E., Harrison B.A. Latta R.K., Lee K.K., Kelly J.F., Thibault P., Galectin3 mediated adherence of Proteus mirabilis to Madin-Darby canine kidney cells., Biochem. Cell Biol 79: 783-788; 2001
39. N. Sharon, I. Ofek., Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. Glycoconjugate J. 17: 659-664; 2000
40. Garduno R.A., Garduno E., Hoffman P.S., Surface –associated Hsp60 Chaperonin of Legionella pneumophilia mediates invasion an a HeLa Cell Model. Infect, Immun. 66(10): 4602-4610; 1998
41. Frisk A, Ison C.A., Lagergard T., GroEl Heat Shock protein of Haemophilus ducreyi:association with cell surface and capacity to bind eukaryotic cells. Infect. Immun. 66(3):1252-1257; 1998
42. Hennequin C., Pocheray F., Waligora-Dupriet, A.J., Collignon A., Barc, M.A, Bourlioux P., Karjalainen T., GroEl (Hsp60) of Clostridium difficile is involved in cell adherence. Microbiology 147 :87-96 ;2001
43. Mathoera R.B., Einerhand A.W.C., Kok D.J., Verbrugh H.A., Mucin mediated cellular adhesion and crystal formation by *Proteus mirabilis* in an enterocystoplasty infection stone model. (*Submitted)*
44. Perez-Vilar J., Hill R., The structure and assembly of secreted mucins. J. Biol. Chem.274:31751-31754;1999

45. N'Dow J, Pearson JP, Bennett MK, Neal DE, Robson CN., Mucin gene expression in human urothelium and in intestinal segments transposed into the urinary tract. J Urol 164(4):1398-404; 2000
46. Copin MC, Devisme L, Buisine MP, Marquette CH, Wurtz A, Aubert JP, Gosselin B, Porchet N. From normal respiratory mucosa to epidermoid carcinoma: expression of human mucin genes. Int J Cancer 86(2):162-8; 2000
47. B.J.W. Van Klinken, A.W.C. Einerhand, L.A. Duits, M.A. Makkink, K.M.A.J. Tytgat, I.B. Renes, M. Verburg, H.A. Buller, J. Dekker. Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am. J. Phys. 276(1): G115-G124; 1999
48. Nordman H, Davies JR, Lindell G, De Bolos C, Real F, Carlstedt I. Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. Biochem J. 364(Pt 1):191-200; 2002
49. Dekker J., Rossen J.W.A., Büller H.A., Einerhand A.W.C., The mucin family: an obituary. Trends Bioch. Sci.27(3):126-131; 2002
50. Aragona F., Re: Mucin gene expression in human urothelium and in intestinal segments transposed into the urinary tract. J Urol 166: 219-220; 2001
51. van den Berg AA, van Buul JD, Tytgat GN, Groen AK, Ostrow JD. Mucins and calcium phosphate precipitates additively stimulate cholesterol crystallization. J Lipid Res 39(9):1744-51; 1998
52. Aronson M, Medalia O, Amichay D, Nativ O. Endotoxin-induced shedding of viable uroepithelial cells is an antimicrobial defense mechanism. Infect Immun. 56(6):1615-7; 1988
53. Mannhardt W, Putzer M, Zepp F, Schulte-Wissermann H., Host defense within the urinary tract. II. Signal transducing events activate the uroepithelial defense. Pediatr Nephrol 1996 Oct;10(5):573-7
54. Parsons CL, Pollen JJ, Anwar H, Stauffer C, Schmidt JD. Antibacterial activity of bladder surface mucin duplicated in the rabbit bladder by exogenous glycosaminoglycan (sodium pentosanpolysulfate). Infect Immun 27(3):876-81; 1980
55. von Bismarck P, Schneppenheim R, Schumacher U. Successful treatment of Pseudomonas aeruginosa respiratory tract infection with a sugar solution--a case report on a lectin based therapeutic principle. Klin Padiatr 213(5):285-287; 2001
56. Ramphal R, Carnoy C, Fievre S, Michalski JC, Houdret N, Lamblin G, Strecker G, Roussel P. Pseudomonas aeruginosa recognizes carbohydrate chains containing type 1 (Gal beta 1-3GlcNAc) or type 2 (Gal beta 1-4GlcNAc) disaccharide units. Infect Immun 59(2):700-704; 1991
57. Lillehoj EP, Kim BT, Kim KC. Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin. Am J Physiol Lung Cell Mol Physiol. 282(4):L751-6; 2002
58. Dohrman A, Miyata S, Gallup M, Li JD, Chapelin C, Coste A, Escudier E, Nadel J, Basbaum C., Mucin gene (MUC 2 and MUC 5AC) up regulation by Gram-positive and Gram-negative bacteria. Biochim Biophys Acta 1406(3):251-9; 1998
59. Schroeder TH, Zaidi T, Pier GB. Lack of adherence of clinical isolates of Pseudomonas aeruginosa to asialo-GM(1) on epithelial cells. Infect Immun 69(2):719-729; 2001
60. King SS, Young DA, Nequin LG, Carnevale EM. Use of specific sugars to inhibit bacterial adherence to equine endometrium in vitro. Am J Vet Res. 61(4):446-449; 2000
61. Riise GC, Qvarfordt I, Larsson S, Eliasson V, Andersson BA. Inhibitory effect of N-acetylcysteine on adherence of Streptococcus pneumoniae and Haemophilus influenzae to human oropharyngeal epithelial cells in vitro. Respiration 67(5):552-558; 2000
62. Hayashi S, Sugiyama T, Yokota K, Isogai H, Isogai E, Shimomura H, Oguma K, Asaka M, Hirai Y. Combined effect of rebamipide and ecabet sodium on Helicobacter pylori adhesion to gastric epithelial cells. Microbiol Immunol 44(7):557-562;2000
63. Munemasa T, Takemoto T, Dahlen G, Hino T, Shiba H, Ogawa T, Kurihara H. Adherence of Bacteroides forsythus to host cells. Microbios 101(399):115-26; 2000
64. Gasser TC, Madsen PO. Influence of urological irrigation fluids on urothelial bacterial adherence. Urol Res. 21(6):401-405; 1993
65. Goodwin, W.E., Winter C.C., Barker W.F., "Cup-patch" technique of ileocystoplasty for bladder enlargement or partial substitution. Surg. Gynecol. Obstet. 108:240-244; 1959
66. Nurse, D.E., McInerney, P.D., Thomas, P.J., Mundy, A.R.: Stones in enterocystoplasties. Br.J.Urol., **77**: 684-687, 1996

Scope of the thesis

Urinary tract infection is a difficult to treat frequent event in both adults and children. Formation of so-called infection stones is a known complication of urinary tract infection. Children with enterocystoplasties are especially at risk for both problems. The opinio communis has been that infection with urease producing uropathogens like *P. mirabilis* is the main culprit for this stone formation. The exact mechanisms behind both problems and their interrelation are still not clear. The purpose of this thesis is to clarify the infection process at the basis of stone formation. The hope is that this will add a new perspective to the intervention of enterocystoplasty infection and stone formation and that it will contribute to existing prophylactic and therapeutic means.

The working hypothesis for this research project is as follows:
Bacterial adhesion to mucins precedes invasion into epithelial cells, leading to safe havens against antibiotic and other treatment, which results in the formation of infection stones by the alkalizing action of urease and inappropriate effect of host defense mechanisms and surgical measures.

In this thesis two basic research models are used: enterocystoplasty patients and *in-vitro* models for enterocystoplasties using several strains of *P. mirabilis*. Enterocystoplasties were chosen as model system as they combine a high incidence of urinary tract infections and of infection stone formation with the presence of very specific conditions of the bladder milieu that can be manipulated *in vitro*.

In *chapter 1* a review is given of the available literature data on urinary tract infection with *P. mirabilis* and stone formation especially in enterocystoplasties. From this review a working hypothesis evolves that describes how the persistence of infection and stone formation depend on a number of variables including bacterial adhesion to the epithelial calls in the enterocystoplasty bladder, invasion of these cells by the bacteria, urease production and interactions of bacteria, bacterial products and cellular products with crystals. In the subsequent chapters the relative importance of these variables to the overall processes of infection and stone formation are investigated.

In *chapter 2* an analysis is given of the clinical risk factors involved in the infection of enterocystoplasties in children and the subsequent stone formation. This retrospective study shows that both processes are more complicated compared to the normal bladder due to the presence of intestinal tissue and their secretions in the bladder. Furthermore the presence of suture materials and other foreign materials and the changed flow of urine all may contribute to the formation of bladder calculi. Subgroups of patients are identified that are at increased risk for infection and stone formation.

The question arises whether all bacterial infections should be treated or whether only certain conditions or bacteria should be treated while regarding others as colonization of the intestinal patch. In *chapter 3* the incidence and nature of bacteriuria and symptomatic infections is evaluated for a group of 56 enterocystoplasty patients to answer this question.

The literature review makes it clear that usually bacterial adhesion is required for a symptomatic infection. For crystal formation to occur, elevation of the urine pH is a second requirement *P.mirabilis* is regarded as the most important uropathogen involved in infection stone formation and is capable of this pH elevation through its production of urease. It is therefore chosen as the pathogen of interest in our model systems. In addition to the urease effect, the environment of

the bladder and especially the enterocystoplasty bladder contains several factors that may effect crystal formation. Urine in a enterocystoplasty thus contains mucins and defoliated cells derived from both bladder and intestinal epithelial cells. Finally, formation of a stone from these crystals may require more factors than mere pH elevation. Somehow the crystals must withstand passage with normal micturation or in the case of most enterocystoplasties, clean intermittent catheterization. Adhesion to the bladder and intestinal epithelium may be required. Overall bacterial characteristics, epithelial cell characteristics and urine composition all combined will decide whether a stone is formed. The contribution of each factor will be discussed in *chapters 4 to 7*.

Chapter 4 addresses the influence of urine conditioning by cell derived mucins and cellular material on crystal growth and the subsequent effect of these mucins on crystal adhesion.

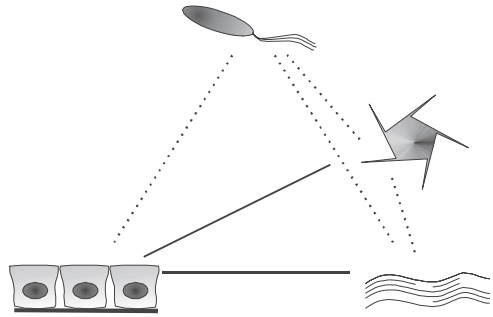
Bacterial adhesion to cells can be followed by invasion of the bacteria into those same cells with intracellular crystal formation. In *chapter 5* it is shown that this invasion has consequences for the survival of *P.mirabilis* in the enterocystoplasty and for the therapeutic effect of antibiotics.

Chapter 6 then addresses the role of mucin mediated cellular adhesion in crystal and stone formation. To enable specific inhibition of the clinically relevant infection it is necessary to clarify this interaction as the first step of an infection.

The surface of the enterocystoplasty interacts with the bacterial surface. This implies an interaction between epithelial surface molecules and bacterial surface molecules as described in chapter 6. This aspect is addressed in detail in *chapter 7*.

Chapter 2

Bladder calculi in Augmentation cystoplasty in children



R.B. Mathoera, D.J. Kok, R.J.M. Nijman.
Urology 56: 482-487; 2000

Abstract

Purpose:
To determine the best preventive strategies for bladder calculi in children with an augmented bladder, the risk factors and prevention strategies for urolithiasis were evaluated.

Materials and methods:
The records of 89 patients following augmentation cystoplasty were reviewed to asses the results of augmentation cystoplasties and in particular the formation and prevention of calculi.

Results:
The median follow-up was 4.9 years after augmentation. Most patients had an ileocystoplasty (71). Bladder calculi occurred in 14 patients (16%) and recurred in 4 patients. Girls had a higher incidence of urolithiasis. Other risk factors were cloacal malformations, vaginal reconstructions, anal atresia, CIC problems and retention, bladder neck surgery and symptomatic urinary tract infections.

Conclusion:
Subgroups with cloacal malformations, vaginal reconstructions, ureter reimplantation and bladder neck surgery were identified that have an increased risk for stone formation and therefore warrant special care in the follow-up after augmentation. This care should include clear emphasis on the role of symptomatic urinary tract infections, especially in patients with cloacal malformations and vaginal reconstructions. Girls have a higher incidence of bladder calculi.

Introduction

The purpose of augmentation cystoplasty is to preserve renal function in case of a small noncompliant high-pressure bladder by creating a large low-pressure reservoir and to promote continence. Augmentation cystoplasty has become a useful surgical option when CIC in combination with anti-cholinergic drugs fails to treat small, noncompliant bladders. However, one of the long-term complications of augmentation cystoplasty is urolithiasis [1, 2], especially in patients infected with urease producing organisms [3, 4].
Depending on the surgical procedure used for the cystoplasty, different prevalence rates of bladder calculi are found. In enterocystoplasties one important factor in the formation of bladder calculi is the production of mucus by the pouch [5]. The mucus may possibly enhance stone formation directly by acting as a heterogeneous nucleator or indirectly by facilitating bacterial growth and retention in calcium, phosphate and ammonium concentrations in urine in combination with alkalinization of urine[1,5,6]. To avoid the problems of mucus formation and high pH, gastrocystoplasty has successfully been used. Indeed, calculi are absent in this procedure[7]. Unfortunately, gastrocystoplasty is hampered by considerable other disadvantages, such as the so-called hematuria-dysuria syndrome and perforation of the gastric segment secondary to peptic ulceration. Some clinical and experimental evidence suggests an increased cancer risk and metabolic complications in gastrocystoplasty and an increased risk for a dumping syndrome after gastrocystoplasty [10-15]. Overall, weighing the urodynamic advantages, complications and limitations, the use of detubularized ileum still seems preferable over the use of sigmoid colon, auto-augmentation or ureterocystoplasty [8-15]. It is therefore worthwhile to try to minimise the complication with stone formation in augmentation cystoplasty. How this should be accomplished is not clear yet. Even for prophylactic treatment with antibiotics it is questioned whether its effects outweighs its disadvantages. Here we have therefore evaluated which factors contribute to the occurrence and prevention of bladder calculi in children following augmentation cystoplasty.

Materials and methods

Between 1987 and 1998, 90 patients underwent surgery in order to obtain a high volume, low-pressure bladder reservoir by bladder augmentation. The records of 89 patients could be reviewed. Augmentation cystoplasty was performed in 38 male and 51 female patients. The mean age was 14 years with a mean period of follow-up of 4.9 years. The mean age at augmentation was 9.5 years, with a range of 9 months to 24 years. The indications for augmentation are mentioned in **Table** 1.
Of all patients 44 had a Mitrofanoff type of continent diversion (49%), most often an appendicovesicostomy constructed at the time of augmentation. On 5 patients (6%) a Mitrofanoff type of continent diversion had been constructed as a secondary procedure at a later stage. Continence enhancing surgery at the bladder neck was performed at the time of augmentation in 45 (57%) of all patients. Concomitant surgical procedures consisted of 21 ureter reimplantations (22%), 2 nephrectomies, 11 vaginal reconstructions using bowel segments and 9 pelvic osteotomies. The different types of bladder augmentation are mentioned in **Table** 2. Only absorbable sutures were used, Vicryl and in the last 6 years also Monocryl.
Follow-up consisted of control of bloodgas analysis, electrolytes, and creatinine every 3 months during the first year and every 6 months thereafter. A cystoscopy was performed every 2 years starting 7 years after augmentation cystoplasty. IVP, ultrasound, urodynamic investigation, and voiding cystourethrography were done according to fixed protocols. All but one patient were on

clean intermittent catheterization; the one patient had a suprapubic catheter for more than 4 years due to reluctance to perform CIC without urolithiasis. Following surgery, all patients were instructed to irrigate their bladder with saline at least once a day, but after some months most patients followed a more liberal regimen. It was stressed that they should irrigate the bladder regularly especially in case of complications, such as pain or infection and large production of mucus. Of all patients 82% regularly irrigated their bladder, and 59% irrigated their bladder at least once daily, according to patient inquiry. Symptomatic and asymptomatic infections associated with bacteriuria were noticed frequently and treated therapeutically in case of a symptomatic infection and often prophylactically in patients with frequent or persistent urinary infections. Symptomatic infections were defined as infections with symptoms such as fever, pain, and production of malodorous urine that require treatment. The occurrence of bladder calculi and other complications of bladder augmentation and their recurrence at several intervals after augmentation were studied for different indications and surgical procedures. Data were analyzed by χ^2 analysis and Fisher exact test.

Results

During the period of follow up 18 stones were found in 14 patients for the first time. In **Table** 3 different risk factors are described. In 3 patients more than one type of stone was found at the first presentation with bladder calculi. Ammonium urate and calcium apatite stones only occurred with struvite stones. None of the stone formers had a history of urolithiasis before augmentation. Two patients, who had had stones prior to augmentation cystoplasty, did not form stones after augmentation. In one patient stones were detected and removed during bladder neck surgery. One patient was known to have recurrent pyelonephritis and developed urolithiasis in both bladder and kidney, from which P.mirabilis was cultured. Stone recurrence occurred in 4 patients in the bladder after a mean period of 1.9 years. Bladder calculi were removed by open surgery in 10 cases, by percutaneous litholapaxy in 2 cases, and transurethral stone extraction was performed in 3 cases. One patient passed the small stones spontaneously with intermittent catheterization. Of the 45 patients who underwent bladder neck surgery 8 of all 10 patients with CIC problems using the transurethral passage and19 of all 27 recurrent urinary tract infections with 9 of 11 calculi were found after bladder neck surgery. Stomal stenosis, although causing CIC problems, was not associated with a higher incidence of stone formation. In 13 patients who had experienced either CIC problems or a urinary retention, a higher incidence of ammonium urate stone formation was found. In 8 of these patients continence enhancing surgical procedures were performed on the bladder neck and 6 also had more bladder neck revisions at a later stage of 16 patients with these revisions. Patients with imperforate anus and patients who underwent reconstructive surgery of the vagina were also more frequent among stone formers than among non-stone formers. There were no differences in faecal soiling with the other groups. Of 11 Patients with a reconstructed vagina 2 of 3 patients with an acute urinary tract infection and a urinary retention were found and 6 of 21 ureter reimplantations were performed.

There were no statistically significant differences found in the frequency of stones between the different surgical procedures for augmentation (**Table** 2). Frequency of bladder irrigation was not related to stone formation. It was not possible to do a reliable evaluation of a Mitrofanoff type of continent diversion, of which 85% have been constructed after 1995, considering the mean period between augmentation and stone occurrence.

Urinary infection of every kind combined showed a distinct relationship with stone formation. Prophylactic treatment with antibiotics reduced the number of struvite stones compared to patients not receiving this treatment, but the total number of stones did not decrease significantly. Prophylactic use of antibiotics was not related to the incidence of infection. Thirteen girls and 1 boy formed stones, which recurred in 3 girls and the boy. Girls also had

more recurrent urinary tract infections (43%), and bladder neck plasties (61%). Of all girls, 10 had a cloacal malformation. After omitting the girls with a vaginal reconstruction, girls still had more recurrent urinary tract infections and formed stones more frequently.

	Number of patients	*Number of Stones (% patients)*
MMC	51	5 (10)
Extrophy	21	3 (14)
Posterior urethral valves	4	-
Cloacal malformation	10	5 (50)
Other obstructive uropathy	3	1
	89	14

***Table** 1. Indication for augmentation cystoplasty in 89 patients and the occurrence of stones*

	Number of patients	*Number of patients with continent diversion*	*Number of patients with stones (%)*	*Mean time lapse between surgery and finding of stones (months)*
ileocystoplasty	71	39	11 (16)	37.6
Kock pouch	2	1	1 (50)	61
Indiana pouch	2	1	0	-
colocystoplasty	9	2	2 (22)	35.3
auto-augmentation	4	0	0	-
ureterocystoplasty	1	1	0	-
total	89	44	14 (16)	39

***Table** 2. The different types of augmentation cystoplasty in 89 patients and the occurrence of stones*

Discussion

Fourteen out of our 89 patients formed 18 bladder calculi for the first time following augmentation. Most often we encountered infection stones. It is known that especially urinary infection with Proteus mirabilis [16] or other urease producing bacteria can cause bladder stone formation by changing urine and bladder mucus composition [5]. In addition fimbriae, adhesins and the glycocalyx have been implicated to increase bladder stone formation [18-21]. In this respect measures that remove bacteria from the bladder may be expected to decrease the risk for bladder stone formation.

The frequency of bladder irrigations was not related to stone formation. From other studies on concrement formation with continent ileal reservoirs [5] it may be concluded that frequent bladder irrigation with a saline solution acts on mucus, bacteria or both, but our data does not support this. Because the patients who irrigate their bladder more than once a day do so because of infection, large amounts of mucus or pain, we believe that this may have influenced our results by revealing no difference in stone formation for different irrigation regimens. A Similar explanation of patient selection applies for the use of antibiotics and the absence of infections. Over the period of follow-up no reliable measurement could be taken of the volume of irrigation fluid used in between consultations. Post irrigation ultrasound was measured at consultations, but is not very reliable due to a high number of false negative results. So far we have only used a saline solution which has proven to be satisfactory in evacuating mucus, but which might be made more effective by adjustments for the bladder volume of the individual child.

	Total number of patients	Patients with stones	Patients without stones	Stone analysis of the detected bladder stones					Total number of patients	Patients with stones	Patients without stones	Stone analysis of the detected bladder stones				R.R.	C.I.$_{95\%}$ RR
				Struvite	Ammonium urate	Calcium Apatite	Unknown					Struvite	Ammonium urate	Calcium Apatite	Unknown		
girls	51	13&	38	6	3	1	7	Boys	38	1	37	1	-	-	0	**9.68**	**71.1-1.3**
vaginal reconstruction	11	7&	4	4&	2&	-	3	No vaginal reconstruction	78	7	71	3	1	1	4	**7.09**	**16.4-3.1**
cloacal malformation	10	5&	5	2	1	-	3	No cloacal malformation	79	9	70	5	2	1	4	**4.38**	**10.5-1.8**
concomitant bladder neck surgery	45	11&	34	6	3	1	5	No concomitant bladder neck surgery	44	3	41	1	-	-	2	**3.58**	**12.0-1.1**
Urinary tract infections	47	11&	36	5	3	1	6	No urinary tract infection	42	3	39	2	-	-	1	**3.27**	**10.9-1.0**
concomitant ureter reimplantation	21	7&	14	4&	2	1	3	No concomitant ureter reimplantation	68	7	61	3	1	-	4	**3.23**	**8.2-1.3**
Retention, CIC problems	13	4	9	2	2&	1&	2	No Retention or CIC problems	76	10	66	5	1	-	5	**2.33**	**6.3-0.9**
Prophylactic antibiotics	58	7	51	2&	1	-	5	No prophylactic antibiotics	31	7	24	5	2	1	2	**0.53**	**1.4-0.2**

Table 3. *Number and stone analysis of the first occurrence of stones (n=18) in overlapping patient subgroups of 89 patients with 14 stone formers. Three patients formed more then one type of stone, ammonium urate and calcium apatite only occurred with struvite stones. R.R.=relative risk, C.I.$_{95\%}$ RR= 95% confidence interval of relative risk.*
&$p \leq 0.05$

Additional note:
The relative risk for stone formation = The risk for stone formation in the index group (left)/ The absolute risk for stone formation in the reference group (right)
For example in the first row: 13/51= 0.25 1/38= 0.03 0.25/0.03= 9.68

Most of the absorbable suture material is used in the pouch or bladder neck area. However, stone formation still occurs more frequently after concomitant bladder surgery. Urinary infections are related to concomitant bladder neck surgery, which may indicate an intermediate role of urinary infections in the formation of stones. Patients with bladder neck plasty may also experience difficulty in bladder emptying. Open surgery was preferred to extract the entire stone without leaving residual fragments and did not require dilating or damaging the bladder neck. To explain the effect of concomitant bladder neck surgery on stone formation, we might have to evaluate the effects on the cystoplasty's mucosa, bacterial adhesion as well as the degradation time of sutures.

The antibiotic treatment has not been changed during the period in which these augmentations were performed. Possible emerging resistance of bacteria against antibiotics may have played a role in stone formation[4]. Virtually all patients have infected bladders preceding the augmentation procedure. Bacteriuria has been regarded as normal in patients with an enterocystoplasty for some time now [22], and the advice has been given not to treat all cases of bacteriuria[23]. The fact that symptomatic urinary infections are significantly more common among stone formers can be seen as a confirmation that infection plays a major part in stone formation in enterocystoplasties and should redirect our attention to this problem or its effects on urine composition and pH.

In the normal bladder the incidence of bladder calculi has been reported to be higher in boys, with a higher incidence of struvite stones. In upper urinary tract infections it is well known that a continuous flow can help to wash out bacteria [24]. It seems possible that in the lower urinary tract such a mechanism may start to fail when voiding is done by CIC. We found more bladder stones in girls than in boys, often in association with cloacal malformation and reconstruction of the vagina and recurrent urinary tract infections, most of them were infection stones.

It is not clear, whether the operation, tissue damage and damage of the GAG layer and mucosa of the enterocystoplasties by CIC have attributed to stone formation similar to a recent theory on kidney stone formation [25, 26]. CIC has been known to be accompanied by bacteriuria, but is usually easily treated and causes no sequellae, however this bacteriuria may also have contributed to calculus formation.

In patients with vaginal reconstructions the indication for surgery most often is a cloacal malformation, requiring many concomitant procedures and may explain the higher incidence of urolithiasis in this group, as does the higher incidence of acute urinary tract infection with urinary retention. The relative risk for urolithiasis is higher then that of most other risk factors. Mucus secretion by the reconstructed vagina and bacterial tropism [27] for small bowel epithelium may have played a role in the increased incidence of urinary tract infection and infection stones. So far only uric acid urolithiasis has been reported in patients with a vaginal reconstruction [28]. Urinary stasis has been identified as an important risk factor for urolithiasis by others and has been regarded as a risk factor for stone formation in cloacal malformation in combination with infection [29]. Considering that patients with an anal atresia or patients with a vaginal reconstruction, both related to cloacal malformations, have a higher incidence of stone formation, concomitant surgical procedures can not be omitted as a possible explanation for the higher incidence of urolithiasis in cloacal malformations. The main prevention method should include perineal hygiene and irrigation.

Conclusion

Infections of the augmented bladder are not benign. Girls are more prone to develop stones in enterocystoplasties, despite CIC. It is clear that concomitant surgical procedures, especially on the bladder neck, increase the risk of urolithiasis. Prophylactic use of antibiotics has no effect on the total number of stones, but seems to decrease the number of struvite stones. Congenital malformations and vaginal reconstructions are an important risk factor and we advise to guard against infections. Perhaps by improving the saline solution for bladder irrigation, we may reduce the incidence of urolithiasis. Further research into improving rinsing fluids and anti-microbiological properties, interactions or damaging of urothelium and intestinal mucosa in augmented bladders and the influence of suture material is needed, to better understand why this gives rise to a higher incidence of stone formation in enterocystoplasties.

References

1. Blyth, B., Ewalt, D.H., Duckett, J.W., Snyder, H.M.: Lithogenic properties of enterocystoplasty. J. Urol., **148**: 575-577, 1992
2. Palmer, L.S., Franco, I., Kogan, S.J., Reda, E., Gill, B., Levitt, S.B.: Urolithiasis in children following augmentation cystoplasty. J. Urol., **150**: 726-729, 1993
3. Ginsberg, D., Huffman , J.L. , Lieskovsky, G., Boyd, S., Skinner, D.G.: Urinary tract stones: a complication of the Kock pouch with continent urinary diversion. J. Urol., **145**: 956-959, 1991
4. Edin-Liljegren, A., Grenabo, L., Hedelin, H., Jonsson, O., Ákerlund, S., Pettersson, S.: Concrement formation and urease-induced crystallization in urine from patients with continent ileal reservoirs. Br. J. Urol., **78**: 57-63, 1996
5. Khoury, A.E., Salomon, M., Doche, R., Soboh, F., Ackerley, C., Jayanthi, R., McLorie, A., Mittelman M.W.: Stone formation after augmentation cystoplasty: the role of intestinal mucus. J. Urol., **158**: 1133-1137, 1997
6. Terai, A., Arai, Y., Kawakita, M., Okada, Y., Yoshida, O.: Effect of urinary intestinal diversion on urinary risk factors for urolithiasis. J. Urol., **153**: 37-41, 1995
7. Kronner, K.M., Casale, A.J., Cain, M.P., Zerin. M.J., Keating, M.A., Rink, R.C.: Bladder calculi in the pediatric augmented bladder. J. Urol., **160**: 1096-1098, 1998
8. Radomski, S.B., Herschorn, S., Stone, A.R.: Urodynamic comparison of ileum vs. sigmoid in augmentation cystoplasty for neurogenic bladder dysfunction. Neurol.Urodyn., **14(3)** : 231-237, 1995
9. Di Benedetto, V., Monfort, G.: Stomach versus sigmoid colon in children undergoing major reconstruction of the lower urinary tract. Ped. Surg. Int., **12(5-6)** : 393-396, 1997
10. Duel, B.P., Gonzalez, R., Barthold, J.S.: Alternative techniques for augmentation cystoplasty. J. Urol., **159(3)** : 998-1005, 1998
11.Nguyen, D.H., Bain, M.A., Salmonson K.L., Ganesan G.S., Burns M.W., Mitchell M.E., The syndrome of dysuria and haematuria in pediatric urinary reconstruction with stomach., J. Urol., **150** :707-709, 1993
12. Khoshoo V., Reifen R.M., Gold B.D., Sherman P.M., Pencharz P.B., Nutrional manipulation in the management of dumping syndrome., Arch. Dis. Child., **66** : 1447-1448, 1991
13. Gold B.D., Bhoopalam P.S., Reifen R.M., Harvey, E., Marcon, M.A., Gastrointestinal complications of gastroplasty., Arch. Dis. Child., **67** : 1272-1276, 1992
14. Buson H., Castro Diaz D., Manivel J.C., Jessurun J., Dayanc M., Gonzalez R., The development of tumors in experimental gastroenterocystoplasty. J. Urol. **150** : 730-733, 1993
15. Gosabez R. J. R., Woodard J. R., Broecker B. H. , Warshaw B., Metabolic complications of the use of stomach for urinary reconstruction., J. Urol., **150** : 710-712, 1993
16. Bellinger, M.F.: Ureterocystoplasty: a unique method for vesical augmentation in children. J. Urol., **149**: 811-813, 1993
17. Diamond, D.A., Menon, M., Lee, P.H., Rickwood, A.M.K., Johnston, J.H.: Etiological factors in pediatric stone recurrence. J. Urol., **142**: 606-608, 1989
18. Wang, Y., Grenabo. L., Hedelin, H., McLean, R.J.C., Nickel, C.J., Pettersson, S.: Citrate and urease-induced crystallization in synthetic and human urine. Urol.Res. **21**:109-115, 1993
19. Rózalski, A., Sidorczyk, Z., Kotelko, K.: Potential Virulence factors of proteus bacilli. Microbiol.Mol.Biol.Rev., **61(1)** : 65-89, 1997
20. Tolson, D.L., Harrison, B.A., Latta, R.K., Lee, K.L., Altman, E.: The expression of nonagglutinating fimbriae and its role in Proteus mirabilis adherence to epithelial cells. Can. J. Microbiol., **43**: 709-717, 1997
21. Dumanski, A.J., Hedelin, H., Edin-Liljegren, A., Beauchemin, D., McLean, R.J.C.: Unique ability of the Proteus mirabilis capsule to enhance miniral growth in infectious urinary calculi. Infect.Immun., **62(7)** : 2998-3003, 1994
22. Akerlund, S., Berglund, B., Kock, N.G., Philipson, B.M.: Voiding pattern, urinary volume, composition and bacterial contamination in patients with urinary diversion via a continent ileal reservoir. Br. J. Urol., **63**: 619-623, 1989
23. Akerlund, S., Campanello, M., Kaijser, B., Jonsson, O.: Bacteriuria in patients with a continent ileal reservoir for urinary diversion does not regularly require antibiotic treatment. Br. J. Urol., **74**: 177-181, 1994
24.Roberts J.A. , Factors predisposing to urinary tract infections in children. Pediatr. Nephrol., **10(4)**: 517-522; 1996

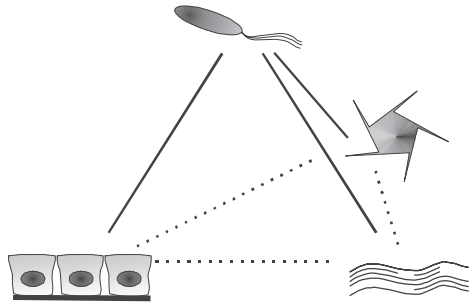
25. Bigelow M.W., Weissner J.H., Kleinman J.G., Mandel N.S., Calcium oxalate crystal attachment to cultured kidney epithelial cell lines. J.Urol. **160**:1528-1532;1998
26.Verkoelen C.F., Romijn J.C., de Bruijn W.C., Boevé E.R., Cao L., Schröder F.H., Association of calcium oxalate monohydrate crystals with MCDK cells. Kidney Int. **48**: 129-138; 1995
27. Raupach, B., Mecsas, J., Heczko, U., Falkow S., Finlay B.B., Bacterial Epithelial Cell Cross Talk. Curr. Top. Microbiol. Immunol. **236**:137-61, 1999
28. Ünüvar, E., Oguz, F., Sahin, K., Nayir, A., Özbey, H., Sidal, M., Coexistence of VATER association and recurrent urolithiasis: a case report. Pediatr. Nephrol., **12**: 141-143, 1998
29. Nurse, D.E., McInerney, P.D., Thomas, P.J., Mundy, A.R.: Stones in enterocystoplasties. Br.J.Urol., **77**: 684-687, 1996

Additional Comment

It seems clear that the differences between a normal bladder and an augmented bladder are such that stone formation is enhanced in certain conditions. Most prevalent risk factor dominating and connecting all the other risk factors seems to be the presence of symptomatic infections. However, prophylactic use of antibiotics seems to have little or no effect on the prevention of stone formation. Children with a cloacal malformation or a vaginal reconstruction (related to the occurrence of a congenital urinary malformation) are at higher risk for stone formation. These children are often treated extensively with antibiotics and are often hospitalised. How this effects the effect of existing antimicrobial options and stone formation will be discussed in the next chapter

Chapter 3

The bacteriology of infections and bacteriuria in enterocystoplasties in children



R. B. Mathoera, C. M. Verduin, R. J.M. Nijman
(Submitted)

Abstract

Introduction:
It is not clear to what extent the incidence of symptomatic urinary tract infections represents the actual bacterial presence in the enterocystoplasty. In this follow-up study, symptomatic and asymptomatic bacterial infections and their antibiotic susceptibility are evaluated with concomitant surgical procedures to identify the relevance of bacteriuria to stone formation.

Patients and Methods:
56 patients were evaluated for a period of 3 years by urine culture and antibiotic susceptibility testing. The complete bacteriological history of the corresponding patient's symptomatic urinary infections were combined with the medical history and asymptomatic bacteriological tests. Overlapping subgroups were used to identify risk factors for stone formation, to evaluate antibiotic treatment and the relevance of bacteriuria compared to symptomatic urinary tract infection in enterocystoplasties.

Results:
13 patients (23%) developed a bladder stone. Most infections were with *E.coli,* but the difference between symptomatic and routine culture was clear for *P.mirabilis* ($p=0.011$, Relative Risk = 2.68). Urease producing strains were more resistant to Amoxicillin, Amoxicillin/ clavulanic acid and Trimethoprim/ Sulfamethoxazole. Resistances often occurred for antibiotics commonly used in prophylaxis and therapy. Catheterization channels and continence enhancing surgery attribute to stone formation and infection, while physical handicaps attribute to the latter.

Conclusion:
Bacterial infection should be regarded as the result of poorly controlled bacteriuria. Bacteriuria are preferentially treated without antibiotics, and eradicated according to a antibiogram. Antibiotic guidelines would be useful. Stone formation is a result of the infection in the susceptible environment of the augmented bladder, after continence enhancing surgery or the construction of catheterization channels.

Introduction

Stone formation in enterocystoplasties is common[1] and generally urolithiasis is ascribed to the rise in pH caused by urease producing bacteria in the enterocystoplasty[2]. In children these enterocystoplasties are constructed to enlarge the bladder and conserve kidney function in an already compromised kidney. In recent years the emphasis of stone prevention has therefore been on treatment and prevention of emergence of urease producing bacteria in the enterocystoplasty and on urease inhibition. *P. mirabilis* infection has thus been associated with the production of urease[3] and urinary stones[4]. Usually, urine cultures are obtained in case of a symptomatic urinary tract infection. These infections are characterized by foul smelling urine, pain and large amounts of mucus, (sometimes accompanied by fever) in the voided enterocystoplasty urine. It is to be expected that these infected urines will yield a urinary pathogen, which lies at the basis of the symptoms.

However, bacteriuria is also found in patients with an enterocystoplasty without symptoms. These infections have been regarded as benign and rather as a colonisation of the augmented bladder than an infection[5]. Antibiotic prophylaxis does reduce the urine nitrosamine levels, which indicates that bacteriuria may have an influence on the incidence of tumours in the augmented blader[6,7]. Especially since colonization is a normal feature of the intestinal tissue present in the augmented bladder. This idea is fueled by the observation that antibacterial treatment is neither effective in the long run nor significantly prevents the complications caused by bacterial infections in the urinary tract. This may be directly attributed to the presence of an intestinal section in the bladder as a means of enlarging the bladder or bacterial overgrowth[8]. There have been variable reports about the incidence of bacteriuria in patients with an enterocystoplasty[5,9]. These infections may be retrospectively evaluated by antibody levels[10,11].

Nevertheless, it is not clear to what extent the incidence of symptomatic urinary tract infections represents the actual bacterial presence in the enterocystoplasty. Also, it has not been decided whether we can actually regard bacteriuria in enterocystoplasties as a colonisation of the incorporated intestinal tissue or if the bacterial presence should still be regarded as a risk factor and real infection even in the absence of obvious clinical/physical symptoms. Finally, evaluation of risk factors and therapeutic measures is necessary to refine the indication for continence enhancing surgical intervention or catheterisation channels, and antibiotic treatment. To answer this we have evaluated both bacteriuria and symptomatic infections in enterocystoplasties.

Materials and Methods

Between 1999 and 2002 patient urine was collected of 56 enterocystoplasty patients without known symptomatic urinary tract infection at the time of investigation at regular follow-up consultations. These urine specimens were collected by clean intermittent catheterisation and cultured on blood agar and McConkey plates.. Researchers were not aware of the patient's history, symptoms or physical condition at the time of the specimen collection. The urinary pH level was measured within 25 minutes after catheterization to ensure reliable measurements. The complete bacteriological history of the corresponding patient was gathered from a database to evaluate the outcome of cultures from urine samples with the clinical indication of symptomatic infections from hereafter referred to as the symptomatic samples. Infections in the patients without clinical indication for further bacteriological testing were evaluated from specimens gathered at consultation and regarded as significant at $>10^5$ colony forming units/ ml, from hereafter referred to as the asymptomatic samples. The patients medical history was combined with the newly constructed database. Infection rates lower then 10^3 colony forming units were not evaluated. All identified pathogens were tested for antibiotic sensitivity using disk diffusion and automated systems. Breaking points for antibiotic sensitivity were acquired from and used according to the methods of the National Committee for Clinical Laboratory Standards

(NCCLS). Following surgery, all patients were instructed to irrigate their bladder with saline at least once a day, but after some months most patients followed a more liberal regimen. It was stressed however that they should irrigate the bladder regularly especially in case of complications, such as pain or infection and large production of mucus. Follow-up consisted of control of bloodgas analysis, electrolytes and creatinine every 3 months during the first year and every 6 months thereafter. A cystoscopy was performed every 2 years, starting 7 years after augmentation cystoplasty. IVP, ultrasound, urodynamic investigation, and voiding cystourethrography were done according to fixed protocols. Symptomatic infections and asymptomatic bacteriuria were noticed frequently and were treated therapeutically in case of a symptomatic infection and often prophylactically in patients with frequent or persistent urinary infections.
Statistical analysis was performed by χ^2 analysis and a Fisher exact test when needed. Differences were considered significant for p-values ≤ 0.05 in 2 sided tests. Correlation direction and Relative Risks were determined in the SPSS 9.0 software package.

Results
In the sample group of 56 patients, 13 (23%) developed a bladder stone. In the 665 specimens from the 56 patients most infections were due to *E.coli*. These did not significantly differ between the symptomatic and asymptomatic group. A bacteriological profile of complicated urinary tract infections could be observed with less frequent bacterial species infecting the augmented bladder (**Table** 1.A).The most prominent difference between the symptomatic samples and asymptomatic samples was the incidence of *Proteus mirabilis* (p=0.011, Relative Risk = 2.68, 95% Confidence interval 5.41 – 1.22). only 20 *Proteus mirabilis* infections (5.3%) were found among 553 specimens from symptomatic urinary tract infections based on clinical observation. Bacterial identifications and antibiotic susceptibility tests were performed for 378 specimens. In bacteriuria specimens, 9 (13.6%) of the 112 identifications were positive for *Proteus mirabilis.* Identified strains are presented in **Table** 1A, antibiotic sensitivity is presented in **Table** 1B. antibiotic resistances of the most frequently used antibiotics closely followed the prescription preferences of clinicians (**Fig**.1). Increased use of cotrimoxazol, augmentin and nitrofurantoine was reflected in an increase in antibiotic resistance in the same or following year. A cyclic pattern occurred as clinicians switched from one antibiotic to another. The formation of bladder calculi was not related to *P.mirabilis* infection in any direct manner. Stone formation was related to urease producing *Enterococcus* and *Providencia* strains but not the typical urease producing bacteria. Furthermore, stone formation was related to antibiotic therapy for symptomatic infections, but mostly to additional procedures to the enterocystoplasty during and after the augmentation procedure (**Table** 2). Medication unrelated to augmentation usually consisted of anti-epileptic medication or vitamin C and seemed to have a beneficial effect on the reduction of the incidence of bladder calculi. Many of the additional procedures were related to the occurrence of specific antibiotic resistances (**Table** 3). This could be explained by the occurrence of other pathogens rather then the normal uropathogens that have a higher incidence following additional surgical procedures. Of these antibiotics Amoxicillin/ clavulanic acid, Nitrofurantoin, Ciprofloxacin and Trimethoprim/ Sulfamethoxazole were commonly used for therapeutic and prophylactic purposes (**Table** 4). Many bacterial strains were exposed to these antibiotics, most of which were capable of producing urease. The number of patients exposed to antibiotic treatment is equally high for patients with symptomatic infections and patient with bacteriuria. Infection with urease producing organisms in enterocystoplasties is the most prevalent. Bacterial strains, found in urine specimens from either symptomatic infections or non-symptomatic bacteriuria were more frequent in patients with specific physical disabilities or additional surgical interventions (**Table** 5), while other interventions significantly reduced

specific bacterial infections. Lower limb paralysis was defined in this particular group of patients as synonymous for the use of a wheel chair, considering the effect of the underlying pathology frequently associated with neurological problems. This disability was associated with an increase in *P.mirabilis* infections, which was more relevant in symptomatic urinary tract infections. Other afflictions affecting the bladder and bladder function, such as the VACTERL-association and to a lesser extent the Arnold-Chiari malformation increased infection with *Enterococcus spp.* and *Enterobacter spp*. respectively Proteus mirabilis . Surgical interventions to reduce vesico-ureteral reflux such as ureter reimplantations and subostial Macroplastique® or Deflux ® injection were also identified as influencing factors. These factors were related to therapeutic interventions at a later stage, presented in **Table** 6. Many of these interventions were identical to the factors correlated to stone formation (**Table** 2).

Overlapping groups based on urine acidity (**Table** 7) correlated with strains and afflictions previously identified as related risk factors in **Table** 5. Although *P.mirabilis* and *Pseudomonades* are not directly correlated with stone formation, they are correlated with many of the factors important for stone formation and could be considered as a confounding factor rather then an intermediate factor for stone formation in enterocystoplasties in children. Typically, these strains and other urease producing bacteria are found more frequently in patients prone to develop resistances to antibiotics used in the urologic field and general practice alike, such as Amoxicillin, Amoxicillin/ clavulanic acid, Trimethoprim/ Sulfamethoxazole and Nitrofurantoin (**Table** 8).

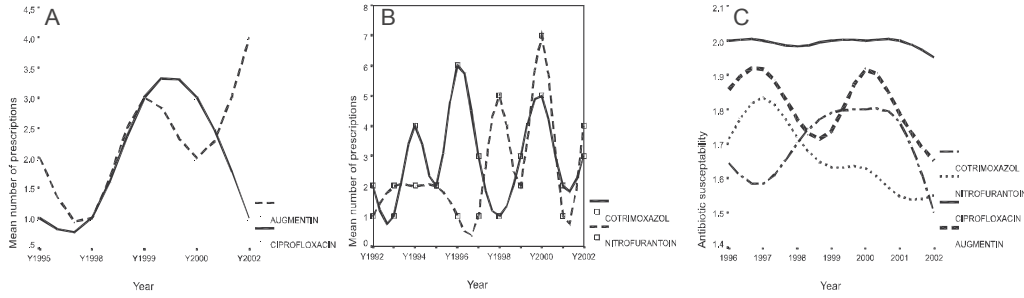


***Fig**.1. Antibiotic use and susceptibility*

A. =Number of amoxicillin/ clavulanic acid (Augmentin) and ciprofloxacin prescriptions for each year in the period from 1995 until 2002., B = The number of trimethoprim/ sulfamethoxazole (cotrimoxazol) and nitrofurantoin prescriptions for each year in the period from 1992 until 2002. C= The resulting antibiotic susceptibility in the period of 1996 until 2002 for amoxicillin/ clavulanic acid (Augmentin), ciprofloxacin, trimethoprim/ sulfamethoxazole (cotrimoxazol) and nitrofurantoin. The antibiotic susceptibility for the separate antibiotics decreases rapidly at the time of highest use. The antibiotic susceptibility was scored as 1 for resistant and 2 for sensitive strains (including urinary sensitivity), The mean of these scores gives an indication of the global fluctuation of antibiotic sensitivity per year.

Discussion

The profile of treated bacterial strains involved in asymptomatic infection of enterocystoplasties is different from the most common strains involved in symptomatic infections. The bacterial profile in bacteriuria and symptomatic infection both correspond with a complicated urinary tract infection. The difference between the incidence of *Proteus mirabilis* in asymptomatic bacteriuria and symptomatic infection is typical in this particular group and may help to explain the incidence of bladder calculi in enterocystoplasties. Except for the *Providencia* strains, no direct correlation with stone formation was found for symptomatic infection with urease producing and other bacteriological strains. The formation of stones in enterocystoplasties is probably more

complicated in enterocystoplasties then in the normal bladder, due to the formation of mucus, additional surgical procedures to enhance continence and ease of catheterisation. Furthermore, Hygiene may play a more important role in the bacterial and antibiotic susceptibility profile and incidence of infections in patients with complex afflictions requiring bladder augmentation.

Bacteriological aspects and relevance to antibiotic treatment

The strains that were treated in case of bacteriuria were most often *E.coli* or *Enterococcus* strains and mixed cultures. E.coli was less frequently treated in symptomatic infections, in contrast to *Enterococcus* strains and mixed cultures. The bacterial profile of these patients is typical for complicated infections and are similar between the bacteriuria evaluation and the symptomatical infections. This leads to the conclusion that the predisposing factors involved in the higher incidence of bacterial infections are more likely to be common for most of the frequent strains rather than strain specific. Strains that are treated less frequent in asymptomatic bacteriuria are more frequent in symptomatic urinary tract infections. Unfortunately many of these less frequently treated strains become more resistant to several antibiotics. The increase in resistance may lead to more symptomatic infections with the most common pathogens and bacteria that have adapted to the urine environment by the production of urease.
Treatment of urinary infections and bacteriuria is usually by antibiotics such as Ciproxin, Amoxicillin/ clavulanic acid, Nitrofurantoin, or Trimethoprim/ Sulfamethoxazole. These last three antibiotics have been advocated by general practitioners in The Netherlands as the antibiotics of choice in most cases of urinary tract infection[12]. In most cases antibiotic treatment is advised without a bacterial identification or antibiotic resistance testing. Bacterial culture and antibiotic resistance testing is only advised by general practitioners in case of complicated urinary tract infections or two failures of treatments based on clinical evaluation only. In all cases antibiotic treatment is started before test results are final and follow-up deemed unnecessary in when symptoms are resolved. This guideline and the failure to recognize infections in enterocystoplasties as complicated urinary tract infections may lead to antibiotic resistance in these patients and failure of treatment or prophylaxis. In our study the most common antibiotics have the highest incidence for resistance, which can be found in several of the most common strains. Guided by their request for a clear definition of the subgroups that cannot be fitted to the general practitioners' guidelines[13], we therefore propose that preferably the group of enterocystoplasty patients with symptomatic infections should receive specific antibiotic treatment after bacterial culture and resistance testing of symptomatic infections followed by a follow-up urine evaluation of the eradication of the responsible bacterial strain. Bacteriuria in enterocystoplasties does not equal a symptomatic infection, although it may lead to some complications where vigilant prevention of recurrent or more pathogenic infections can be considered. Clearly in these patients the presence of symptoms due to a cellular reaction to infection is the indication for testing instead of the presumption that bacteriuria must be consistent with a urinary infection. In Western Europe, the most common uropathogen is *E.coli* with 80% of all isolates in normal bladders[14] and 50% in augmented bladders[6], which is too high to fit the profile of bacteriuria. In the more southern regions of Europe *E.coli* is still most frequent, but other bacterial strains such as *Proteus, Klebsiella, Enterobacter* and *Citrobacter* have a higher incidence[14]. Prevalence of different uropathogens and antibiotic resistances do not resemble that of the general population but are more comparable to those of nursing home patient in the same region in The Netherlands[15]. The prevalence of *P.mirabilis* in our patients is lower, due to vigilance of the urologists and general practitioners alike, although mixed cultures may include *P.mirabilis*. Similar to our patients resistance to Trimethoprim/ Sulfamethoxazole is frequent and can be attributed to prescribing habits.

However, *P.mirabilis* was not directly associated with stone formation, but could be correlated with several other factors that were correlated with stone formation. Hygiene and catheterisation problems could explain a higher incidence of urease producing bacteria. Infection with *P.mirabilis, Pseudomonas spp.* and other urease producing bacteria could be enhanced by lower limb paralysis, catheterisation problems or due to urine reflux in the absence of ureter reimplantation or other anti-reflux surgery leading to urinary stasis. These infections lead to a pH elevation that could induce stone formation. Medication unrelated to augmentation, such as vitamin C could have an influence on stone formation. The prescription preferences of clinicians influenced the overall antibiotic resistance profile.

Urological aspects and relevance to treatment

The most common risk factor for stone formation seems the presence of additional or concomitant surgical procedures to the augmented bladder as was previously described[16]. Vaginal reconstructions could not be confirmed in this group as a risk factor of stone formation. They could however be related to surgical procedures on the bladder neck which were correlated to stone formation. Mitrofanoff appendicovesicostomy could be related to stone formation. This should probably be attributed to catheterization problems and additional infections and regarded as a concomitant surgical procedure, as described above. The continence enhancing surgical procedures could be related to specific antibiotic resistances against common antibiotics such as Amoxicillin/ clavulanic acid and Trimethoprim/ Sulfamethoxazole. The continence enhancing procedures may enhance the incidence of resistance by creating urinary stasis in a prophylacticaly treated patient[1]. Antibiotic prophylaxis or treatment of patient with or without symptomatic infections is very similar and may lead to resistance and treatment failure when bacteriuria evolves into symptomatic infection. This could play a role for *P.mirabilis* infections when evaluating the number of symptomatic and asymptomatic infections of *P.mirabilis* in patients with lower limb paralysis. Furthermore, groups of afflictions and surgical procedures seem to involve a higher incidence of specific bacterial infections. This could mean a predisposition due to a changed anatomical structure on top of the already raised incidence of infections.

The pH level is raised by the presence of intestinal tissue in the bladder but will increase further by the presence of urease. We propose that the incidence of stone formation in enterocystoplasties in the augmented bladder is not only due to a predisposition to infection but also to the smaller difference between the initial urine pH and the pH level needed for struvite and brushite precipitation.

Conclusion

Bacterial infection should be regarded as the result of poorly controlled bacteriuria. Bacteriuria should be treated preferentially without antibiotics, but not eradicated unless a clear antibiogram is available as for all complicated infections. Clean intermittent catheterization and bladder rinsing to prevent urinary stasis may have a more valuable role in this case. Antibiotics for enterocystoplasty patients should be separated into antibiotics for clinical treatment and a limited number of one or two antibiotics for prophylaxis and regularly evaluated for their efficacy as a prophylactic. Stone formation is related to urease producing bacteria and bacterial infection which are related to additional and concomitant surgical procedures. Surgical procedures in the augmented bladder are especially a risk for stone formation in case of continence enhancing surgery. A more effective method of bacteriuria limitation either by enhancing antibiotic effectivity or inhibiting bacterial adhesion and infection to limit antibiotic use may be required to decrease antibiotic resistance and stone formation.

Appendix of Tables

Identification U.T.I.	Cases	% of Total Sum	% of Identification	Identification Bacteriuria	Cases	% of Total Sum	% of Identification
Unidentified/Negative	175	33.1%		*Unidentified/Negative*	46	41.1%	
Acinetobacter spp.	1	0.1%	0.3%	*Acinetobacter spp.*	1	0.9%	1.5%
Acinetobacter baumannii	1	0.1%	0.3%				
Acinetobacter lwoffii	1	0.1%	0.3%				
Aeromonas hydrophila/caviae	1	0.2%	0.3%	*Aeromonas hydro/caviae*	3	2.7%	**4.5%**
Burkholderia. Cepacia				*Burkholderia. Cepacia*	1	0.9%	1.5%
Chrysemonas lutea				*Chrysemonas lutea*	1	0.9%	1.5%
Citrobacter spp.	1	0.1%	0.3%				
Citrobacter amalonaticus	1	0.0%	0.3%				
Citrobacter freundii	11	1.8%	**2.9%**	*Citrobacter freundii*	1	0.9%	1.5%
Comamonas acidovorans	1	0.2%	0.3%				
Corynebacterium spp.	1	0.2%	0.3%				
Enterobacter					1	0.9%	1.5%
Enterobacter avium					1	0.9%	1.5%
Enterobacter cloacae	8	1.3%	**2.1%**	*Enterobacter cloacae*	2	1.8%	**3.0%**
Enterobacter intermedius	1	0.2%	0.3%				
Enterobacter sakazakii	1	0.2%	0.3%				
Enterococcus spp.	5	1.1%	1.3%				
Enterococcus avium	3	0.6%	0.8%				
Enterococcus faecalis	15	2.4%	**4.0%**	*Enterococcus faecalis*	1	0.9%	1.5%
Enterococcus faecium	1	0.2%	0.3%				
Escherichia coli	143	25.1%	**37.8%**	*Escherichia coli*	21	18.8%	**31.8%**
Klebsiella oxytoca	14	2.3%	**3.7%**	*Klebsiella oxytoca*	2	1.8%	**3.0%**
Klebsiella pneumoniae	15	2.0%	**4.0%**	*Klebsiella pneumoniae*	1	0.9%	1.5%
Mixed Cultures	35	6.6%	**9.3%**	*Mixed Cultures*	10	8.9%	**15.2%**
Morganella morganii	6	0.8%	**1.6%**	*Morganella morgangii*	1	0.9%	1.5%
Ochrobactrum anthropi	1	0.3%	0.3%	*Orchobacter antropii*	1	0.9%	1.5%
Pantoea spp.				*Pantoea spp.*	1	0.9%	1.5%
Pasteurella heamolytica				*Pasteurella heamolytica*	1	0.9%	1.5%
Pasteurella pneumotropica				*Pasteurella pneumotropica*	1	0.9%	1.5%
Proteus mirabilis	20	2.7%	**5.3%**	*Proteus mirabilis*	9	8.0%	**13.6%**
Proteus vulgaris	2	0.1%	0.5%				
Providencia alcalifaciens	1	0.1%	0.3%				
Providencia rettgeri	3	0.2%	0.8%				
Pseudomonas spp.	5	1.2%	1.3%	*Pseudomonas spp.*	1	0.9%	1.5%
Pseudomonas aeruginosa	14	1.7%	**3.7%**	*Pseudomonas aeruginosa*	1	0.9%	1.5%
Pseudomonas pseudoalc				*Pseudomonas pseudoalc.*	1	0.9%	1.5%
Pseudomonas stutzeri	2	0.5%	0.5%				
Serratia marcescens	4	0.7%	1.1%	*Serratia marescens*	1	0.9%	1.5%
Shigella dysenteriae				*Shigella dysenteriae*	1	0.9%	1.5%
Staphylococcus spp.	13	2.5%	**3.4%**				
Staphylococcus aureus	14	2.8%	**3.7%**				
Staphylococcus epidermidis	6	0.8%	**1.6%**				
Stenotrophomonas maltophilia				*Stenotrophomonas maltophilia*	1	0.9%	1.5%
Streptococcus spp.	14	2.8%	**3.7%**				
Streptococcus bovis	2	0.2%	0.5%				
Streptococcus intermedius	2	0.4%	0.5%				
Streptococcus intestinalis	1	0.3%	0.3%				
Streptococcus mitis	3	0.3%	0.8%				
Streptococcus oralis	2	0.4%	0.5%				
Streptococcus pneumoniae	2	0.2%	0.5%				
Streptococcus sanguinis	1	0.0%	0.3%				
Total identification	378		100.0%	Total identification	66		100.0%
Total	553	100.0%		Total	112	100.0%	

***Table** 1.A. Bacteriological identification of strains from symptomatic urinary tract infections and bacteriuria specimens in 56 children with an enterocystoplasty*

	PEN	FLU	AMO	AUG	PIP	TAZ	CFZ	CFX	CAX	CAZ	ATM	IMP	GEN	TOB	AMI	NOR	CIP	FUR	COT	ERY	CLI
Acinetobacter spp.	-	-	-	-	-	100.0%	-	-	-	100.0%	-	100.0%	-	0.0%	100.0%	-	100.0%	-	100.0%	-	-
Acinetobacter baumannii	-	-	-	-	-	100.0%	-	-	-	100.0%	-	100.0%	-	100.0%	100.0%	-	100.0%	-	100.0%	-	-
Acinetobacter lwoffii	-	-	0.0%	100.0%	100.0%	100.0%	-	0.0%	100.0%	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	0.0%	100.0%	-	-
Aeromonas hydrophila/caviae	-	-	0.0%	100.0%	100.0%	-	0.0%	100.0%	-	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	-	-
Citrobacter spp.	-	-	0.0%	0.0%	100.0%	-	100.0%	100.0%	-	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	-	-
Citrobacter amalonaticus	-	-	0.0%	0.0%	100.0%	100.0%	-	0.0%	100.0%	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	-	-
Citrobacter freundii	-	-	0.0%	0.0%	87.5%	100.0%	0.0%	90.9%	100.0%	90.9%	-	100.0%	87.5%	100.0%	100.0%	100.0%	100.0%	90.9%	81.8%	-	-
Corynebacterium spp.	100.0%	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	100.0%	100.0%
Enterobacter cloacae	-	-	0.0%	0.0%	100.0%	80.0%	0.0%	100.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	75.0%	75.0%	-	-
Enterobacter intermedius	-	-	0.0%	0.0%	-	100.0%	-	100.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	-	-
Enterobacter sakazakii	-	-	0.0%	0.0%	-	0.0%	-	100.0%	100.0%	100.0%	-	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	-	-
Enterococcus spp.	0.0%	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	-	40.0%	0.0%
Enterococcus avium	0.0%	-	100.0%	-	-	-	-	-	50.0%	-	-	-	-	-	-	-	-	100.0%	-	100.0%	66.7%
Enterococcus faecalis	0.0%	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	93.3%	-	41.7%	0.0%
Enterococcus faecium	-	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	-	100.0%	0.0%
Escherichia coli	-	-	46.2%	93.0%	61.9%	97.2%	97.4%	99.3%	100.0%	100.0%	-	100.0%	96.7%	100.0%	100.0%	95.8%	95.8%	86.0%	57.3%	-	-
Klebsiella oxytoca	-	-	0.0%	100.0%	91.7%	100.0%	66.7%	100.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	78.6%	100.0%	-	-
Klebsiella pneumoniae	-	-	0.0%	100.0%	83.3%	100.0%	80.0%	100.0%	100.0%	100.0%	-	100.0%	91.7%	100.0%	100.0%	100.0%	100.0%	26.7%	80.0%	-	-
Morganella morganii	-	-	0.0%	0.0%	80.0%	100.0%	0.0%	20.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	0.0%	83.3%	-	-
Ochrobactrum anthropi	-	-	-	-	0.0%	0.0%	-	-	-	0.0%	0.0%	100.0%	-	0.0%	100.0%	100.0%	100.0%	-	100.0%	-	-
Proteus mirabilis	-	-	70.0%	100.0%	88.2%	100.0%	66.7%	100.0%	100.0%	100.0%	-	100.0%	94.1%	100.0%	100.0%	95.0%	100.0%	0.0%	70.0%	-	-
Proteus vulgaris	-	-	0.0%	0.0%	100.0%	100.0%	0.0%	0.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	0.0%	100.0%	-	-
Providencia alcalifaciens	-	-	0.0%	0.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	0.0%	100.0%	-	-
Providencia rettgeri	-	-	0.0%	0.0%	66.7%	100.0%	50.0%	100.0%	100.0%	100.0%	-	100.0%	100.0%	-	100.0%	100.0%	100.0%	0.0%	100.0%	-	-
Pseudomonas spp.	-	-	-	-	100.0%	100.0%	-	-	-	100.0%	75.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	-	100.0%	-	-
Pseudomonas aeruginosa	-	-	-	-	92.3%	100.0%	-	-	-	100.0%	85.7%	100.0%	-	92.9%	92.9%	92.9%	92.9%	-	85.7%	-	-
Pseudomonas stutzeri	-	-	-	-	100.0%	100.0%	-	-	-	100.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	-	100.0%	-	-
Serratia marcescens	-	-	0.0%	0.0%	100.0%	100.0%	-	25.0%	100.0%	100.0%	-	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	0.0%	75.0%	-	-
Staphylococcus spp.	7.7%	7.7%	-	-	-	-	-	7.7%	-	-	-	-	61.5%	-	-	-	53.8%	-	38.5%	76.9%	61.5%
Staphylococcus aureus	15.4%	100.0%	-	-	-	-	-	100.0%	-	-	-	-	100.0%	-	-	-	100.0%	-	92.3%	100.0%	100.0%
Staphylococcus epidermidis	16.7%	16.7%	-	-	-	-	-	16.7%	-	-	-	-	50.0%	-	-	-	50.0%	-	16.7%	66.7%	100.0%
Streptococcus spp.	100.0%	-	100.0%	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	100.0%	-	100.0%	100.0%
Streptococcus bovis	100.0%	-	100.0%	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	100.0%	-	50.0%	50.0%
Streptococcus intermedius	100.0%	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	-	100.0%	100.0%
Streptococcus intestinalis	100.0%	-	100.0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0%	-	100.0%	100.0%
Streptococcus mitis	100.0%	-	100.0%	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	100.0%	-	100.0%	100.0%
Streptococcus oralis	100.0%	-	-	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	-	-	100.0%	100.0%
Streptococcus pneumoniae	100.0%	-	100.0%	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	100.0%	-	100.0%	100.0%
Streptococcus sanguinis	100.0%	-	-	-	-	-	-	-	100.0%	-	-	-	-	-	-	-	-	-	-	100.0%	100.0%

***Table** 1.B. Antibiotic susceptibility of identified strains. PEN= Penicillin, FLU= Fluconazole, AMO=Amoxicillin, AUG= Augmentin. PIP= Piperacillin, TAZ= Piptazobactam, CFZ= Cefazoline, CFX= Cefuroxim, CAX= Ceftriaxon, CAZ= Ceftazidime, ATM= Aztreonam, IMP= Imipenem, GEN= Gentamycin, TOB= Tobramycin, AMI= Amikacin, NOR= Norfloxacin, CIP= Ciprofloxacin, FUR= Nitrofurantoin, COT=Cotrimoxazole (Trimethoprim/ Sulphamethoxazole), ERY= Erythromycin, CLI= Clindamycin (Susceptibility <95% in gray, intermediate resistance is counted as susceptible*

	χ^2 value	p-value (χ^2/ Fisher exact)	Correlation direction	Relative Risk	95% Confidence Interval
Appendicovesicostomy	3.83	0.047	~ Calculi	**6.74**	0.8 – 56.9
Bladder neck surgery	5.17	0.022	~ Calculi	**5.71**	1.1 – 28.8
Redo of the bladder neck	5.13	0.037	~ Calculi	**5.13**	1.1 – 23.1
Antibiotic therapy	3.84	0.050	~ Calculi	**3.64**	0.96 – 13.84
Medication unrelated to augmentation	5.17	0.022	÷ Calculi	**0.18**	0.04 – 0.88
Enterococcus spp.	10.5	0.007	~ Calculi	**7.22**	1.89 – 27.48
Providencia spp.	12.6	0.003	~ Calculi	-	-

***Table**.2. Risk factors for stone formation in enterocystoplasties*
13 of 56 patients developed bladder stones.
~ Calculi = positive correlation with bladder stone formation
÷ Calculi = negative correlation with bladder stone formation

	Anti – reflux surgery		Ureter reimplantation		Macroplastique ®	
		p-value		p-value		p-value
Augmentin		0.862		0.296	R	0.036
Ceftriaxon		0.333	R	0.021		0.057
Norfloxacin	S	0.014		0.578		0.233
Ciprofloxacin	S	0.046		0.811		0.514
Imipenem	S	0.041		0.411		0.651
	Concomitant nephrectomy		**Concomitant Mitrofanoff**		**Mitrofanoff after augmentation**	
Cotrimoxazol		0.694	R	0.016		0.074
Nitrofurantoin	S	0.045		0.799		0.721
Piperacillin	R	0.031		0.155		0.714
Imipenem		1.00		0.155	R	0.045

***Table** 3. Antibiotic susceptibility in relation to concomitant surgery*
p-values are determined by χ^2- analysis. Shaded areas are significant ($p<0.05$)
R= Resistant, S = Sensitive

		AUG	FUR	CIP	COT	CFX	TOB	GEN	Total
Bacterial strains from symptomatic urinary tract infection	*Acinetobacter spp*	1	1	1	1				1
	Citrobacter spp.	1	4		4	2	1	1	7
	Enterobacter spp.	2	2	1	3	1			5
	Enterococcus spp.	2	7	3	4	4	1		12
	Klebsiella spp.	7	7	3	6	2	1		11
	Mixed cultures	4	10	6	9	4	2	2	19
	Proteus spp.	3	4	2	5	1			7
	Providencia spp.	1	2	1	2	2	1	1	4
	Pseudomonas spp.	4	4	2	5	1	1	1	7
	Staphylococcus spp	5	5	5	6	2	2	1	11
	Streptococcus spp.	3	4	3	7	1	2	3	10
	Urease positive I	9	15	6	12	6	3	1	25
	Urease positive II	9	15	6	12	6	3	1	25
	Urease positive III	9	13	6	11	5	2		22
	Total	11	20	10	19	6	5	4	35
Bacterial strains from asymptomatic bacteriuria	*E.coli*	6	8	3	8	2	2	2	13
	Mixed cultures	3	2	1	3	2	1	1	6
	Aeromonas hydro/ caviae		1		1				1
	Proteus mirabilis	3	2	2	4			1	5
	Klebsiella spp.			1	1			1	1
	Pasteurella spp.	1			1				1
	Citrobacter spp.	1		1					1
	Pseudomonas spp.	1	1	1	1	1			3
	Other Strains	2	6	2	6	1	2	1	8
	Total	12	18	7	20	6	4	5	32

***Table**.4. Antibiotic prescriptions by clinicians.*
Bacterial strains in symptomatic and asymptomatic urinary tract infection for enterocystoplasty patients previously under antibiotic therapy and prophylaxis. Bacterial infections occur despite antibiotic treatment or prophylaxis. Numbers represent the number of patients. AUG= Augmentin, FUR= Nitrofurantoin, CIP =Ciprofloxacin, CFX= Cefuroxim, TOB = Tobramycin, GEN = Gentamycin. Urease positive I = Urease positive III + Citrobacter spp. +Providencia spp. , Urease positive II = Urease positive III + Citrobacter spp. , Urease positive III = Enterobacter spp. + Enterococcus spp. + Klebsiella spp. + Proteus spp. + Serratia spp.

U.T.I. Strains	Surgical factors	χ^2 value	p-value (χ^2/ Fisher exact)	Correlation direction	Relative Risk	95% Confidence Interval
Proteus mirabilis	*Lower Limb paralysis*	11.5	0.004	~ factors	**14.6**	2.3 – 90.8
Providencia spp.	*Macroplastique ®*	8.73	0.037	~ factors	**16.0**	1.6 – 156.5
Pseudomonas spp.	*Ureter reimplantation*	4.69	0.026	÷ factors	**-**	-
	All anti-reflux surgery	19.3	< 0.001	÷ factors	**0.026**	0.003 – 0.228
Urease positive	*Appendicovesicostomy*	4.44	0.036	~ factors	**3.71**	1.1 – 13.0
Bacteriuria Strains	**Surgical factors**	**χ^2 value**	**p-value (χ^2/ Fisher exact)**	**Correlation direction**	**Relative Risk**	**95% Confidence Interval**
E.coli	*Ureter reimplantation*	8.44	0.006	~ factors	**6.47**	1.7 – 24.6
	All Anti-reflux surgery	4.10	0.040	~ factors	**4.80**	0.95 – 24.16
Proteus mirabilis	*Lower Limb paralysis*	4.70	0.063 (N.S.)	~ factors	**6.83**	0.99 – 47.04
	Arnold-Chiari malformation	6.35	0.060 (N.S.)	~ factors	**10.4**	1.2 – 88.4

***Table** .5. Correlation between surgical factors and bacterial infections or bacteriuria.*
Bacterial strains were determined in routine urine specimens to evaluate symptomatic urinary tract infections. Separate urine specimens were routinely evaluated for bacteriuria. ÷ factors= negative correlation with surgical factors, ~ factors= positive correlation with surgical factors. Macroplastique ® or Deflux ® instillation is used as a bulk substance in the submucosa to form an obstruction of the lumen in the urinary tract.

Therapy	Prior concomitant surgery and affliction	χ^2 value	p-value (χ^2/ Fisher exact)	Correlation direction	Relative Risk	95% Confidence Interval
Redo Augmentation	*Lower Limb paralysis*	5.69	0.030	~	**5.42**	1.23 – 23.81
	Appendicovesicostomy after augmentation	8.31	0.012	~	**8.00**	1.69 – 37.67
Redo Bladder neck	*Appendicovesicostomy after augmentation*	4.98	0.047	~	**5.33**	1.11 – 25.63
	Vaginal reconstruction	10.10	0.006	~	**10.25**	2.05 – 51.25
	Bladder calculi	5.13	0.037	~	**5.14**	1.14 – 23.10
Abdominal surgical procedures (not bladder)	*Lower Limb paralysis*	7.46	0.011	~	**6.12**	1.53 – 24.36
	Anti-reflux surgery	5.91	0.025	÷	**0.22**	0.06 – 0.78
	Concomitant ureter reimplantation	5.75	0.024	÷	**-**	-
Antibiotic prophylaxis	*Appendicovesicostomy*	5.91	0.025	~	**4.50**	1.28 – 15.81
Anticholinergic medication	*Arnold-Chiari malformation*	6.91	0.022	~	**12.66**	1.29 – 124.50
	All tethered cord	6.97	0.018	~	**8.40**	1.43 – 49.49
Other medication	*Lower Limb paralysis*	4.54	0.047	~	**4.14**	1.06 – 16.12
	Bladder calculi	5.17	0.029	÷	**0.18**	0.04 – 0.88

***Table**.6. afflictions and surgical interventions affecting the bladder and bladder function related to therapeutic interventions at a later stage. ~ = positive correlation.*
÷ = negative correlation

pH - groups	*afflictions*	χ^2 *value*	*p-value* (χ^2/ *Fisher exact)*	*Correlation direction*	*Relative Risk*	*95% Confidence Interval*
≥pH 7.0	*Lower Limb paralysis*	5.40	0.027	~	**4.62**	1.2 – 17.7
	Arnold-Chiari malformation	6.20	0.028	~	**11.3**	1.2 – 111.3
	Proteus mirabilis	6.20	0.028	~	**11.3**	1.2 – 111.3
	Pseudomonas spp.	7.09	0.026	~	**-**	-
≥pH 6.5	*Lower Limb paralysis*	5.79	0.015	~	**9.56**	1.1 – 80.7
	Mixed cultures	5.81	0.026	÷	**0.09**	0.01 – 0.90

Table *.7. Overlapping subgroups of enterocystoplasty patients based on urine acidity correlated with physical and bacteriological afflictions. Bacterial strains were determined in routine urine specimens to evaluate bacteriuria and pH level. ~ = positive correlation. ÷ = negative correlation*

Antibiotics	*Bacterial strains*	χ^2 *value*	*p-value* (χ^2/ *Fisher exact)*	*Correlation direction*	*Relative Risk*	*95% Confidence Interval*
Amoxicillin	Urease positive**	7.65	0.009	Res. ~ strain	**9.1**	1.6 - 51.4
	Klebsiella spp.	6.20	0.011	Res. ~ strain	**-**	-
Augmentin	*Enterobacter spp.*	5.64	0.024	Res. ~ strain	**1.29**	1.0 – 1.6
	Urease positive*	6.70	0.011	Res. ~ strain	**6.3**	1.4 – 27.7
	Citrobacter spp.	9.77	0.004	Res. ~ strain	**-**	-
Cotrimoxazol	Urease positive**	4.69	0.031	Res. ~ strain	**3.8**	1.1 – 13.4
	Enterococcus spp.	4.32	0.038	Res. ~ strain	**1.4**	1.0 – 1.8
	Pseudomonas spp.	7.82	0.004	Res. ~ strain	**-**	-
	Staphylococcus spp	5.51	0.018	Res. ~ strain	**6.1**	1.2 – 31.7
Erythromycin	Urease positive**	4.41	0.042	Res. ~ strain	**8.9**	0.9 – 85.7
	Enterococcus spp.	6.64	0.015	Res. ~ strain	**9.8**	1.5 – 63.8
Piperacillin	Staphylococcus spp.	5.53	0.019	Res. ~ strain	**6.4**	1.2 – 33.9
Piptazobactam	Enterococcus spp.	6.15	0.024	Res. ~ strain	**8.1**	1.3 – 50.2
	Mixed culture	10.7	0.001	Res. ~ strain	**-**	-
Cefazoline	Enterococcus spp.	6.5	0.017	Res. ~ strain	**2.2**	1.0 – 4.8
Cefuroxime	Citrobacter spp.	5.12	0.028	Res. ~ strain	**9.0**	1.0 – 80.8
	Staphylococcus spp	5.56	0.019	Res. ~ strain	**4.7**	1.2 – 18.1
Gentamycine	Citrobacter spp	5.47	0.039	Res. ~ strain	**6.4**	1.1 – 34.2
	Staphylococcus spp	8.75	0.006	Res. ~ strain	**10.5**	1.8 – 60.0
Tobramycine	Staphylococcus spp	10.4	0.003	Res. ~ strain	**-**	-
Amikacine	Enterococcus spp.	6.0	0.040	Res. ~ strain	**3.2**	0.6 – 17.6
	Staphylococcus spp	9.72	0.007	Res. ~ strain	**-**	-
Furadantine	Proteus spp.	5.52	0..020	Res. ~ strain	**-**	-
Clindamycine	Enterococcus spp.	20.4	<0.001	Res. ~ strain	**11.2**	9.3 – 2936.2

Table*.8. Antibiotic resistances in patients related to incidence of bacterial strains.*
Res. ~strain= positive correlation between resistance and incidence of infection with the particular strain.
*Urease positive *= Enterobacter spp. + Enterococcus spp. + Klebsiella spp. + Proteus spp. + Serratia spp.+ Citrobacter spp. + Providencia spp*
*Urease positive **= Enterobacter spp. + Enterococcus spp. + Klebsiella spp. + Proteus spp. + Serratia spp.+ Citrobacter spp.*

References

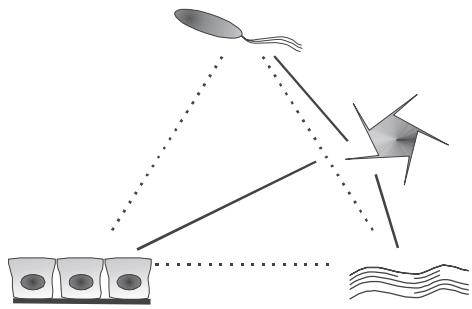
1. Stones in enterocystoplasties. Nurse DE, McInerney PD, Thomas PJ, Mundy AR. Br J Urol 77(5):684-687; 1996
2. Johnson DE, Russell RG, Lockatell CV, Zulty JC, Warren JW, Mobley HL. Contribution of Proteus mirabilis urease to persistence, urolithiasis, and acute pyelonephritis in amouse model of ascending urinary tract infection. Infect Immun 61(7):2748-2754; 1993
3. McLean RJ, Cheng KJ, Gould WD, Nickel JC, Costerton JW. Histochemical and biochemical urease localization in the periplasm and outer membrane of two Proteus mirabilis strains. Can J Microbiol 32(10):772-778; 1986
4. Rodman JS. Struvite stones. Nephron 81 (Suppl 1):50-59; 1999
5. Akerlund S, Campanello M, Kaijser B, Jonsson O., Bacteriuria in patients with a continent ileal reservoir for urinary diversion does not regularly require antibiotic treatment. Br J Urol. 74(2):177-181; 1994
6. Lauvetz RW, Monda JM, Kramer SA, Husmann DA. Urinary pH and urea concentration correlate to the bacterial colonization rate in gastric, colonic, ileal and myoperitoneal bladder augmentation. J Urol 154(2 Pt 2):899-902; 1995
7. Chan RC, Reid G, Bruce AW, Costerton JW. Microbial colonization of human ileal conduits. Appl Environ Microbiol 48(6):1159-1165; 1984
8. Bergman B, Kaijser B, Nilson AE , Conduit urinary diversion and urinary-tract infection. II. Raised serum antibody titers against Escherichia coli and Proteus mirabilis in relation to bacteriologic findings Scand J Urol Nephrol. 13(1):71-7 7; 1979
9. Bergman B, Kaijser B, Nilson AE , Conduit urinary diversion and urinary-tract infection. I. Raised serum antibody titers against Escherichia coli and Proteus mirabilis in relation to bacteriologic findings. Scand J Urol Nephrol. 1979;13(1):65-70
10. Wiersma TJ, Timmermans AE., Summary of the 'Urinary tract infections' guideline (first revision) of the Dutch College of General Practitioners. Ned Tijdschr Geneeskd. 145(15):735-739; 2001
11. Meijman F.J., The Dutch College of General Practitioners' guideline on urinary tract infections: response from the viewpoint of family practice. Ned Tijdschr Geneeskd. 145(15):716-171; 2001
12. Kahlmeter G., The ECO-SENS Project: a prospective, multinational, multicentre epidemiological survey of the prevalence and antimicrobial susceptibility of urinary tract pathogens – interim report. J. Antimicob. Chem. 46 Suppl 1 :15-22; 2000
13. Vromen M., Van der Ven A.J.A.M., Knols A., Stobberingh E.E., Antimicrobial resistance patterns in urinary isolates from nursing home residents. Fifteen years of data reviewed. J. Antimicob. Chem. 44:113-116;1999
14. R.B. Mathoera, D. J. Kok, R. J. M. Nijman, Bladder calculi in augmentation cystoplasty in children. Urology 56(3): 482-487; 2000

Additional comment

From the findings in the comparative study on bacteriological infections of the enterocystoplasty and bacteriuria, it becomes clear that community acquired infections should be checked for the correct bacteriological strain and treated specifically instead of the empirical treatment, often used in general practice. The use of broad spectrum antibiotics and the preferential use of certain antibiotics such as cotrimoxazol and amoxicillin (/clavulanic acid) to treat the urinary tract infections seen in enterocystoplasties have a tendency to transform the characteristics of a community acquired infection into a nosocomial infection due to the increased resistance among bacterial strains. Furthermore, the high degree of mixed cultures and low frequency of detected P.mirabilis infections may be explained by intracellular infection and increased resistance may thus explain the formation of stones; this will be discussed in chapter 4. In the next chapter the interaction between the enterocystoplasty and the urine content will be discussed in relation to bacterial/crystal adhesion and crystal formation as it seems that infection of the enterocystoplasty increases the pH level of the urine content conditioned by the enterocystoplasty, which correlates with stone formation The high number of mixed cultures may mask a number of P.mirabilis infections. Further research has to be done whether a specific search for urease producing bacterial strains among patients with an enterocystoplasty will benefit stone prevention by more specific antibiotic therapy of these strains. At this point we can not advocate a specific search for urease producing bacteria with current laboratory testing to identify them in mixed cultures, but clearly identification of uropathogens should preceed specific antibiotic treatment of symptomatic urinary tract infections in enterocystoplasties

Chapter 4

Cellular membranes as mediators of crystal formation



R.B. Mathoera, D.J. Kok, W.J. Visser, C.M. Verduin, R.J.M. Nijman.
J Urol. 166: 2329-2336; 2001

Abstract

Purpose:
Cells are a major component of mucus in enterocystoplasties. In this study we evaluated the role of secreted mucins on cells and cellular membranes as crystal adhesion and agglomeration mediators in infected artificial urine.

Materials and methods:
Five human intestinal cell lines, HT29, HT29-18N2, HT29-FU, HT29-MTX, Caco-2 and one ureter cell line, SV-HUC-1 were incubated for 3 hours in artificial urine with *P.mirabilis* (ATCC49565) in monolayer and scraped conditions. We isolated Triton X-100 soluble membrane proteins from cells to evaluate the effect of MUC2 and MUC5AC as membrane associated proteins on crystal formation and crystal adherence. Scanning electron microscopy , Light microscopy, Coulter Counter measurements and X-ray Microanalysis (XRMA) were used to evaluate crystal formation.

Results:
Brushite crystals where found adhered to cellular surface sites rich in sulphur as crystal agglomerates. Smaller and more numerous crystals were observed in the presence of scraped cells. Crystal formation and agglomeration was inhibited by the presence of MUC5AC, whereas MUC2 had the opposite effect. Both are present on cellular membranes and are rich in sulphur. Cellular invasion by bacteria occurred in all cell lines.

Conclusion:
Membrane associated cellular secretions such as MUC2 and 5AC are important crystal adhesion molecules on cells. The effects of secreted MUC2 and MUC5AC on stone formation by crystal adhesion are similar, but different for crystal agglomeration. The effects of MUC2 and MUC5AC may explain the high incidence of bladder calculi in enterocystoplasties and emphasize the role of cellular surfaces in urine.

Introduction

Calcium phosphate and struvite stone formation is well known in enterocystoplasties. Important contributing factors are bacterial colonization and mucus formation[1-5]. Bacterial derived urease production is implicated in making urine conditions favourable for the formation of bladder calculi in enterocystoplasties [4,6]. Bacterial capsules have been suspected to concentrate and induce struvite crystals[7]. Mucus has been regarded to contribute both by providing a basis for bacterial and crystal retention and by effecting crystal formation and growth[8-11]. Mucus clearance has thus been regarded as an important means for prevention of bladder calculi in enterocystoplasties[5]. Urine collected from enterocystoplasties has a high content of mucins that, given their biochemical structure, can enhance crystal formation[8]. In addition there are numerous cells, due to the high turn over of cells in the enterocystoplasty and bacterial infection. It is not clear how these cells and their membrane proteins influence crystal growth and agglomeration. Finally it is not known which of these factors, cells, cell membranes and mucins, are involved in the attachment of crystals to the bladder wall in enterocystoplasties. In this study we therefore evaluated the influences of cell membranes and mucins on crystal formation, adhesion and agglomeration in infected artificial urine *in-vitro* to determine the role of cells in urine as a major component of mucus in enterocystoplasties.

Materials and Methods

Bacterial culture

One *Proteus mirabilis* strain was attained from the ATCC (49565) and stored in 15% glycerol at -80°C until needed. *P. mirabilis* was cultured in Luria broth 0.05% glycerol until late lag-phase growth before use.

Cell culture

We used 5 intestinal cell lines, caco-2, HT-29, HT29-18N2, HT29-FU, HT29-MTX and one ureter cell line, SV-HUC-1. Caco-2 and HT-29 were obtained from the ATCC and used within 15 passages. SV-HUC-1 was obtained from the ATCC and used from passage 30 to passage 34. The HT29-FU and HT29-MTX were gifts from Dr.T.Lesuffleur (INSERM U505, Centre de recherche Biomédicales des cordeliers, Paris, France), HT29-18N2 was a gift from Dr.D.Louvard (UMR 144 CNRS-Institut Curie, 25 rue d'Ulm, 75248 Paris, Cedex 05). All intestinal cell lines were cultured on DMEM-high glucose with glutamine, 10% FBS and 5mM NEAA. SV-HUC-1 was cultured on Ham's F12 supplemented with 10% FBS.

Cell Culture and lysed cells infection:

The cell lines were cultured in 6 wells plates and upon reaching confluency divided in two groups. One group was scraped, one left intact. To both groups 3 ml artificial urine was added and incubated for 3 hours. After 3 hours both groups were infected with 100µl bacterial suspension, corresponding with $1.7x10^5$ bacteria/ml, and incubated for 3 hours before microscopic evaluation.

^{35}S Pulse-Chase cell labeling and Immunoprecipitation

Cells were preincubated for 1 hour with L-glutamine, methionine and cysteine free DMEM (Sigma- Aldrich D0422), labeled with 7 MBq/ml Redivue Promix L-[^{35}S] in vitro cell labelling mix in culture medium for 45 min, followed by a 2 hour chase with DMEM supplemented with 10%FBS to label mucin precursors. Membrane proteins were isolated and 100 µl was precipitated for 2 hours at 4°C with 100µl 1:4 diluted Goat-anti-mouse agarose in PBS (Sigma-Aldrich A6531) preincubated for 2 hours with monoclonal anti-MUC-2 (NCL-MUC-2, Novocastra Laboratories Ltd.) or anti-MUC-5AC (NCL-MUC-5AC, Novocastra Laboratories Ltd.) antibodies. The ^{35}S- labeled mucin precursors were quantified in a liquid scintillation counter by measuring the relative ^{35}S contribution of the mucin precursors to the total ^{35}S incorporation. Cells were grouped according to mucin production.

Protein isolation

Cell membranes were isolated from confluent cell cultures to ensure differentiation. Cells were washed with ice cold PBS and scraped with a protease inhibitor cocktail (Complete ™, Boehringer Mannheim), centrifuged at 3000 rpm for 10 min. The pellet was washed in PBS/ protease inhibitor and centrifuged at 2000 rpm for 5 min. For every 10^7 cells 200 µl RIPA buffer was added containing 10mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS and 5 mM EDTA. Triton X-100 soluble membrane proteins (TXSP) were acquired to contain membrane (glyco)proteins including membrane associated and intrinsic membrane proteins. The pellet was resuspended and placed on ice for 15-20 minutes, and centrifuged at 14000 rpm for 25 minutes at 4°C. The supernatants were stored at –20°C as 5 mg/ml stock solutions. The protein concentration was measured using a protein assay as described by Bradford (Bio-Rad), measuring the absorption at 595 nm.

SDS-PAGE

We used SDS-PAGE to identify pulse chase labeled immunoprecipitates of MUC2 and MUC5AC. Ten µl of immunoprecipitated labeled MUC2 and MUC5AC from different cell lines, homogenized human colon mucosa and samples of centrifuged and dialysed urine from enterocystoplasties was added to 10µl of 2x Laemli buffer. Samples were boiled for 5 minutes before analysis on 3% stacking/ 4% separating SDS-PAGE containing glycine. Samples were run for 2.5 hours at 100 mV in a mini-protean II dual Slab Cell (Biorad). Some gels were stained with Coomassie brilliant blue, Alcian blue -periodic acid Schiff (AB-PAS), or Alcian Blue - silver to determine the presence of (sulphated) glycoproteins.

Artificial Urine Composition

Two separate solutions were made to ensure the stability of the solutions. Solution A consisted of: 1.3 g/l sodium citrate, 1.3 g/l KH_2PO_4 , 2.0 g/l sodium oxalate and 25 g/l urea in distilled water. Solution B consisted of: 1.3 g/l $CaCl_2$, 1.3 g/l $MgCl_2$, 9.8 g/l NaCl, 4.6 g/l $NaSO_4$, 3.2 g/l KCl, 2 g/l NH_4Cl and 25 g/l urea in distilled water. Both solutions were combined in equal volumes, less than one hour before starting experiments. Artificial urine of pH 6.5 without NaCl, $NaSO_4$, and KCl and with 12.5 g/l urea, resulting in a lower osmolarity of 316 mosm, was used on cells to prevent artefacts due to high osmolarity.

Coulter Counter measurements

Four dilutions of TSXP-proteins were made in artificial urine, 0.5mg/ml, 0.125mg/ml, 0.031mg/ml, 0.007mg/ml TXSP, and 100 µl of bacterial solution in Luria broth/0.05% glycerol was added. To achieve pH 7.3 and higher, sufficient for precipitation of calcium phosphate and struvite, they were incubated at 37°C for 3 hours. Effects on particle size, number and bacterial association was evaluated by the distribution at 5%, 1%, and 0.1% cut-off values of controls and comparing the area under the curve below and above 3.9 µm in a Beckman Coulter Counter Multisizer. To measure crystal formation, the infected solutions were diluted in distilled water filtered three times over a 0.2 µm pore size filter. A second measurement after addition of 20 µl 37% hydrochloric acid to dissolve calcium apatite and struvite crystals served as the background measurement containing bacteria, proteins and debris. In addition by selecting an appropriate range of 3.90 µm and larger, the bacterial contribution to the particle number was eliminated and only true crystal formation and agglomeration effects were studied. All experiments were performed in triplicate. Measurements were evaluated by Z* distribution, Friedman's test, Wilcoxon signed ranks tests, Pearson and Spearman correlation, and paired samples t-tests where appropriate.

Light Microscopy

Infected dilutions were evaluated at 200x and 400x magnification to identify crystal types and crystal formation. A second observer confirmed all light microscopic evaluations of crystals. The second observer was not blinded for two observations and blinded for one. One observer did not have previous knowledge crystal morphology. Difference in assessment led to re-evaluation of samples.

Scanning Electron Microscopy

Cells were cultured on coverglass until confluency. Infected TXSP-protein solutions were filtered over 0.05 µm pore size millipore filters and fixed in 2.5% glutaraldehyde/ 0,15M cacodylate pH 7.4 for 2 hrs. Filters and cells were washed in 0.1M cacodylate pH 7.3 twice for 15 minutes. One percent OsO_4 /0.1 M cacodylate pH 7.3 was added and incubated at room temperature for 1 hour. Samples were washed overnight in 0.1M cacodylate pH 7.3 at 4°C. One percent tannic acid in 0.05M cacodylate pH 7.0 was added and incubated for 1hour at room temperature, washed in distilled water for 10 minutes, followed by alcohol dehydration. The samples were washed with ethanol 100% and 1ml hexamethyldisilazane (HMDS) and dried under 0.5 ml HMDS for optimal preservation of cell morphology. They were mounted on aluminium stubs with colloid silver and gold-palladium sputter coated before viewing under a JEOL-JSM25 electron microscope with a blinded second and third observer. Two observers had no previous knowledge of crystal morphology. The experiment was done twice with cells and once with filters.

X-ray micro analysis (XRMA)

To verify morphological determination of crystal composition and determine characteristics of the crystal adhesion site, X-ray microanalysis was performed. Cells were prepared similar to SEM samples but were mounted on carbon stubs with colloid silver and coated with carbon before viewing under a Philips 525M SEM with an energy dispersive spectrometry system

(EDAX-DX4). A Z-MAX light element detector was used for quantification. For the X-ray microanalysis (XRMA) with a Voyager XRMA System (NORAN Instruments Inc.) the acceleration voltage was adjusted to 10 keV using a point analysis during a live time of 100 seconds. Elements were identified by X-ray spectra emitted due to changed electron energy levels. The used magnifications depended on the size of the target. Measurements were made from at least 3 sites with brushite crystals and control measurements of bacteria, cells and coverglass in one experiment. A blinded second and third observer confirmed observations. One observer had no previous knowledge of crystal morphology.

Confocal Laser Scanning Microscopy (CLSM)

Cells were cultured on coverglass until confluency. For evaluation of bacterial adhesion and invasion, cells were infected with *P.mirabilis* for 3 hours. Cells were maintained at 37°C and the viable stain Syto 16 was applied. The first antibody against *P.mirabilis* (Biogenesis, Nuclilab) was added and incubated for 15 min. A TRITC labeled anti-mouse-antibody was applied and incubated for 15 min. For evaluation of mucin location cells were incubated with NCL-MUC-2 or NCL-MUC-5AC, washed with DMEM, incubated with the FITC labeled secondary antibody and washed with DMEM. Incubation steps were 45 minutes in DMEM. Images were made with a Zeiss LSM 410 laser scanning confocal microscope (Zeiss, Oberkochen, FRD). A 488 nm Ar-laser was used to excite the TRITC labeled anti-Proteus mirabilis antibodies. The TRITC signal was passed through a 510-540 nm band-pass filter. An overlay of the Syto 16 or FITC signal was done using the 488 nm laser and a 560 nm beam splitter to separate the Syto 16 signal from the TRITC signal, showing bacterial infection or mucin location. The experiment was repeated twice and results were confirmed on representative samples by a blinded second observer.

Results

Immunoprecipitation and SDS-PAGE

All the cell lines produce both MUC2 and MUC5AC, but differ in the produced amounts (**Table** 1.). SDS-PAGE of immunoprecipitates shows smears of mucin dimers and monomers, larger than 250kDa and corresponding to Alcian blue-Silver staining of MUC2 and MUC5AC immunoprecipitates from homogenized human colon and patient urine (**Fig**.1.B) and TXSP's on AB-PAS staining (**Fig**1.A). Many are sulphated glycoproteins, mucins or mucin-like, as demonstrated by AB-PAS staining of SDS-PAGE and larger than 250kDa. Both MUC2 and MUC 5AC contain sulphated groups and sugar residues and are larger than 250 kDa. Two equal groups representing MUC2 producing cell lines HT29, HT29-18N2, HT29-FU and MUC2 negative cell lines were made. Two similar groups were made for MUC5AC producing cell lines SV-HUC-1, HT29, Caco-2, and MUC5AC negative cell lines.

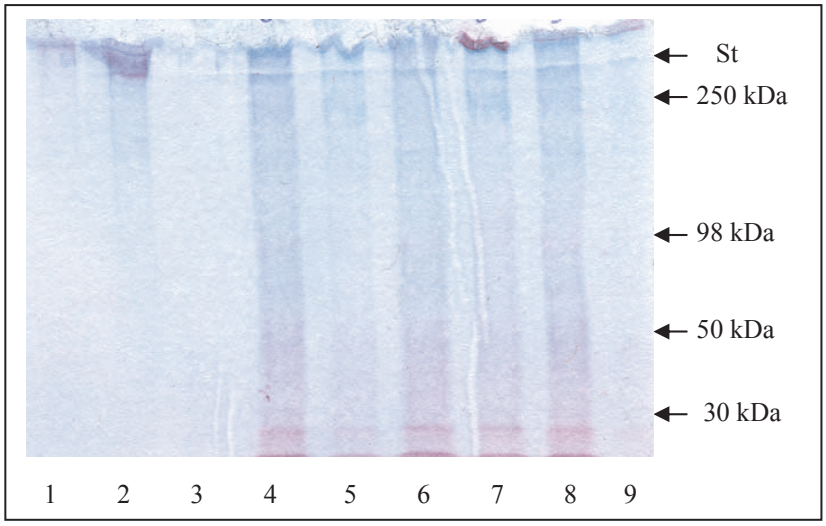


Fig. 1.A. *Alcian b1ue/ Periodic acid-Schiff staining of 10% .SDS-PAGE. Strongest staining of sulphated and glycosylated protein precursors above 250 kDa can be found in all cell lines, gastric and colonic mucosa. St, border between stacking and separating gel, 1, human gastric mucin. 2, human colonic mucin. 3, P. mirabilis capsule. 4, Caco-2, 5, HT29-MTX. 6, HT29-FU. 7, HT29-18N2. 8, HT-29, 9, SV-HUC-1*

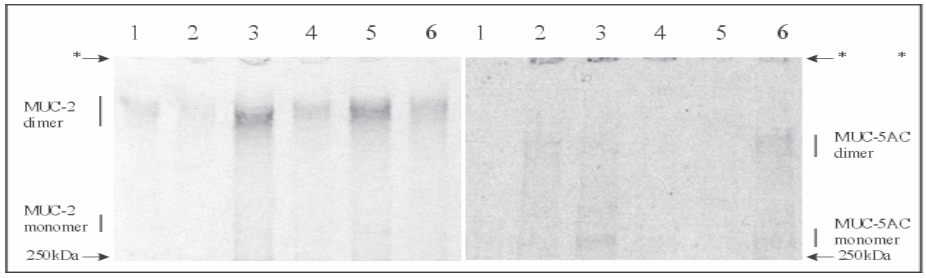


Fig 1.B. *SDS-PAGE of immunoprecipitated MUC2 and 5AC and ^{35}S-Pulse-Chase labeled MUC2 and 5AC precursors. 1, Caco2. 2, SV-HUC-1 3, HT29 4, HT29-18N2. 5, HT29-FU 6, HT29-MTX*
Left : MUC2 immunoprecipitates of ^{35}S labeled Mucin precursors
Right :MUC5AC immunoprecipitates of ^{35}S labeled mucin precursors

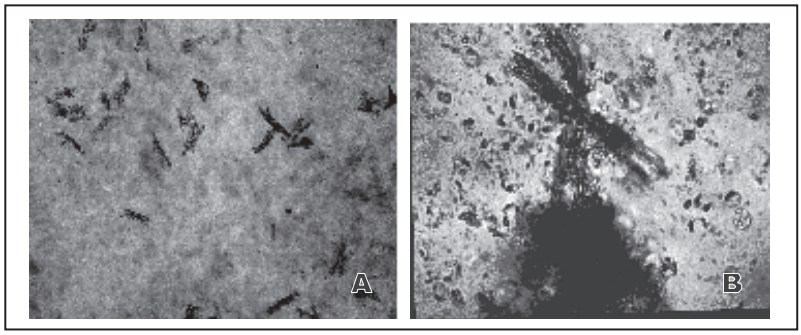


Fig 2, *Light microscopy of Caco-2 cells after infection with P. mirabilis, A, Small twinned crystals occurred in artificial urine with scraped cells and incubated for 3 hours with P. rnirabilis, B, large crystals formed when crystals were not scraped comparable to wells without cells (not shown), Reduced from x400.*

Light microscopy

Lysed and non-lysed cells:

Light microscopically crystals formed after 2.5 hours in artificial urine at pH values between 7.0 and 7.5. They appeared as calcium phosphate twinned crystals, and often agglomerated with scraped cells. Wells containing scraped cells expressed more and smaller crystals at an earlier time than the wells containing adherent cells (**Fig**. 2). Crystals in wells containing adherent cells were similar to crystals without cells. The use of an artificial urine with a lower osmolarity gave similar results but with an increased crystallization induction time.

TXSP- protein dilutions:

We found larger typically twinned and amorphous crystals at increasing dilutions in most cell lines. This was difficult to evaluate objectively by light microscopy and we therefore rely on coulter counter data for quantification of TXSP and mucin effect. An increase in bacterial growth on the bottom of the wells was observed with increasing protein concentration, microscopically as a decrease in transparency (**Fig**. 3).

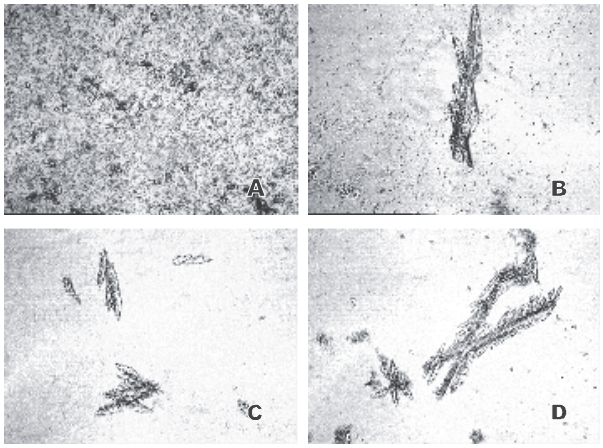


Fig 3.
Infected membrane dilution series of HT29-FU in artificial urine incubated for 3 hours A,1/10 dilution: 0.5 mg/ml TXSP B, 1/40 dilution: 0.125 mg/ml TXSP C, 1/160 dilution: 0.031 mg/ml TXSP, 1/640 dilution: 0.007 mg/ml TXSP.
Decrease in transparency can be observed due to bacteria, with smallest crystals in part A and largest crystals in part D Reduced from x200

Confocal Laser Scanning Microscopy (CLSM)

Patches of bacterial biofilm were formed on the non-detached cells and exposed glass surface. Bacterial invasion of cells was frequent in all cell lines(**Fig**4.A.). MUC2 and MUC5AC were expressed mainly on the cellular membrane. In cases of high expression of mucins, staining also occurred in the cytosol, mainly as granules (**Fig**.4).

EVALUATION PURPOSE	METHOD	CACO-2	SV-HUC-1	HT-29	HT29-18N2	HT29-FU	HT29-MTX
BACTERIAL INVASION AND ADHESION	C-LSM: Syto16-TRITC (2x)	+	+	+	+	+	+
RELATIVE MUC2 AND MUC5AC CONTENT IN RIPA ISOLATION	Immunoprecipitation of Pulse-Chase labeled mucin precursors (^{35}S): MUC-2 measured in Liquid Scintillation Counter (CPM/ % of total ^{35}S-label)	**551** / 0.26%	**396** / 0.08%	**7326** / 0.64%	**6410** / 0.39	**2848** / 0.49%	**2921** / 0.28%
	Immunoprecipitation of Pulse-Chase labeled mucin precursors (^{35}S): MUC-5AC measured in Liquid Scintillation Counter (CPM/ % of total ^{35}S-label)	**889** / 0.44%	**2096** / 0.44%	**3209** / 0.28%	**2640** / 0.16%	**981** / 0.17%	**1749** / 0.17%
	Ratio MUC2: MUC5AC	0.59	0.18	2.26	2.40	2.90	1.64
	MUC2+ MUC5AC (% of total ^{35}S- label in RIPA)	0.71%	0.51%	0.93%	0.55%	0.66%	0.45%
QUALITATIVE EVALUATION OF CRYSTALS	Scanning Electron Microscopy Crystal/ Largest agglomerate size range (μm)	2.6 / 36.0	4.2 / 17.0	3.4 / 17.4	3.6 / 23.4	4.0 / 13.3	4.5 / 58.5
QUANTITIVE EVALUATION OF CRYSTALS	Average Coulter Counter measurements: Mean crystal size (μm) *	**6.6**	**7.8**	**8.9**	**6.6**	**6.1**	**7.2**
	Average Coulter Counter measurements: Mean crystal number in 3.9 to 20 μm range over 70 channels	**1617**	**752**	**994**	**1213**	**3353**	**587**
	Average Coulter Counter measurements: Maximum Particle number in 3.9 to 20 μm range°	**17367**	**17197**	**9021**	**11247**	**18889**	**20060**

Table *1. Individual cellular and crystal characteristics. Coulter Counter *Significantly correlated with ($p<0.05$) with 1/mean crystal number °Significantly correlated with counts (CPM) of ^{35}S in pulse-chase labeled MUC2 and MUC5AC Immunoprecipitations ($p<0.05$) but not with the ^{35}S- count of unprecipitated ^{35}S-labeled proteins*

TXSP (mg/ml)	1/10 dilution (0.5)$^{++}$	1/40 dilution (0.125)	1/160 dilution (0.031)	1/640 dilution (0.007)	Average number of crystals**	Average total crystal surface (μm^2)	Average total crystal volume (μm^3)$^{+++}$	Average number of crystals with bacteria**		Bacterial/ crystal surface area*** (%)
BSA (mg/ml)	2891 (0.400)	3898 (0.100)	554 (0.025)	846 (0.007)				number	%	
Caco-2*	76184	74628	257118	66544	118618.5	67012.00	17555402	37442.3	31.57	37.4
SVHUC-1	107589	86465	103734	84239	95506.75	78382.00	5203390	67866.3	71.06	10.9
HT29^{+}	197811	120430	86030	65861	117533.0	105978.0	16737596	74236.0	63.16	8.1
HT29-18N2	129186	90700	106909	86279	103268.5	75654.00	24960915	66546.1	64.44	8.1
HT29-FU*	153222	100545	114213	367040	183755.0	143044.0	10822694	27414.5	14.92	38.4
HT29MTX	128475	103082	143102	101459	119029.5	72801.00	39714443	23618.5	19.84	40.0
Average	132077	95974	135183	128069	122825.8	92404.00	3034931	46828.3	38.13	23.8

Table *5.Particle number effect of TXSP's compared to BSA. Mean total particle number of three measurements. TXSP's from these cell lines all increase the mean total crystal number (Friedman's two way analysis of variance $\chi^2=15.4$,$p=0.031$, mainly due to the second dilution compared to the first and third but not due to variance between cell lines, according to Wilcoxon Signed ranks tests)*

**Average numbers represent the mean raw count over the four dilutions corrected for the background assuming that all particles below 3.9μm after subtraction of background are bacteria.***Bacterial surface area is represented as a percentage of the crystal surface area, assuming that all particles below 3.9μm after subtraction of background are bacteria and correlated to the ^{35}S count from MUC5AC precipitates ($p<0.05$) $^{+}$Crystal particle numbers are significantly correlated with concentration of TXSP. $^{++}$ The 1/10 dilutions (0.5mg/ml TXSP) are significantly correlated with the average total crystal surface and MUC2 concentration ($p<0.05$). All TXSP's enhance the mean total particle number significantly in paired samples t-test except * ($p<0.05$, all are significant in one-sided paired samples t-test with BSA as a negative control). $^{+++}$Average total crystal volume is correlated with the MUC5AC concentration.

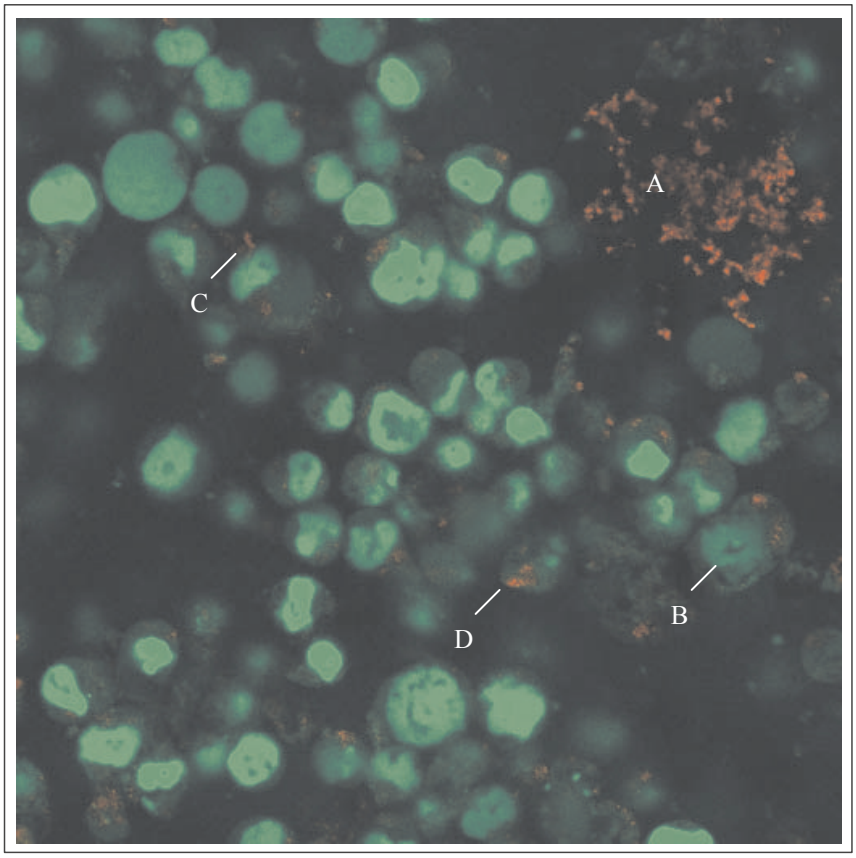


***Fig**.4.A. Confocal Laser Scanning Microscopy image of P.mirabilis infected HT-29 cells stained with viable stain (Syto 16, green) and bacteria stained with a primary anti-P.mirabilis antibody and a secondary TRITC labeled antibody (red). A) Bacterial colony, bacterial patches were found on glass and cellular surface. B) Apoptotic cell, C) Invading P.mirabilis, D)cytoplasmatic infection and invasion of HT-29 cell. Reduced from x800*

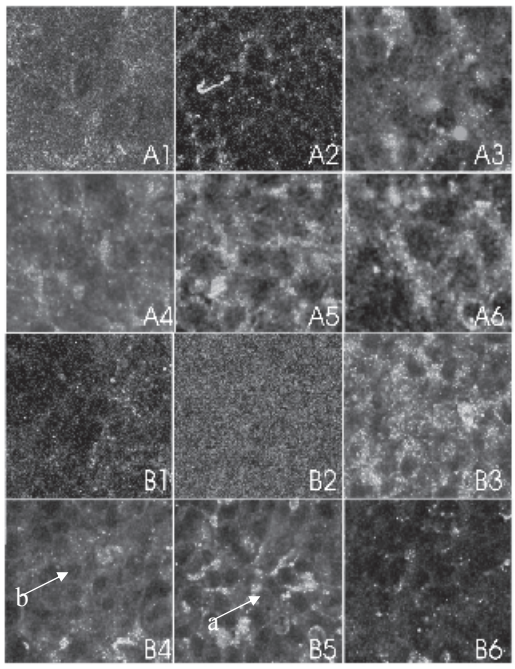


Fig. *4.B. CLSM picure of cells stained with anti-MUC2 (A) and anti-MUC5AC (B) antibodies and secondary fluorescein isothiocyanate – labelled antibody. Staining occurs primarily on granules (arrow a) and membranes (arrow b). MUC2 staining of Caco-2 and MUC5AC staining sre more diffuse but include cellular membrane 1, Caco-2, 2, SV-HUC-1, 3, HT-29, 4, HT29-18N2, 5, HT29-FU, 6, HT-29-MTX*

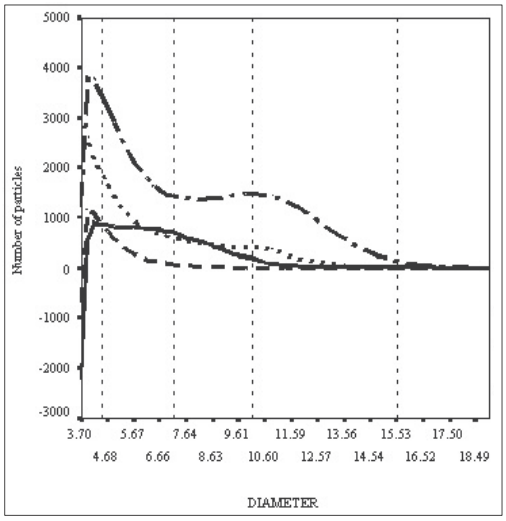


Fig. *5. Average coulter counter measurements of all protein dilutions. Concentration dependent effect can be observed for distribution of large particles. Highest peak corresponds with single crystals while plateau corresponds with crystal agglomerates. Solid line indicates 1/10 dilution: 0.5 mg/ml TXSP. Dashes indicate 1/40 dilution: 0.125 mg/ml TXSP. Dots represents 1/160 dilution: 0.031 mg/ml TXSP. Intermittent dashes and dots indicate 1/640 dilution: 0.007 mg/ml TXSP.*

Coulter Counter Measurements

After dissolution of the crystals by HCl, the bacteria that previously adhered to the crystal mass were freed, apparent as a particle number increase between 1.97 to 3.70 μm. The number of liberated bacteria was sufficient to cover an average of 23.8% (sd= 14.8%) /2= 11.9% of the original crystal surface, ranging from 0 to more than 100%. This was correlated with the ^{35}S count after MUC5AC (p=0.023) but not MUC2 precipitation.

A concentration dependent effect on particle size and number was observed for the average particle numbers for the dilutions of the different cell lines (**Fig**. 5). A correlation between sulphur incorporation and particle size and maximum number (**Table** 1.) was demonstrated.

MUC2 induced the formation of larger crystals (**Table** 2.) whereas MUC5AC induced smaller crystals (**Table** 3). The effect of MUC2 and 5AC on the mean particle number over all channels in the 3.9 to 20 μm range is presented in **Table** 4. The concentration dependent effect of TXSP's on the mean total particle number is mainly due to TXSP's from HT29 (**Table** 5.), which also produces the most MUC2 and 5A

	Diameter(μm)	Concentration MUC2 (mg/100ml)	Mean Total Count	cut-off value (%)	MUC2+ (% outside 95%, 99% and 99,9% distribution of the Muc2- control)			
MUC2-	6.792	0.103	104082	5	81.26	11.56	15.44	45.18
	9.081			1	38.22	4.90	6.91	23.59
	14.36			0.1	8.06	0.68	0.48	1.15
	7.101	0.0258	88058	5	87.00	11.98	16.40	48.83
	10.42			1	31.62	3.75	4.65	16.02
	16.15			0.1	2.53	0.44	0.17	0.27
	10.01	0.0064	167984	5	18.32	**2.24**	**2.93**	10.14
	12.59			1	9.95	**0.85**	**0.79**	2.56
	17.06			0.1	0.50	0.13*	**0.04**	**0.06**
	7.937	0.0016	83080	5	69.82	8.98	13.09	41.21
	10.81			1	30.58	3.45	4.11	13.97
	16.34			0.1	2.20	0.43	0.16	0.24
	Mean Total Count				160072	103891	102838	173060
	Concentration MUC2 (mg/100ml)				0.253	0.063	0.016	0.004

***Table** 2. Coulter Counter measurements of MUC2 effect on crystal size. MUC2- cell lines: SV-HUC-1, Caco-2, HT29-MTX; MUC2+ cell lines:HT-29, HT29-18N2, HT29-FU. Mucin concentrations were estimated from to the percentage of ^{35}S incorporation in mucin precursors compared to the total amount of incorporated ^{35}S and the measured amount of proteins isolated in RIPA. The mean total count is the corrected mean particle count over three measurements.*
*All differences are significant ($p<0.001$ except *, $p<0.05$) in Z*-distribution. MUC2 enhances the formation of larger crystals (Friedman test $\chi^2=26.5$, $p<0.01$)*

	Diameter(μm)	Concentration MUC 5AC (mg/100ml)	Mean Total Count	cut-off value (%)	MUC5AC- (% outside 95%, 99% and 99,9% distribution of the Muc5AC+ control)			
MUC5AC+	9.998	0.1939	127194	5	24.06	**1.80**	**4.11**	16.31
	13.39			1	11.51	**0.50**	**0.72**	2.42
	17.49			0.1	0.39	**0.08**^{NS}	**0.05**	**0.06**
	7.586	0.0484	93841	5	49.98	5.16^{NS}	13.03	46.50
	11.39			1	26.18	1.51	2.90	11.13
	16.44			0.1	1.86	0.22	0.14**	0.23
	10.27	0.0121	148960	5	19.73	**1.41**	**3.12**	12.35
	12.73			1	12.15	**0.55**	**0.89****	3.23
	17.3			0.1	0.42	**0.07***	**0.05**	**0.07****
	7.999	0.0030	71214	5	60.21	5.90	15.06	55.47
	10.52			1	39.74	2.72	5.84	22.95
	15.87			0.1	4.45	0.37	0.28	0.53
	Mean Total Count				137000	98109	121400	184900
	Concentration MUC 5AC(mg/100ml)				0.0834	0.0209	0.0052	0.0013

***Table** 3. Coulter Counter measurements of MUC5AC effect on crystal size. MUC5AC- cell lines: HT29-18N2, HT29-FU,HT29-MTX; MUC5AC+ cell lines:HT-29, Caco-2, SV-HUC-1. Mucin concentrations were estimated from to the percentage of ^{35}S incorporation in mucin precursors compared to the total amount of incorporated ^{35}S and the measured amount of proteins isolated in RIPA. The mean total count is the corrected mean particle count over three measurements.*
All differences are significant ($p<0.001$ except=$p<0.05$, **= $p<0.01$ and ^{NS}=non-significant) in Z*-distribution. MUC5AC inhibits the formation of larger crystals (Friedman test $\chi^2=30.3$, $p<0.01$)*

	Concentration (mg/100ml)	Mean particle number		Concentration (mg/100ml)	Mean particle number	Paired differences					Significance (2-tailed) p
						Mean difference	Sd	se	C.I. 95% Lower	C.I. 95% Upper	
MUC 5AC+	0.1939	688.6	MUC 5AC-	0.0834	690.6	-2.0	868.7	103.8	-209.1	205.1	0.985
	0.0484	**516.1**		0.0209	174.1	342.0	787.1	93.4	155.7	528.3	**<0.001**
	0.0121	**1411.2**		0.0052	650	761.1	1334.3	157.2	447.5	1074.6	**<0.001**
	0.0030	571.3		0.0013	**3702.5**	-3131.2	2585.0	306.8	-3743.1	-2519.4	**<0.001**
MUC2 +	0.253	**1409.3**	MUC2 -	0.1030	364.2	1191.7	1503.8	179.7	833.1	1550.3	**<0.001**
	0.063	197.5		0.0258	**503.3**	-363.0	904.8	107.4	-577.1	-148.8	**0.001**
	0.016	498.9		0.0064	**1491.2**	-992.3	1159.5	136.7	-1264.8	-719.9	**<0.001**
	0.004	**1685.6**		0.0016	515.2	1082.1	939.0	111.4	859.9	1304.4	**<0.001**
MUC 5AC+	0.1939	**688.6**	BSA	200.00	382.3	458.2	1057.6	135.4	187.3	729.1	**<0.001**
	0.0484	**516.1**		50.00	191.3	363.9	1015.3	125.0	114.3	613.5	**<0.001**
	0.0121	**1411.2**		12.50	314.9	1144.4	1707.5	210.2	724.6	1564.1	**0.002**
	0.0030	571.3		6.25	**610.1**	917.6	1488.2	317.3	257.8	1577.5	**0.038**
MUC2 +	0.253	**1409.3**	BSA	200.00	382.3	1439.9	1116.9	144.2	1154.4	1728.4	**<0.001**
	0.063	**197.5**		50.00	191.3	3.9	240.0	29.5	-55.1	62.9	**<0.001**
	0.016	498.9		12.50	314.9	138.9	1021.9	125.8	-112.3	390.1	0.885
	0.004	**1685.6**		6.25	610.1	2757.9	1344.5	286.7	2161.8	3354.0	**0.003**
MUC 5AC-	0.0834	**690.6**	BSA	200.00	382.3	420.7	363.0	46.9	326.9	514.5	**<0.001**
	0.0209	174.1		50.00	**191.3**	-4.0	316.4	39.0	-81.8	73.8	**<0.001**
	0.0052	650		12.50	314.9	228.2	1402.2	172.6	-116.5	572.9	0.550
	0.0013	**3702.5**		6.25	610.1	6283.3	2168.7	462.4	5321.7	7244.8	**0.005**
MUC2 -	0.1030	364.2	BSA	200.00	**382.3**	109.2	1130.0	144.7	-180.2	398.6	0.126
	0.0258	**503.3**		50.00	191.3	394.4	1132.9	139.4	115.9	672.9	**<0.001**
	0.0064	**1491.2**		12.50	314.9	1199.6	1868.6	230.0	740.2	1658.9	**0.008**
	0.0016	515.2		6.25	610.1	861.5	1509.1	321.7	192.4	1530.6	0.0510

***Table** 4. Coulter Counter measurements of particle number. Mean differences over 70 channels in particle number in a 3.9 to 20 μm range evaluated in paired samples t-test. Sd=standard deviation, se=Standard error of the mean, C.I.$_{95\%}$= 95% confidence interval.*

Scanning Electron Microscopy

Infected cells on coverglass

Scanning Electron Microscopy showed agglomeration of brushite crystals on cells. This was found for all the infected samples. No crystals were found on the non-infected controls. Patches of bacterial colonies adhered to the cellular surface of the intestinal and between cells of the ureter cell line (**Fig**. 6). The composition of the crystals was confirmed by XRMA and in addition to calcium and phosphorus a relatively high sulphur peak at the site of crystal adhesion was observed (**Fig**. 7)

Filtered infected protein dilutions

Scanning Electron Microscopy showed that the larger crystals were all agglomerates of smaller crystals. Crystals were similar to the crystals found on the surface of infected cells. In most cases no bacteria were found on crystals except on agglomerates. Most large agglomerates were composed of flat trapezoid crystals agglomerated to form a rosette, which can appear as an amorphous crystal when viewed laterally (**Fig**. 6.C.).

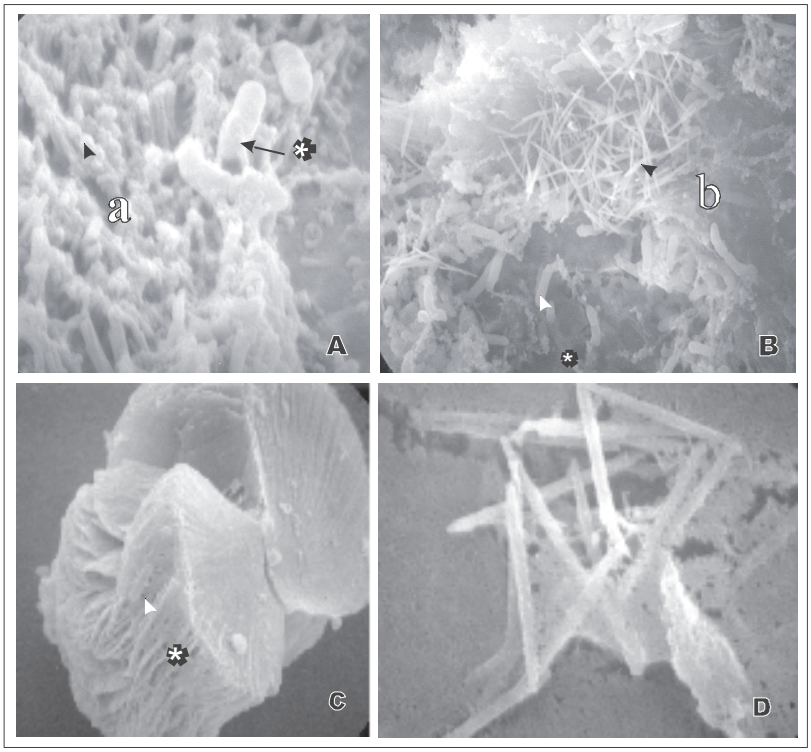


***Fig**.6. Scanning Electron Microscopy. A) Bacterial adhesion to microvilli on HT29-MTX. Arrow a indicates microvilli. Reduced from x 15.000 (bacterial size 1.7 by 0.2 micron). B) crystal adhesion to cellular surface as crystal agglomerates. Arrow b indicates crystal agglomerate. Reduced from x 3.000. C)Agglomerated crystals (rosette) in 0.5 mg/ml TXSPs from Caco-2 consisting of smaller trapezoid crystals. Reduced from x 7.000. D) Brushite needles (3 μm) agglomerated by 0.007 mg/ml TXSP from Caco-2. Reduced from 15.000. Asterisk indicates bacteria.*

Discussion

In this study we show that bacterial infection first induces the formation of brushite crystals in artificial urine instead of struvite, adhered to the cellular surface at sites relatively rich in sulphur.

The effect of smaller and numerous crystals for all scraped cell types, indicates some similarity in characteristics. Caco-2 is a colonic epithelial cell line regarded as ileum like for its membrane characteristics[12]. HT-29 is regarded a true colonic epithelial cell type and easily forms mucin producing subclones [13-15]. SV-HUC-1 can be regarded as a non-MUC2 producing, non-secreting cell line stable within 5 passages[16], while HT29-18N2 and HT29-MTX produce mucins only in certain culture conditions[13,15]. MUC2 and to a lesser extent MUC5AC, can be found in the entire small and large intestine. When secreted, much of the secreted mucins still remain on the cellular surface, simplifying isolation of soluble mucins in the TSXP solution with RIPA buffer. These mucins are known to contain differences in sulphate groups and O-glycosylated sugar residues[17]. Many of the TXSP's are membrane associated and integrated membrane proteins from the cellular membrane, some are from the cystosol. They are freed from the cell by rigorously disrupting the cellular membrane. In cultured cell lines the production of mucins is small, even in the so-called mucin producing cell lines, compared to the artificial urine volume. Differences in the oligosaccharide chains and the number of tandem repeats cause the main differences in the

molecular weight between MUC2 and MUC5AC. But there are great similarities in the relatively large and rigid molecular structure, secreted at the luminal side as dimers with a typical general structure of sulphate rich domains to form a gel-like layer[18]. Considering the characteristics of the crystal adhesion sites, the cellular location of mucins and their high sulphur content, we choose to quantify these small amounts of mucins by measuring the sulphur incorporation. Crystal formation and agglomeration in infected TXSP dilutions was tested in crystallization experiments using solubilized membrane components instead of whole membranes to reduce background. MUC2 and MUC5AC, which are not only present on the cellular membrane but are also present in urine from enterocystoplasties may adhere crystals on the cellular membrane but act differently in solutions.

The isolated TXSP's of mucin producing cells show a distinct effect on crystal size when compared to the TXSP's of non-mucin producing cells. However, all TXSP's induce both an increase in crystal size and crystal number compared to BSA. This indicates an effect of mucins despite the presence of other cellular proteins. MUC2 increases whereas MUC5AC decreases particle size. It becomes clear that soluble MUC5AC inhibits agglomeration. This indicates that MUC5AC adheres to the calcium phosphate crystals without spanning more than one crystal. MUC2 enhances agglomeration and must therefore adhere to more than one crystal. The agglomeration of crystals reduces the number of particles, resulting in an inverse correlation of the mean particle size with the mean particle number.

With detached cells, the entire cellular membrane is in contact with the artificial urine and

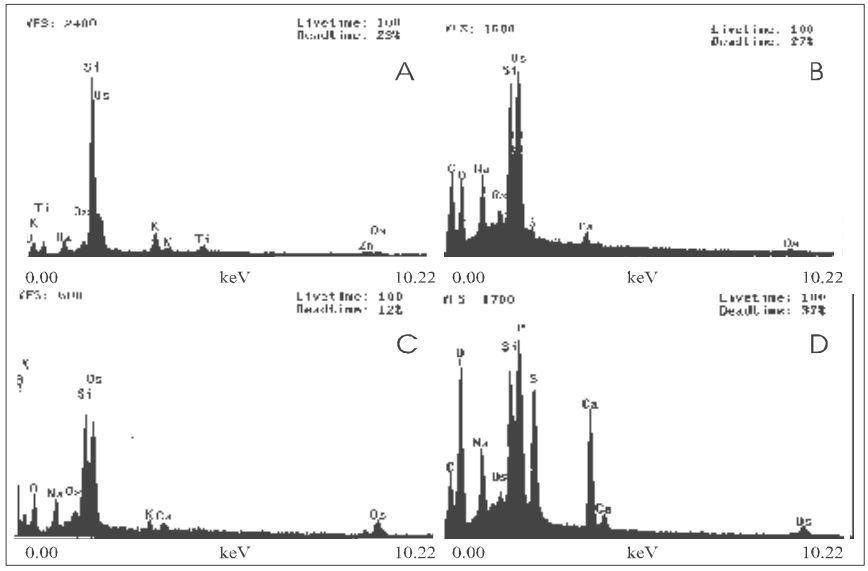


***Fig**.7. X-ray microanalysis spectra. Measurements; all had 100 seconds live time. A) Spectrum of coverglass without sulphar peak. B) Spectrum of cellular surface of HT29-MTX with a small sulphur peak, but no phosphate and small calcium peak. C) Spectrum of bacteria with small calcium peak but no sulphar or phosphate. D) Spectrum of crystals adherent to cellular surface, with high sulphar peak, high calcium and high phospate. Excitation curve of phosphate slopes and causes phosphate peak to overlap with voltages designated to Ytrium (corrected as P for phosphate). VFS, Vertical full scale.*

smaller crystals are formed. Both MUC2 and MUC5AC are present on the entire cellular membrane in different local concentrations. Molecules capable of adhering to crystals and present on the cellular membrane such as MUC2 and 5AC can function as cellular crystal adhesion molecules. In ileocystoplasties with similar cellular surfaces, detached cells might thus increase the number of crystals adhering on the cell surface and the formation of small crystals, while soluble MUC5AC inhibits and MUC2 enhances the formation of large agglomerates. Unfortunately the production of MUC2 by ileal and colonic cells is larger than the production of MUC5AC and large agglomerates of small crystals may adhere to the cellular surface as in our model. From our model we speculate that mucus clearance from enterocystoplasties will be most effective if membrane associated MUC2 and 5AC and soluble MUC2 is cleared from the bladder whereas soluble MUC5AC would act similar to glycosaminoglycans, protecting the enterocystoplasty from stone formation.
Other attributing factors may be either nucleation or crystal growth into the measuring range and bacterial invasion by *P. mirabilis* and bacterial adherence to the cell surface, which may involve MUC5AC as suggested by the correlation with bacterial adhesion to crystals. In the 316 mosm artificial urine, decreased urea concentration and absence of NaCl, may have inhibited bacterial growth or differentiation[20], with a longer crystal induction time.
In enterocystoplasties where both attached and detached mucin producing cells are present in infected alkaline urine and cell turn-over is high, this model may in part explain the high incidence of urolithiasis.

Conclusion: Membrane associated cellular secretions such as MUC2 and 5AC may be important crystal adhesion molecules in stone formation. These mucins influence crystal agglomeration, when present in urine and crystal adhesion when membrane associated. The effect on crystal agglomeration depends on the mucin type. These effects may play a role in the high incidence of bladder calculi in enterocystoplasties, thus emphasizing the role of cellular membranes in urine.

Acknowledgements
The authors wish to acknowledge Ing. J.M. van't Noordende and Dr.H.K. Koerten at the Sylvius Laboratory for Electron Microscopy of Leiden University Medical Centre for their expert assistance with XRMA and Dr.A.W.C.Einerhand at the department of Pediatrics of the Erasmus University for her assistance with anti-mucin antibody selection.

References

1. Mathoera, R.B., Kok, D.J., Nijman, R.J.M.: Bladder calculi in augmentation cystoplasty in children. Urology, **56**(3): 482-487, 2000
2. Silver, R.I., Gros, D.A., Jeffs, R.D., Gearhart, J.P.: Urolithiasis in the exstrophy-epispadias complex. J Urol, **158**(3 Pt 2): 1322-6, 1997
3. Griffith, D.P.: Struvite stones. Kidney Int, **13**: 372-382, 1978
4. Mobley, H.L.T., Islan,d M.D., Hausinger, R.P.: Molecular biology of microbial ureases. Microbiol Rev, **59**(3): 451-480, 1995
5. Khoury. A.E., Salomon, M., Doche, R. et al.: Stone formation after augmentation cystoplasty: the role of intestinal mucus. J Urol, **158**(3 Pt 2): 1133-7, 1997
6. Blyth, B., Ewalt, D.H., Duckett, J.W., Snyder, H.M. 3d.: Lithogenic properties of enterocystoplasty. J Urol, **148**(2 Pt 2): 575-7, 1992
7. Dumanski, A.J., Hedelin, H., Edin-Liljegren, A., Beauchemin, D., Mc Lean, R.J.C.: Unique ability of the Proteus mirabilis capsule to enhance mineral growth in infectious urinary calculi. Infect Immun, **62**(7): 2998-3003, 1994
8. Mann, S.: Molecular recognition in biomineralization. Nature, **332**: 119-124, 1988
9. Gillon, G., Mundy, A.R.: The dissolution of urinary mucus after cystoplasty. Br J Urol, **63**(4): 372-374, 1989
10. Sherman, P., Fleming, N., Forstner, J., Roomi, N., Forstner, G.: Bacteria and the mucus blanket in experimental small bowel bacterial overgrowth. Am J Pathol, **126**(3): 527-34, 1987
11. Bruce, A.W., Reid, G., Chan, R.C., Costerton, J.W.: Bacterial adherence in the human ileal conduit: a morphological and bacteriological study. J Urol, **132**(1): 184-8, 1984
12. Duizer, E., Gilde, A.J., Versantvoort, C.H., Groten, J.P.: Effects of cadmium chloride on the paracellular barrier function of intestinal epithelial cell lines. Toxicol Appl Pharmacol, **155**(2): 117-126, 1999
13. Philips, T.E., Huet, C., Bilbo, P.R. et a;.: Human intestinal goblet cells in monolayer culture: characterization of a mucus-secreting subclone derived from the HT29 colon adenocarcinoma cell line. Gastroenterology, **94**(6): 1390-1403, 1988
14. Lesuffleur, T., Barbat, A., Luccioni, C. et al.: Dihydrofolate reductase gene amplification-associated shift of differentiation in methotrexate adapted HT-29 cells. J Cell Biol, **115**(5): 1409-1418, 1991
15. Lesuffleur, T., Porchet, N., Aubert, J.P. et al.: Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. J Cell Sci, **106**(Pt 3): 771-783, 1993
16. Meisner, L.F., Wu, S.Q., Christian, B.J., Reznikoff, C.A.: Cytogenetic instability with balanced chromosome changes in an SV40 transformed human uroepithelial cell line. Cancer Res, **48**(11): 3215-3120, 1988
17. Neutra M.R., Forstner J.F., Mucucs: synthesis, secretion and function. In: *Physiology of the Gastrointestinal Tract* (2nd ed.) edited by L.R. Johnson New York: Raven, 1987, p 975-1009.
18. Perez-Vilar J., Hill R.L., The structure and assembly of secreted mucins. J.Biol.Chem. **274**(45): 31751-31754; 1999
19. Allison, C., Lai, H.C., Gygi, D., Hughes, C.: Cell differentiation of Proteus mirabilis is initiated by glutamine, a specific chemoattractant for swarming cells. Mol Microbiol, **8**(1): 53-60, 1993
20. Jones, H.E., Park, R.W.A.: The influence of medium composition on the growth and swarming of Proteus. J Gen Microbiol, **47**(3): 369-378, 1967

Additional comment

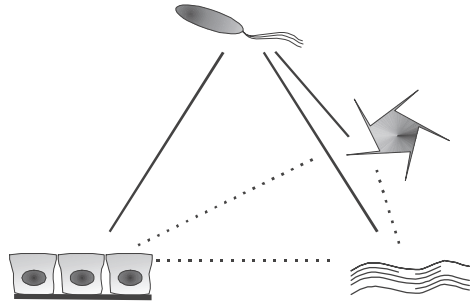
No hyaluronic rich extracellular matrix as proposed for MDCK cells was considered as an explanation for the smaller size of the more numerous crystals, but rejected after consistent negative results in initial testing for a shielding extracellular matrix by use of fixed erythrocytes in a particle exclusion assay according to Knudson e.a.[1]

In intestinal tissue, the classic protective molecules of the epithelium that make up the unstirred water layer, are mucins rather then polysaccharides and should be considered the most probable interacting molecule. Although some similarities in regard to crystal interaction may exist, the effect on crystal number and size is best explained by the effect of molecules, capable of starting nucleation in saturated solutions or inhibiting crystal growth. Cellular lysis often occurs with the formation of smaller crystals. This will be discussed further in the next chapter. Similar reports of altered crystal size have been made for several urinary proteins (THP) and other mucins such as MUC1 [2].

1. Knudson W., Knudson C.B. Assembly of a chondrocyte-like pericellular matrix on non-chondrogenic cells. Role of the cell surface hyaluronan receptors in the assembly of a pericellular matrix. J. Cell Sci. 99: 227-235; 1991
2. Nagatsuma K., Asakura H., Nakamura S., Murai M., The effect of mucin on the nucleation and growth of struvite crystals in vitro. A.L. Rodgers, B.E. Hibbert, B. Hess, S.R. Khan, G.M. Preminger, Proceedings of the 9th International Symposium on Urolithiasis 2000 p202-203

Chapter 5

Pathological and therapeutical significance of cellular invasion by Proteus mirabilis in an enterocystoplasty infection stone model.



R. B. Mathoera, D. J. Kok, C. M. Verduin, R. J.M. Nijman
Infection and Immunity 70(12): 7022-7032; 2002

Abstract

Proteus mirabilis infection often leads to stone formation. We evaluated how bacterial-mucin adhesion, invasion and intra-cellular crystal formation are related to antibiotic sensitivity and may cause frequent stone formation in enterocystoplasties. Five intestinal (Caco-2, HT29, HT29-18N2, HT29-FU, HT29-MTX) and one ureter cell line (SV-HUC-1) were incubated in artificial urine with 5 *Proteus mirabilis* strains. Flowcytometry (FACS), laser-scanning microscopy and electron microscopy evaluated cellular adhesion/invasion, pathological changes to mitochondria and *P.mirabilis*-mucin colocalisation (MUC2/ MUC5AC). An MTT (thiazolyl blue tetrazolium bromide)-assay and FACS-analysis of caspase 3 evaluated the cellular response. Infected cells were incubated with antibiotics at dosages representing the expected urinary concentrations in a 10 year old, 30 kg child to evaluate bacterial invasion and survival. All cell lines showed colocalisation of *P.mirabilis* with Human Colonic Mucin i.e. MUC2 and Human Gastric Mucin i.e. MUC5AC. Correlation between membrane mucin expression and invasion was significant and opposite for SV-HUC-1 and HT29-MTX. Microscopically, invasion by *P.mirabilis* with intra-cellular crystal formation and mitochondrial damage was found. Double membranes surrounded bacteria in intestinal cells. Relative resistance to cotrimoxazol and augmentin was found in the presence of epithelial cells. Ciprofloxacin and gentamycin remained effective. Membrane mucin expression was correlated with relative antibiotic resistance. Cell invasion by *P.mirabilis* and a mucin and cell type related distribution and response difference, indicate bacterial tropism which affects crystal formation and mucosal presence. Bacterial invasion seems to have cell type dependent mechanisms and prolong bacterial survival in antibiotic therapy, giving a new target for therapeutic optimalisation of antibiotic treatment

Introduction

Proteus mirabilis has been designated the most important bacterial agent in the formation of infection stones, both in normal and augmented bladders (14, 23, 26). In the period following augmentation of the bladder the pH rises and stones may be formed consisting of ammonium magnesium phosphate, calcium phosphate and calcium apatite. This stone formation has been attributed to the rise in pH as a result of urea splitting by urease (13) or crystal formation on the bacterial capsule (9, 10). When urine pH rises crystals will form in vitro in normal human urine and in artificial urine above pH 7.3 (11, 16). For crystals to mature into calculi they also must be protected against washout from the bladder. Adhesion of crystals and or bacteria to bladder wall cells and crystal formation inside invaded cells could be of importance. In addition, adhesion/ invasion may be a target in fighting the recurring cycles of infection and stone formation. Mucins such as MUC2 and MUC5AC play a part in the interaction between crystals and cells in our cellular model and are secreted to the cellular surface (22). Their role in the incorporation of bacteria into the cell is as yet unclear. Regular irrigation of the bladder in this respect should have a beneficial effect preventing stone formation by clearing crystals, mucus and bacteria. However patients on clean intermittent catheterization (CIC) appear to be at risk for bladder stone formation (2). Further study of the occurrence of these adhesion/ invasion processes and their dependence on cell/bacterial/ crystal characteristics is warranted.

Enterocystoplasties in children are well suited for this study. Mucus formation, bacteriuria and stone formation in these cystoplasties are common. The cystoplasties are usually constructed to enlarge small non-compliant bladders and consist of an ileal or colonic pouch attached to the remaining bladder (9). Infecting bacteria will encounter multiple types of epithelium that differ in surface characteristics. In girls with both vaginal reconstructions and an augmented bladder the incidence of bladder stones is especially high (23). Therefore bacterial tropism may play a role in enterocystoplasties and differences in adhesion properties could be involved. *P.mirabilis* has been noted to invade intestinal INT 407, HCT-8, Caco-2 and HT-29 cells, Monkey Kidney cells and T24 bladder cells and several other urothelial cell lines in culture, which in some cases can be inhibited (3, 25, 32). For the invasive properties of *P. mirabilis* there are many proposed mechanisms and influencing factors among which LPS (17), hemolysin and urease in the presence of urea (24). Bacterial invasion may give rise to a relative resistance to antibiotics such as aminopenicillins and persistent infection, due to the save haven provided by the epithelial cells. Here we used an enterocystoplasty *in-vitro* model to study the complex of: infection with urease producing bacteria, intra and extra-cellular crystal formation, adhesion to and invasion of epithelial cells, as well as their influence on antibiotic resistance.

Materials and Methods

Bacterial culture

One *Proteus mirabilis* strain was attained from ATCC (49565) and stored in 15% glycerol at -80°C until needed. Bacteria where cultured in Luria broth 0.05% glycerol until late log-phase growth before use. Four *P.mirabilis* strains were isolated from patients with an enterocystoplasty (AB129, AB474, AB780, AB964) using the API- system of identification (**Table** 1) and gram staining and stored in 15% glycerol at -20°C. Genomic DNA was isolated from the 5 strains using the Wizard® Genomic DNA Purification Kit (Promega, Madison WI, USA) and a single primer RAPD-PCR to rule out identical strains. The single 10 nucleotide RAPD PCR primer (5'-GTGGATGCGA-3') is routinely used in strain identification. PCR was carried out in 50 µl

volumes with 5-30 ng genomic DNA, 0.4 u SuperTaq DNA polymerase and SuperTaq buffer (Stratech Scientific Ltd), 0.5mM of each deoxynucleoside triphosphate with 0.5μM primer. At least 4 fragments were amplified for each sample in a GeneAmp PCR System 9700 thermocycler programmed for 5' 94° and 40 cycles of 94° 30'', 25° 30'', 72° 45''. Amplification products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidiumbromide (**Fig**. 1).

Cell culture

We used 5 intestinal cell lines, caco-2, HT-29, HT29-18N2, HT29-FU, HT29-MTX and one ureter cell line, SV-HUC-1 to study the interactions most common in enterocystoplasties. Caco-2 and HT-29 cell lines were obtained from ATCC and have been passaged for an unknown number of times but all cells used were within 15 passages. SV-HUC-1 was obtained from the ATCC and has been used from passage 30 to passage 34 to ensure stability within the cell line. All intestinal cell lines were cultured on Dulbecco's minimal Eagle's medium (DMEM)-high glucose with glutamine, 10% fetal bovine serum (FBS) and 5mM non-essential amino acids (NEAA). SV-HUC-1 was cultured on Ham's F12 supplemented with 10% FBS.

MTT-assay

MTT-assay was performed as described by others to asses cellular survival in artificial urine in the absence of culture medium by measuring hexosaminidase activity (15,16). Cells were cultured in 24 wells plates at 50000 –100000 cells per well for 4 days. The cells were overlaid with artificial urine for time periods between 5 hours and 30 minutes, with or without conditioned medium from a 3 hours *P.mirabilis* culture in Luria broth at a 1:5 broth to urine ratio. The supernatant was removed and 200μl (MTT) thiazolyl blue tetrazolium bromide (Applichem, Darmstadt) was added at 5mg/ml. The cells were incubated for 4 hours with the MTT solution. Then the supernatant was removed and dimethyl-sulphoxide added and shaken for 5 minutes to extract the formazan formed by the cellular metabolism. The supernatant was transferred to a 96 wells flat-bottom plate and light absorption by formazan was measured in a BioRad platereader with a 570nm test wavelength and a 690nm reference wavelength. Similarly comparisons were made between 3 hour incubation with bacterial secretions in Luria broth compared to Luria broth as an artificial urine supplement for 5 strains. All MTT assays were performed in triplicate.

Artificial Urine Composition

Two separate solutions were made and combined at the start of experiments, to prevent premature precipitation. Solution A consisted of: 1.3 g/l sodium citrate, 1.3 g/l KH_2PO_4 , 2.0 g/l sodium oxalate and 25 g/l urea in distilled water. Solution B consisted of: 1.3 g/l $CaCl_2$, 1.3 g/l $MgCl_2$, 9.8 g/l NaCl, 4.6 g/l $NaSO_4$, 3.2 g/l KCl, 2 g/l NH_4Cl and 25 g/l urea in distilled water. Both solutions were combined in equal volumes, less than one hour before the experiments. Artificial urine of pH 6.5 without NaCl, $NaSO_4$ and KCl and with 12.5 g/l urea with a lower osmolarity of 316 mosm was also used as a control for osmotic effects on the cell lines.

Cell Culture infection

Cells were cultured in 6 or 24 wells plates and infected after reaching confluency. For transmission electron microscopy cells were cultured on 100μm melinex (Du Pont Teijin Films

Netherlands BV, Rozenburg, The Netherlands), for confocal laser scanning microscopy on glass coverslips, before infection. Artificial urine was added and incubated for 3 hours to rise above pH 7.3, infected with 150µl bacterial suspension (2.5-3McF ≈ 10^5-10^6 bacteria) for 3ml of artificial urine before microscopic evaluation.

Confocal Laser Scanning Microscopy (C-LSM)

Infected cells were washed three times with DMEM and maintained at 37°C. A rabbit-anti-Human Colonic Mucin (HCM) or rabbit-anti-Human Gastric Mucin (HGM) antibody was applied for 15 minutes and washed three times with DMEM. Anti-HCM and anti-HGM characteristics were previously described by Tytgat e.a.(29). Anti-HCM (Human Colonic Mucin) is a rabbit polyclonal antibody raised against purified human colonic mucin it recognizes mainly the mature, fully glycosylated MUC2. Anti-HGM (Human Gastric Mucin) is a rabbit polyclonal antibody raised against purified human gastric mucin it recognizes mainly the mature, fully glycosylated MUC5AC. A monoclonal antibody against P.mirabilis (Biogenesis, Nuclilab) was added, incubated for 15 min and washed three times with DMEM. A fluorescein-isothiocyanate (FITC) labeled anti-rabbit antibody (Caltac laboratories, Burlingame, CA) was applied, incubated for 15 min, washed thoroughly with DMEM and followed by a thio-rhodamine-isothiocyanate (TRITC) labeled anti-mouse-antibody (DAKO A/S, Denmark). Images were made with a Zeiss LSM 410 laser scanning confocal microscope (Zeiss, Oberkochen, FRD). A 488 nm Ar-laser was used to excite TRITC labeled antibodies and a 633 Kr-laser without a filter to visualize the reflection of the crystals. The TRITC signal was passed through a 510-540 nm band-pass filter. An overlay of the FITC signal was done using the 488 nm laser and a 560 nm beam splitter to separate the FITC from the TRITC signal, showing bacterial infection and crystal formation. Viable stain Syto16 was used instead of anti-mucin antibodies, omitting the last washing step, to show viable epithelial cells and determine bacterial invasion. Experiments were performed in triplicate except bacterial invasion assessment, which was performed twice to confirm previous findings. A second observer evaluated a representative selection of images.

Transmission Electron Microscopy

Cells were cultured on melinex sheet to attain transferable monolayers until confluency and fixed in 1% glutardialdehyde/4% formaldehyde in 0.1M PBS pH 7.2/ 4˚C for at least 2hrs before processing. Detached cells were collected and centrifuged to a pellet before processing. Samples were washed in 0.1M PBS pH7.2/ 4˚C for 12 hours followed by a secondary fixation in 1% osmium tetra-oxide/ 1.5% $K_4Fe(CN)_6$ in 0.1M PBS pH 7.2. Samples were washed twice in distilled water for 30 minutes and dehydrated in ethanol 50%, 50%, 70%, 70%, 90%, 90%, 96%, 96%, for 10 minutes and twice in ethanol 100% for 15 minutes. Samples were impregnated in equal volumes of Epoxy resin (LX112) and ethanol 100% for 60 minutes at room temperature followed by pure Epoxy resin (112) for 60 minutes at 37˚C. Resin was allowed to polymerize for 12 hours at 60˚C after which the melinex sheet was removed.
Sectioning of blocks was performed on a Type LKB IV ultramicrotome at 40 nm and collected on a copper 200 mesh grid. Sections were incubated with 6% uranyl acetate for 10 minutes followed by lead citrate for 1 minute before viewing under a Philips Morgangi ™ 268 transmission electron microscope connected to a CCD camera (Mega view II).

Gentamycin Invasion Assay

Cells were cultured in 24 wells plates until confluency, incubated with *P.mirabilis* in HBSS for 3 hours. Cells were washed twice with HBSS to remove free bacteria and incubated with Gentamycin 0.01% in PBS for 1 hour to kill accessible bacteria. The monolayers were washed 8 times with PBS, to remove all gentamycine and lysed in poly-L-Lysine (Sigma-Aldrich P1524) for 5 min. A 1/10 dilution series was made, plated on blood-agar plates and incubated at 37°C for 24 hrs. Experiments were performed in triplicate to reveal bacterial survival in epithelial cells.

Relative Antibiotic Susceptibility Assay (RASA)

The RASA procedure followed the gentamycin invasion assay except for three major changes. First the incubation time with the antibiotic was increased from 1 to 18 hours. Second, a panel of commonly used antibiotics was applied at concentrations reflecting the calculated expected urinary excretions in pediatric urology for a 10 year old child weighing 30 kg. Amoxicillin: 450 µg/ml, Augmentin: 600 µg/ml Amoxicillin / clavulanic acid 60 µg/ml, Gentamycin: 90 µg/ml Cotrimoxazol: 46 µg/ml trimethoprim /230 µg/ml sulfamethoxazole, Ciprofloxacin 300 µg/ml, Nitrofurantoin: 50 µg/ml, Metronidazole: 20 µg/ml. Third, lysates were plated on McConkey agar instead of blood-agar to enable colony counting and incubated at 37˚C for 24 hours. For a panel of five bacterial strains the resistance against a specific antibiotic (n=8 including DMEM control) was tested both in the presence and the absence of a specific cell-line (n=6). Overall the number of colony forming units, cfu, was counted for 5x2x6x8=480 different situations. For each combination of bacterial strain and antibiotic we calculated the ratio of cfu in the presence (cfu_{cell+}) and absence of cells (cfu_{cell-}), cfu_{cell+}/ cfu_{cell-}. This ratio was considered to be increased at a cut-off point of 10. For the statistical analysis of the differences between the situations cells present and cells absent we used the average of the ratio's obtained for the five bacterial strains in a paired samples T-test. These stringent conditions answer the questions if bacteria can resist antibiotic treatment by invading cells and which antibiotics would still be suitable.

Flowcytometric analysis of infected epithelial cells

All flowcytometric measurements of Human Colonic Mucin, Human Gastric Mucin, caspase3, and bacteria were performed separately using material from the same infected sample. Infected cells were incubated in 70% ethanol for 30' and scraped followed by overnight incubation at room temperature in 2% paraformaldehyde/ 2% formaldehyde to fixate cells and bacteria. Fixed cells were washed twice with PBS and the first antibody in 0.5%BSA/PBS was applied against *P.mirabilis*, Human Colonic Mucin, Human Gastric Mucin, or caspase 3, and incubated for 1 hour at 37˚C. Cells were washed with PBS twice and a secondary FITC labeled antibody was applied and incubated for 1 hour at 37˚C after which the cells were washed twice with PBS before measurement. After the first measurement Trypan Blue was added to quench an intra-cellular FITC signal and incubated for 30' after which cells were washed twice with PBS and measured again. The decrease in signal determines the contribution of membrane bound and intra-cellular signal. 1:200 antibody dilutions were used. Pearson correlation was used to analyze the mean FL-1 count in relation to the RASA.

Results

Bacterial identification

Bacterial strains with similar API codes (**Table** 1) were identified as unique strains in RAPD-PCR (**Fig**. 1).

Confocal Laser Scanning microscopy

In confocal laserscanning microscopy epithelial cells of approximately 7 microns, expanded in size after infection with *P.mirabilis* and a colocalisation of mucins expressed on the cellular membrane and bacterial adhesion was demonstrated for Human Colonic Mucin (HCM) and for Human Gastric Mucin (HGM) (**Fig**. 2C, D). Furthermore intra-cellular crystal formation was demonstrated for all cell lines and intra-cellular invasion confirmed (**Fig**. 2A, B). In the intestinal cell lines cytoplasmic colonies are observed (**Fig**. 2B) opposed to single cell invasion in the ureter cell line (**Fig**. 2A). Both HCM and HGM appeared as granules in cells and membrane associated mucins on the cells, indicating the mature mucin form (**Fig**. 2C,D). Colocalisation of HCM and HGM with *P.Mirabilis* occurred in all cell lines and is visible in **Fig** 2C and D as a yellow/ orange signal. For a better assessment of this colocalisation, the single colocalisation signal is shown in black for the same section areas are in **Fig** 2E and F. Large cells were observed for HT29-18N2 with a large number of cytoplasmic bacteria.

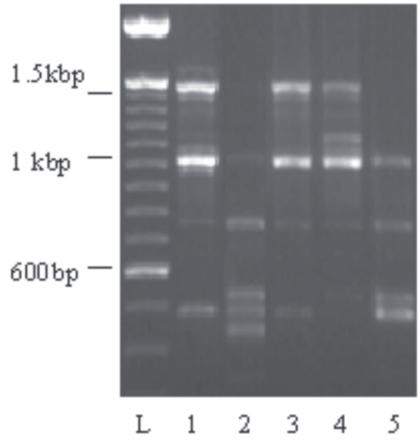


***Fig**. 1*
RAPD –PCR products on 1.5% ethidiumbromide agarose gel. L=100bp Standard, 1= AB129, 2= AB474, 3= AB780, 4= AB964, 5= ATCC49565. All strains have different patterns

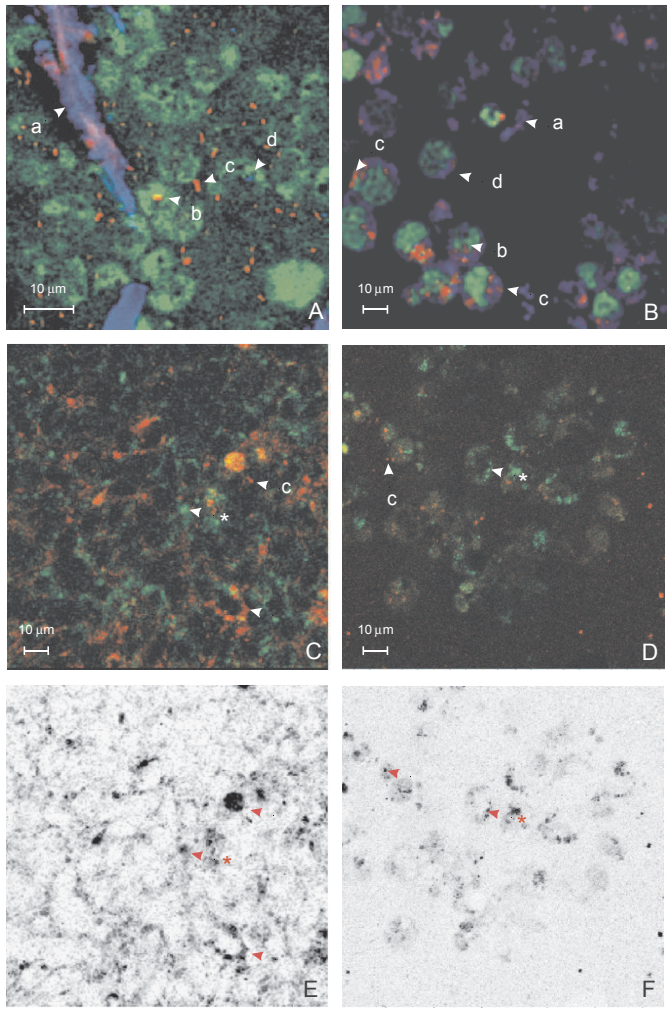


Fig. 2

Confocal Laserscanning Microscopy Images (all images at 800x magnification). Bacteria are always red. A=SV-HUC-1 cell line stained with viable stain Syto 16 with reflecting crystals (blue/purple) . B=HT29-MTX stained with viable stain Syto 16 (green), with reflecting crystals (blue) . The symbols (a), (b), (c), (d), indicate extracellular crystals, intracellular bacteria, extracellular bacteria, and intracellular crystals respectively. C=HT29-MTX cell line showing HCM in green and colocalisation in yellow (=HCM along the cellular membrane, arrow =bacterial /HCM colocalisation). D= HT29-MTX cell line showing HGM in green and colocalisation in yellow (*=HGM without bacterial colocalisation, arrow =HGM with bacterial colocalisation). The yellow colocalisation signal is produced by a simultaneous red TRITC and green FITC signal.*

E= the colocalisation signal of P.mirabilis and HCM of C indicated in black. F= the colocalisation signal of P.mirabilis and HGM of D indicated in black. (Produced in CorelDraw 9.0 for Microsoft ® Windows)

	ATCC49656	AB129	AB474	AB780	AB964
API	0737000	0336000	0737000	0737000	0733000
Amikacin	<=2	<=2	4	<=2	4
Amoxicillin/ Clavulanic acid	<=8	<=8	<=8	<=8	16 (I)
Amphicillin	>=32 (R)	2	4	>=32 (R)	4
Cefotaxime	<=4	<=4	<=4	<=4	<=4
Ceftazidime	<=8	<=8	<=8	<=8	<=8
Cefuroxime-sodium	<=4	<=4	<=4	<=4	<=4
Cefuroxime -axetil	<=4	<=4	<=4	<=4	<=4
Cephalothin	>=32 (R)	4	4	4	4
Ciprofloxacin	<=0.5	<=0.5	<=0.5	<=0.5	<=0.5
Gentamycin	<=0.5	<=0.5	1	<=0.5	1
Imipenem	<=4	<=4	<=4	<=4	<=4
Meropenem	<=2	<=2	<=2	<=2	<=2
Nitrofurantoin	>=128 (R)	>=128 (R)	>=128 (R)	>=128 (R)	>=128 (R)
Norfloxacin	<=4	<=4	<=4	<=4	<=4
Pipercillin/Tazobactam	<=8	<=8	<=8	<=8	<=8
Tobramycin	<=0.5	1	<=0.5	>=0.5	1
Trimethoprim/ Sulfamethoxazole	<=10	<=10	>=320 (R)	>=320 (R)	>=320 (R)

***Table** 1.*

Antibiotic susceptibility of 5 P.mirabilis strains. Resistance (R) and intermediate resistance (I) are indicated. Minimal Inhibitory Concentrations are represented in μg/ml

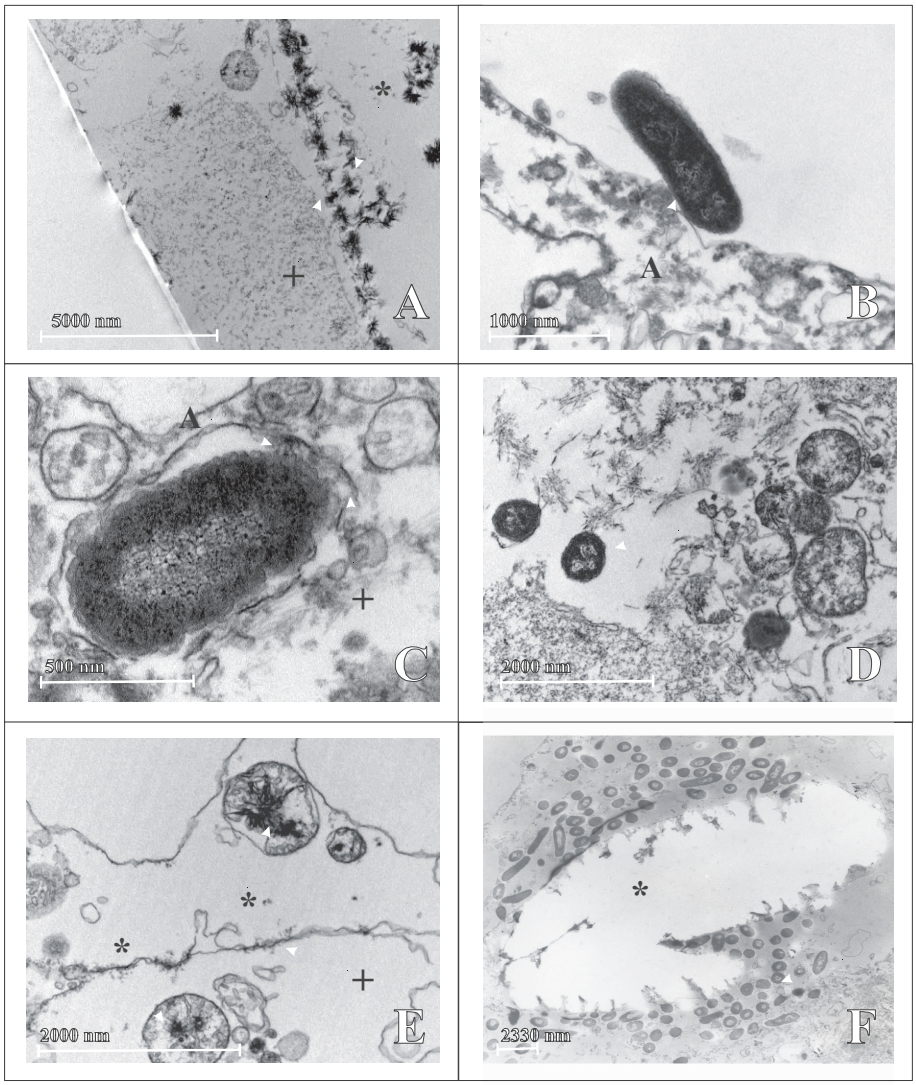


Fig. 3.

Transmission Electron microscopy
A= crystals on SV-HUC-1 cell surface (2800x), B= bacterial adhesion to the cellular surface of HT29-MTX, condition without artificial urine (22000), C= invasion in HT29-FU with double membrane (44000x), D= bacterial invasion in SV-HUC-1, without a double membrane (14000x), E= crystal formation inside cellular organelle of HT29-FU, probably destroyed mitochondrion (18000x).F= Crystal ghost in HT29-MTX , with large cytoplasmatic colony of P.mirabilis surrounding the crystal (3000x). Arrows without any symbols indicate bacteria. Arrows with a symbol (A) indicate bacterial adhesion to the double membrane. The double membrane is indicated by (+), and crystals are indicated by (*). (Produced in CorelDraw 9.0 for Microsoft ® Windows)

Evaluation parameters	Cell line	Culture and infection conditions			
		DMEM	Artificial urine	DMEM *+P.mirabilis*	Artificial urine+ *P.mirabilis*
Crystal adhesion	Caco2	-	+	-	+
	SV-HUC-1	-	-	-	+
	HT29	-	+	-	-
	HT29-1 8N2	-	-	-	-
	HT29-FU	-	-	-	-
	HT29-MTX	-	-	-	+
Intra-cytoplasmatic crystal formation	Caco2	-	+	-	+
	SV-HUC-1	-	-	-	+
	HT29	-	+	-	+
	HT29-1 8N2	-	-	-	+
	HT29-FU	-	+	-	+
	HT29-MTX	-	-	-	+
Intra-mitochondrial crystal formation	Caco2	-	-	-	+
	SV-HUC-1	-	-	-	+
	HT29	-	-	-	-
	HT29-1 8N2	-	-	-	-
	HT29-FU	-	-	-	+
	HT29-MTX	-	-	-	+
Bacterial adhesion	Caco2	-	-	+	+
	SV-HUC-1	-	-	+	+
	HT29	-	-	+	-
	HT29-1 8N2	-	-	-	+
	HT29-FU	-	-	-	+
	HT29-MTX	-	-	+	-
Bacterial invasion	Caco2	-	-	-	+
	SV-HUC-1	-	-	+	+
	HT29	-	-	-	+
	HT29-1 8N2	-	-	-	+
	HT29-FU	-	-	-	+
	HT29-MTX	-	-	-	+

***Table** 2. Transmission electron microscopy results with varying culture and infection conditions. Cell pellets and monolayer cultures were used in infected artificial urine conditions, all other conditions were evaluated in monolayer conditions. (+=present, -=absent)*

Transmission Electron Microscopy

Cellular adhesion (**Fig**. 3B), cytoplasmatic colonies and invasion by *P.mirabilis* surrounded by a double membrane was observed in intestinal cell lines (**Fig**. 3C) with intra- cytoplasmatic crystal formation and crystal adhesion to the cellular membrane. The urothelial cell line showed fewer and always single bacteria invaded into the cells without a double membrane (**Fig**. 3D) and large crystal deposits on and in the cell (**Fig**. 3A). Occasionally intra-mitochondrial (**Fig**. 3E) crystal formation was observed. Results are presented in **Table** 2. Results indicate that bacterial infection in artificial urine of 316 mosm. enhances the formation of crystals (intra-cellular and in mitochondria) in conjunction with enhanced bacterial invasion. For HT29-18N2 large-scale destruction of cells was noted with large cells and bacterial colonies contained by a double membrane. Infected epithelial cells showed loss of nuclear staining and density, indicating damage.

Relative antibiotic susceptibility assay (RASA) and gentamycin invasion assay

After vigorous washing to remove all antibiotic and free bacteria; viable bacteria were found upon cell lysis for all cell lines tested, confirming bacterial invasion. The RASA test showed that this cellular invasion provides the bacteria with a relative protection against the antibiotics, cotrimoxazol and amoxicillin/ clavulanic acid. DMEM acts as a control for bacterial growth without any inhibition in the presence of cells. Amoxicillin acts as a control for the Amoxicillin content in Augmentin, to assess clavulanic acid. Amoxicillin does not show diminished effectivity in the presence of cells and neither Metronidazole nor nitrofurantoin exhibits a significant change in effectivity according to a paired samples T-test, although Augmentin (amoxicillin/ clavulanic acid) does. Ciprofloxacin and gentamycin still seem suitable antibiotics in the presence of epithelial cells.
Results of the Relative antibiotic susceptibility assay are presented in **Table** 3.

MTT-assay

Cell survival decreased in time in the presence of artificial urine instead of normal culture medium. The hexosaminidase activity dropped to approximately 50% in all cell lines with or without the added preconditioned Luria broth in 3 hours. The cells showed a hexosaminidase activity at a 3 hour period which was comparable in conditions with (**Fig**. 4) or without the preconditioned Luria broth. (**Fig**.. 5), although some cell lines with conditioned medium from certain bacterial strains showed differences compared to a blank sample without conditioned medium. These readings were always higher in the Caco-2, HT29, and HT29-FU cell lines and always significantly higher in the HT29-FU cell line, while only significant for the SV-HUC-1 cell line in combination with the ATCC49565 strain. Significant differences are marked in **Fig**. 5.

Flowcytometric analysis of infected epithelial cells

On average over all epithelial cell lines 23% of the bacteria were located intra-cellular. 23.2% was found in Caco-2, 26.7% in SV-HUC-1, 22.7% in HT29, 0.5% in HT29-18N2, 31.8% in HT29-FU, 24.1% in HT29-MTX. The HT29-18N2 values for bacterial invasion were very low due to large-scale destruction of the epithelial cells and high permeability for Trypan Blue, which shows aberrant results for the AB780 strain only. Results are presented in **Fig**. 6. Of all Human Colonic Mucin and Human Gastric Mucin expressed by the cells, most was present on the cellular surface, only a small fraction was contained as granules in the cell. 60%-95% of HCM and HGM was expressed on the cellular surface, but did not show significant differences among cell lines. Of the more then 5000 correlations analyzed between flowcytometric data, RASA and the MTT-assay, the relevant significant results are presented as a meta-analysis in **Table** 4. Caspase 3 expression was not correlated with hexosaminidase activity, bacterial invasion, MUC2 or MUC5AC expression. Hexosaminidase activity was not correlated with bacterial invasion.

		Relative Antibiotic Susceptibility Assay																	
	Strain	DMEM		Amoxicillin		Augmentin		Cotrimoxazol		Ciprofloxacin		Gentamycin		Nitrofurantoin		Metronidazole	
Caco-2	AB129	+	M= 0.60 Se= 0.51 P= 0.305	-	M= 0.80 Se= 0.66 P= 0.294	-	M= 0.80 Se= 0.58 P= 0.242	+	M= 1.0 Se= 0.32 P= 0.034*	-	M= -0.40 Se= 0.68 P= 0.587	-	M= 0.60 Se= 0.40 P= 0.208	-	M= 1.0 Se= 0.63 P= 0.189	+	M= 1.0 Se= 0.32 P= 0.034*
	AB474	+		+		+		+		+		+		+		+	
	AB780	-		-		+		+		-		-		+		+	
	AB964	+		+		+		+		-		+		+		+	
	ATCC 49565	-		+		-		-		-		-		+		-	
SV-HUC-1	AB129	-	M= 0.62 Se= 0.39 P= 0.189	-	M= 0.02 Se= 0.44 P= 0.966	+	M= 1.42 Se= 0.24 P= 0.004*	-	M= 0.02 Se= 0.01 P= 0.178	+	M= -0.18 Se= 0.36 P= 0.646	-	M= 0.02 Se= 0.01 P= 0.178	-	M= 0.02 Se= 0.01 P= 0.178	+	M= 0.02 Se= 0.01 P= 0.178
	AB474	+		+		+		-		+		+		+		-	
	AB780	+		+		+		-		+		+		+		+	
	AB964	-		+		+		+		-		-		-		-	
	ATCC 49565	-		-		+		-		-		-		-		-	
HT29	AB129	+	M= 0.40 Se= 0.24 P= 0.178	-	M= 0.60 Se= 0.24 P= 0.070	-	M= 0.60 Se= 0.40 P= 0.208	-	M= 0.60 Se= 0.24 P= 0.070	-	M= -0.20 Se= 0.73 P= 0.799	-	M= 0.20 Se= 0.66 P= 0.778	-	M= 0.20 Se= 0.86 P= 0.828	+	M= 0.80 Se= 0.37 P= 0.099
	AB474	+		-		+		+		+		+		+		+	
	AB780	-		+		+		+		+		-				+	
	AB964	-		+		-		+		-		+		-		-	
	ATCC 49565	-		+		-		-		-		-		+		-	
HT29-18N2	AB129	-	M= 1.0 Se= 0.44 P= 0.086	-	M= 1.0 Se= 0.44 P= 0.086	-	M= 4.20 Se= 0.58 P= 0.002*	-	M= 0.60 Se= 0.39 P= 0.202	-	M= 0.00 Se= 0.02 P= 1.0	-	M= 0.20 Se= 0.19 P= 0.347	-	M= 0.60 Se= 0.39 P= 0.202	-	M= 0.60 Se= 0.67 P= 0.424
	AB474	+		+		-		-		-		-		-		-	
	AB780	+		+		+		-		-		-		-		-	
	AB964	-		+		+		-		-		-		-		+	
	ATCC 49565	-		-		+		-		-		-		-		-	
HT29-FU	AB129	+	M= 0.80 Se= 0.37 P= 0.099	-	M= 0.40 Se= 0.24 P= 0.178	+	M= 1.0 Se= 0.55 P= 0.142	+	M= 1.20 Se= 0.20 P= 0.004*	-	M= -0.20 Se= 0.49 P= 0.704	-	M= 0.20 Se= 1.02 P= 0.854	-	M= 0.20 Se= 0.86 P= 0.828	+	M= 1.0 Se= 0.55 P= 0.142
	AB474	+		+		+		+		+		+		+		+	
	AB780	-		+		+		+		-		-		-		+	
	AB964	-		-		-		+		-		-		-		+	
	ATCC 49565	+		-		-		+		-		-		+		-	
HT29-MTX	AB129	+	M= 0.20 Se= 0.37 P= 0.621	-	M= 0.00 Se= 0.55 P= 1.0	-	M= 0.20 Se= 0.58 P= 0.749	+	M= 0.80 Se= 0.20 P= 0.016*	-	M= -0.40 Se= 0.68 P= 0.587	-	M= 0.20 Se= 0.86 P= 0.828	-	M= -0.40 Se= 0.75 P= 0.621	+	M= 0.60 Se= 0.51 P= 0.305
	AB474	-		+		+		+		+		+		+		-	
	AB780	-		+		+		+		+		+		-		+	
	AB964	+		-		-		+		-		-		-		+	
	ATCC 49565	-		-		-		-		-		-		-		-	

***Table** 3. Relative antibiotic resistance assay: logarithmic number of cfu's of lysed cells /logarithmic number of cfu's in cell-free controls were divided into two groups <=1 (presented as -) and >1 (presented as +) and used in a binomial test to evaluate possible effectivity of antibiotics. The number of colony forming units were read from McConkey agar plates. The logarithmic colony counts were used in paired T-tests with the corresponding cell-free control to asses P.mirabilis resistance to antibiotic therapy in the presence of cells. M= The mean of paired differences, Se= Standard error of the mean. P=p-value in paired T-test, *=significant in paired T-test (right column gray) Left column gray= possible effective antibiotic in this cell line (binomial test, all controls as negative, samples are positive when colony count exceeds the corresponding control)*

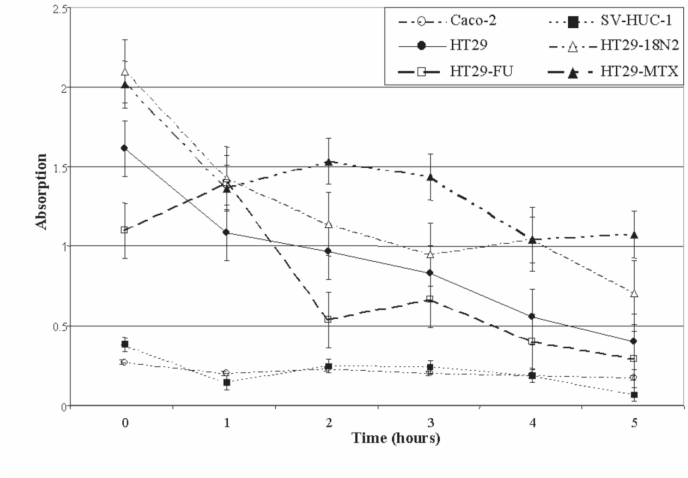


Fig. *4. A) MTT assay of epithelial cells incubated with artificial urine followed in time. Error bars represent the Standard error of the mean.*

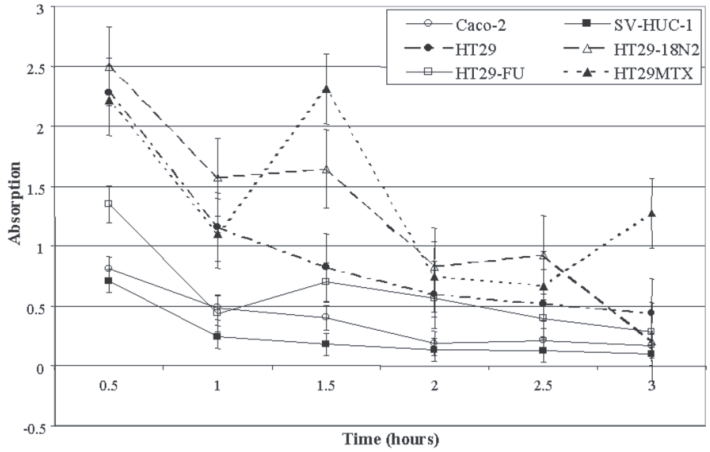


Fig. *4. B) MTT assay of epithelial cells incubated with artificial urine and conditioned Luria broth by P.mirabilis ATCC49565 followed in time. Error bars represent the Standard error of the mean.*

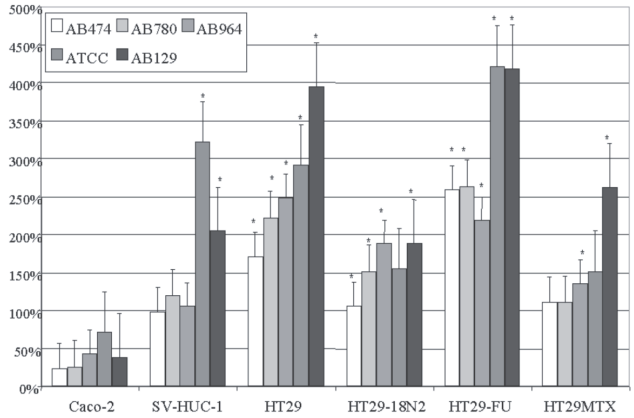


***Fig**. 5. MTT assay of epithelial cells incubated for 3 hours with artificial urine supplemented with conditioned Luria broth by 5 P.mirabilis strains for 3 hours compared with artificial urine and unconditioned Luria broth. Incubation with unconditioned Luria broth and artificial urine for 3 hours was set as 100%. Values over 100% outside the standard error bars are indicated as significant (*). Histograms represent average values of three MTT-assays. Error bars represent the Standard error of the mean.*

First Factor		Direction of correlation	Second Factor		*p-value*
	Cell-line			Antibiotic	
MUC5AC	HT29-MTX	negative	Bacterial invasion	-	0.048
Human Colonic Mucin	HT29-MTX	negative	Bacterial invasion	-	0.023
MUC5AC	HT29-MTX	negative	Bacterial survival	Ciprofloxacin	0.025
MUC5AC	HT29-MTX	negative	Bacterial survival	Gentamycin	0.042
Hexosaminidase	HT29-MTX	positive	Bacterial survival	Metronidazole	0.029
MUC5AC	HT29	positive	Human Colonic Mucin	-	0.049
Caspase3	HT29	positive	Bacterial survival	DMEM	0.007
Caspase3	HT29	negative	Bacterial survival	Amoxicillin	0.007
MUC5AC	SV-HUC-1	positive	Bacterial invasion	-	0.042
Caspase3	SV-HUC-1	positive	Bacterial survival	Nitrofurantoin	0.008
Bacterial invasion	SV-HUC-1	positive	Bacterial survival	Metronidazole	0.032
Human Colonic Mucin	Caco-2	negative	Hexosaminidase	-	0.038
Human Colonic Mucin	Caco-2	positive	Bacterial survival	Amoxicillin	0.006
Bacterial invasion	HT29-18N2	negative	Bacterial survival	DMEM	0.026
Bacterial invasion	HT29-18N2	negative	Bacterial survival	Amoxicillin	0.027
Bacterial invasion	HT29-18N2	negative	Bacterial survival	Augmentin	0.027
Bacterial invasion	HT29-18N2	negative	Bacterial survival	Ciprofloxacin	0.025
Bacterial invasion	HT29-18N2	negative	Bacterial survival	Metronidazole	0.027

***Table** 4. Meta-analysis of infection and survival data. Bacterial invasion, Caspase 3 expression, MUC5AC and Human Colonic Mucin were determined by Flowcytometric Analysis. Bacterial survival was determined by RASA and the Hexosaminidase activity was determined by the MTT-assay. The meta-analysis of data was performed by Pearson correlation. Correlations were significant ($p<0,05$) in a 2-tailed test.*

Discussion

Conditions as they exist in the augmented bladder, especially the presence of intestinal cells and their secretions in the presence of urine, enhance bacterial infection and invasion. Often *Proteus mirabilis* infection of the augmented bladder is difficult to treat and leads to stone formation. *P.mirabilis* can invade epithelial cells and survive intracellular (3,5,25,32,33,34). It is not known if this effects antibiotic effectivity. We studied the effect of bacterial invasion by *Proteus mirabilis* on antibiotic susceptibility and intracellular crystal formation in conditions modeled to enterocystoplasties. The relative antibiotic susceptibility assay was developed in artificial urine for this purpose. Artificial urine is a useful defined culture medium for the growth of *P.mirabilis (4)*. The meta-analysis of a number of control parameters for bacterial survival in cells including the MIC values help to evaluate the data gathered from our relative antibiotic susceptibility assay. The data allow us to propose the scheme for bacterial invasion/ survival shown in **Fig** 7. Interventional studies must be performed to test the validity of the scheme

In this study artificial urine enhances invasion of bacteria and intra-cellular crystal formation. The presence of crystals in cellular organelles i.e. intra-mitochondrial crystals confirms their intra-cellular origin. The large Ca^{2+} stores in mitochondria and the endoplasmatic reticulum (21,31) may explain the cellular location of crystals when the pH level rises above 7.3 and the solubility of calcium phosphate is exceeded. Mitochondrial damage may then lead to increased cytosolic calcium levels and precipitation at other cell sites. Bacterial activity and mechanical damage by crystals eventually leads to nuclear destruction and a disruption of calcium levels (12,21) with a more diffuse distribution of crystals throughout the epithelial cytoplasm as was observed in electron microscopy. The rise in pH level was caused by urease producing *P. Mirabilis* strains. These strains invaded the cells and formed a micro-environment, which supported the formation of crystals.

We hypothesized that micro-colony formation inside epithelial cells protects against antibiotic treatment. Especially for antibiotics which have a concentration dependent activity, a low membrane permeability or for which *P.mirabilis* has already acquired a relative resistance. *In vivo* epithelial invasion would create a bacterial pool from which the next cycle of infection can originate. An additional double membrane surrounded bacteria in the intestinal cell lines providing an extra barrier for antibiotics to pass in addition to the cellular cytoplasm, suggesting that *P.mirabilis* was better protected inside intestinal cells. The RASA test (where extracellular bacteria were removed) shows that intracellular bacteria better survive treatment with the antibiotics cotrimoxazol and amoxicillin/ clavulanic acid, for which one or more strains were already (relatively) resistant. Invasion was confirmed in flowcytometric data. On average 23% of the bacteria were located inside intact cells for all cell lines tested accept HT29-18N2, that showed massive cell destruction. This may be beneficial for the epithelial cells as increased cell membrane permeability makes *P.mirabilis* lose their safe haven, as in HT29-18N2.

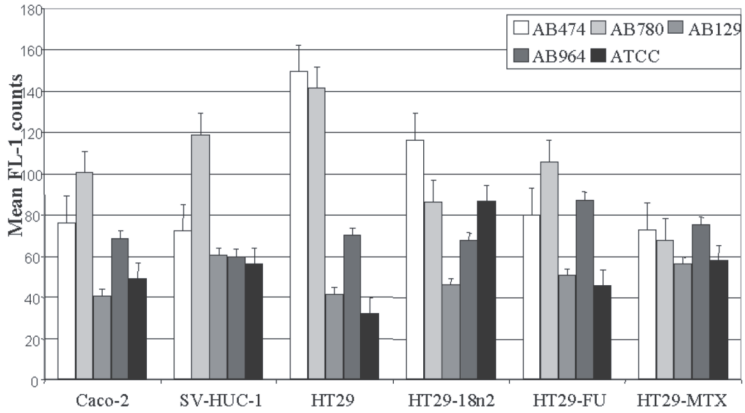


***Fig**. 6 A) Flowcytometric analysis of caspase 3 expression in epithelial cells infected with 5 P.mirabilis strains. Error bars represent the Standard error of the mean.*

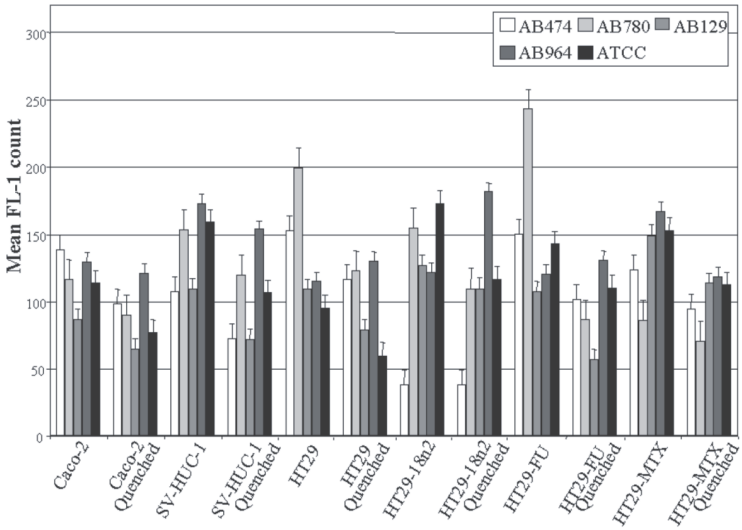


Fig. 6 *B) Bacterial adhesion (quenched) and total bacterial infection of epithelial cells. Bacterial invasion was regarded to be the difference between the quenched (extra-cellular) data and the total P.mirabilis infection. Error bars represent the Standard error of the mean.*

Self-destruction could be a way for the cells to deprive the bacteria of their protection. In infected cells mitochondrial swelling and destruction was visible. This suggests an apoptosis mechanism involving the radical oxygen species (ROS) →cytochrome c →caspase 9 →caspase 3 pathway (28).
Expression of caspases due to infection would decrease bacterial survival for amoxicillin in HT29 but according to our data does not significantly increase bacterial invasion. Only in control antibiotics and DMEM did caspase 3 correlate with increased bacterial survival. This suggests that cellular membrane permeability is increased. Overall neither a significant effect of apoptosis on RASA or a direct correlation between caspase 3 and bacterial invasion was found. It may be that *P.mirabilis* counteracts the effect of apoptosis as it produces an iron dependent superoxide dismutase that handles radical oxygen species (ROS). Also, caspase 3 can promote nuclear membrane permeability without increasing cellular permeability (12) as is observed in electronmicroscopy.
The presence of artificial urine and absence of normal culture medium affected the cellular metabolism and increased the invasion of epithelial cells. However bacterial secretions that constitute a stress-situation for the cell only marginally effected the metabolism of epithelial cells. When invasion is very efficient, like for the HT29-18N2 cell line, there is rapid cell destruction. Thus we investigated the cellular reaction to invasion, using hexosaminidase activity as a marker for cellular survival and metabolic activity (6, 8). Hexosaminidase, a detoxification enzyme (15) in the lysosomal degradative pathway, may be activated by proinflammatory stimuli (27). When hexosaminidase function was enhanced, lysosomal degradation and bacterial survival with Metronidazole increased.

Epithelial mucins have a dual effect in the invasion of *P.mirabilis,* depending on the cell line. In HT29-MTX bacterial invasion is decreased by MUC2 and MUC5AC and colony formation occurs, which could indicate phagocytosis of the cellular membrane with the bacteria in which the mucins act as a bacterial barrier. In the ureter cell line SV-HUC-1 bacterial invasion occurs as single bacteria and is positively correlated with MUC5AC expression indicating a receptor function. Mucins such as MUC-2 could also function as a barrier to antibiotics such as amoxicillin in Caco-2. Bacterial secretions seem to induce metabolic activity in the cells, which may help the destruction of *P.mirabilis* and detoxification of antibiotics. It seems that excretions from *P.mirabilis* induce a cellular response in certain HT29 cell lines that requires a higher metabolic activity of the epithelial cell. The HT29, and HT29-FU subclones and Caco-2 that are activated in the presence of bacterial secretions are known to produce relatively more MUC2 and MUC5AC (22). Mucin production may be involved as a defense mechanism against interaction with *P.mirabilis* LPS as has been reported for other bacteria (21) and may be regulated similarly (19). Recycling of the cellular membrane, unrelated to the bacterial agent incorporates substances from the cellular membrane such as mucins, as described for MUC1 (1). On the other hand mucins may inhibit phagocytosis by polymorphonuclear leukocytes in the lung (30). It is not clear from this study how different mucin types interact at a molecular level with bacteria, but there seems to be a difference in the invasion capability of *P.mirabilis* according to the type of epithelium i.e. urothelium or intestinal epithelium related to mucin expression.

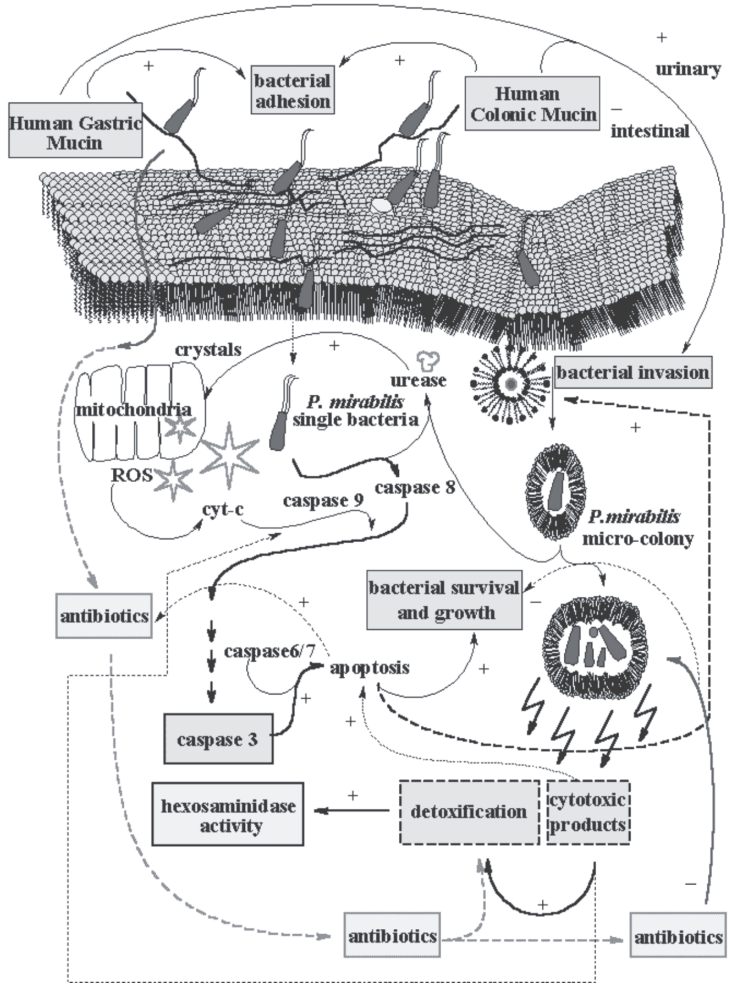


***Fig**. 7 Schematic presentation of our interpretation of the data and meta-analysis. The diagram shows bacterial adhesion and invasion of epithelial cells with possible mechanisms of influencing factors on the outcome of the relative antibiotic susceptibility assay. Both bacteria and crystals can start apoptosis by inducing cellular stress. Bacterial invasion is either inhibited or enhanced by adhesion to HGM or HCM. Bacterial invasion and apoptosis may enhance antibiotic effectivity by increasing the permeability of the cellular membrane after increasing the permeability of the nuclear membrane. Mitochondrial damage will enhance the bacterial induced apoptosis of the epithelial cell. P.mirabilis will invade the cell and form microcolonies, protected from antibiotics in the cytoplasm by the microenvironment formed within the double membrane formed in intestinal cell lines. In SV-HUC-1 single bacteria are found without the protection of a double membrane (left), whereas in the intestinal cell lines a double membrane was found surrounding invaded P.mirabilis bacteria and microcolonies (right).*

Conclusion

The overall interpretation of our data is depicted in **Fig**. 7. Bacterial invasion in epithelial cells is enhanced by the enterocystoplasty environment with urine, mucin and intestinal cells. The invasion into urothelial and intestinal cells show different aspects, resulting in a single membrane respectively double membrane protection. This protection induces a relative resistance of *P. mirabilis* to the antibiotics Cotrimoxazol (TMP/SMX) and Amoxicillin/ clavulanic acid. Gentamycin and ciprofloxacin are the best antibiotics in the presence of epithelial cells and nitrofurantoin also seems more effective in the presence of cells. *P.mirabilis* thrives better inside the cell when there are antibiotics in the culture medium and better outside the cell when there is no selection pressure from antibiotics. Resistance to Cotrimoxazol and of course nitrofurantoin are also often found clinically (7). Resistance to Amoxicillin/ clavulanic acid and ciprofloxacin is less frequent. *P.mirabilis* appears to interact with mucins such as MUC2 and MUC5AC in the adhesion to epithelial cells preceding invasion, but is not necessarily internalized with these mucins. These mucins act differently according to the origin of the cell type and may relate to the different microbiological function of intestinal and urinary epithelium. Human Colonic Mucin is equivalent to the mature form of MUC-2 and Human Gastric Mucin is equivalent to the mature form of MUC-5AC (29). *P.mirabilis* appears to reproduce more effectively in intestinal cells than in ureter cells by the formation of cytoplasmic colonies. This may help explain the persistence of *P.mirabilis* in enterocystoplasties under antibiotic therapy. The infection stones in the augmented bladder may start as intra-cellular crystals (approximately 7 microns according to CLSM) at the surface of the bacterial capsule. These crystals are protected from wash out by voiding and also enhance bacterial survival when they reach a larger size, according to the generally accepted safe-haven theories for biofilms and urinary calculi.
Further research, e.g. on interactions of bacterial molecules with MUC2 and MUC5AC, must show if fighting cell invasion can provide a more effective treatment of *P.mirabilis* infection.

Acknowledgement

The authors wish to acknowledge the expert assistance of Dr. P. Van den Heul and especially ing. A.W. De Jong with the Transmission Electron Microscopy sectioning and imaging and Dr. A. Houtsmüller for his assistance with confocal laserscanning microscopy imaging. ing. C. van der Schee provided assistance with the RAPD-PCR. Dr. Hop provided assistance with statistical analysis of data. Anti-HCM and anti-HGM were gifts from Dr.A.W.C. Einerhand at the dept. of pediatrics at the Erasmus University Rotterdam. The HT29-FU and HT29-MTX were gifts from Dr. Lesuffleur (INSERM U505, Centre de recherché Biomédicales des cordeliers, Paris, France), HT29-18N2 was a gift from Daniel Louvard (UMR 144 CNRS-Institut Curie, 25 rue d'Ulm, 75248 Paris, Cedex 05).

References

1. Altschuler, Y., C.L. Kinlough, P.A. Poland, J.B. Bruns, G. Apodaca, O.A.Weisz, R.P. Hughey. 2000. Clathrin-mediated endocytosis of MUC1 is modulated by its glycosylation state. Mol Biol Cell. 11: 819-831
2. Barroso, U., Jr., R. Jednak, P. Fleming, J.S. Barthold, R. Gonzalez. 2000. Bladder calculi in children who perform clean intermittent catheterization. BJU Int. 85: 879-884
3. Braude, A.I., J. Siemienski. 1960. Role of bacterial urease in experimental pyelonephritis. J. Bact. 80: 171-179
4. Brooks, T., C.W. Keevil. 1997. A simple artificial urine for the growth of urinary pathogens. Lett. Appl. Microbiol. 24: 203-206
5. Chippendale, G.R., J.W. Warren, A.L. Trifillis, H.L.T. Mobley. 1994. Internalization of Proteus mirabilis by human renal epithelial cells. Infect Immun. 62; 3115-3121
6. Cole, S.P.C. 1986. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother. Pharmacol. 17; 259-263
7. Daza, R., J. Gutierrez, G. Piedrola. 2001. Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections. Int J Antimicrob Agents. 18: 211-215
8. Denizot, F., R. Lang. 1986. Rapid colorimetric assay for cell growth and survival., J.Immunol. Meth. 89: 271-277
9. Duel, B.P., R. Gonzalez , J.S. 1998. Barthold, alternative techniques for augmentation cystoplasty. J. Urol. 159: 998-1005
10. Dumanski, A.J., H. Hedelin , A. Edin-Liljegren, D.Beauchemin , R.J.C. Mc Lean. 1994. Unique ability of the Proteus mirabilis capsule to enhance mineral growth in infectious urinary calculi. Infect Immun. 62: 2998-3003
11. Ebisuno, S., T. Komura, K. Yamagiwa, T. Ohkawa. 1997. Urease induced crystallizations of calcium phosphate and magnesium ammonium phosphate in synthetic urine and human urine. Urol Res. 25: 263-267
12. Faleiro, L., Y. Lazebnik. 2000. Caspases disrupt the nuclear-cytoplasmic barrier., J Cell Biol 151: 951-959
13. Griffith, D.P. 1978. Struvite stones. Kidney Int. 13: 372-382
14. Kaefer, M., M.S. Tobin, W.H. Hendren, S.B. Bauer, C.A. Peters, A. Atala, A.H. Colodny, J. Mandell, A.B. Retik. 1997. Continent urinary diversion: the Children's Hospital experience. J Urol 157: 1394-1399
15. Karkkainen, P., M. Salaspuro. 1991. beta-Hexosaminidase in the detection of alcoholism and heavy drinking. Alcohol Alcohol Suppl 1: 459-464
16. Kok, D.J., J. Poindexter, C.Y.C. Pak. 1993. Calculation of titratable acidity from urinary stone risk factors. Kidney Int. 44: 120-126
17. Korn, A., Z. Rajabi , B. Wassum , W. Ruiner , K. Nixdorff. 1995. Enhancement of uptake of lipopolysaccharide in macrophages by the major outer membrane protein OmpA of gram-negative bacteria. Infect Immun. 63: 2697-2705
18. Leite, A.Z., A.M. Sipahi, A.O. Damiao, A.M. Coelho, A.T. Garcez, M.C. Machado, C.A. Buchpiguel, F.P. Lopasso, M.L. Lordello, C.L. Agostinho, A.A. Laudanna. 2001. Protective effect of metronidazole on uncoupling mitochondrial oxidative phosphorylation induced byNSAID: a new mechanism. Gut 48: 163-167
19. Li, D., M. Gallup, N. Fan, D.E. Szymkowski, C.B. Basbaum. 1998. Cloning of the amino-terminal and 5'-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. J Biol Chem. 273: 6812-6820
20. Li, J.D., W. Feng, M. Gallup, J.H. Kim, J. Gum, Y. Kim, C. Basbaum. 1998. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells. Proc. Natl. Acad. Sci. U S A. 95: 5718-5723
21. Luo, Y., J.D. Bond, V.M. Ingram. 1997. Compromised mitochondrial function leads to increased cytosolic calcium and to activation of MAP kinases. Proc. Natl. Acad. Sci. 94: 9705-9710
22. Mathoera, R.B., D.J. Kok, W.J. Visser, C.M. Verduin, R.J.M. Nijman. 2001. Cellular membrane associated mucins in artificial urine as mediators of crystal adhesion: an in vitro enterocystoplasty model. J Urol. 166: 2329-2336

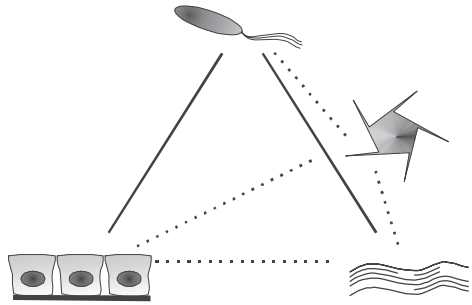
23. Mathoera, R.B., D.J. Kok, R.J.M. Nijman. 2000. Bladder calculi in augmentation cystoplasty in children. Urology 56: 482-487
24. Mobley, H.L.T., G.R. Chippendale, K.G. Swihart, R.A. Welch. 1991. Cytotoxicity of the HpmA hemolysin and urease of Proteus mirabilis and Proteus vulgaris against cultured human renal proximal tubular epithelial cells. Infect Immun 59: 2036-2042
25. Oelschlaeger, T.A., B.D. Tall. 1998. Uptake pathways of clinical isolates of Proteus mirabilis into human epithelial cell lines. Microb Pathog. 21: 1-16
26. Schwartz, B.F., M.L. Stoller. 2000. The vesical calculus.Urol. Clin. North Am. 27: 333-346
27. Shikhman, A.R., D.C. Brinson, M. Lotz. 2000. Profile of glycosaminoglycan-degrading glycosidases and glycoside sulfatases secreted by human articular chondrocytes in homeostasis and inflammation. Arthritis Rheum 43: 1307-1314
28. Skulachev, V.P. 1998. Cytochrome c in the apoptotic and antioxidant cascades. FEBS Lett. 423: 275-280
29. Tytgat K.M., L.W. Klomp, F.J. Bovelander, F.J. Opdam, A. Van der Wurff, A.W. Einerhand, H.A. Buller, G.J. Strous, J. Dekker, 1995.Preparation of anti-mucin polypeptide antisera to study mucin biosynthesis. Anal Biochem. 226: 331-341.
30. Van Klinken, J.W., A.W.C. Einerhand, H.A. Büller, J. Dekker. 1998. Strategic biochemical analysis of mucins. Anal. Biochem. 265: 103-116
31. Vishwanath, S., R. Ramphal, C.M. Guay, D. DesJardins, G.B. Pier. 1988. Respiratory-mucin inhibition of the opsonophagocytic killing of Pseudomonas aeruginosa. Infect Immun. 56: 2218-2222
32. Waters, S.L., J.K. Wong, R.G. Schnellmann. 1997. Depletion of endoplasmic reticulum calcium stores protects against hypoxia- and mitochondrial inhibitor-induced cellular injury and death. Biochem Biophys Res Commun. 240: 57-60
33. Wells, C.L., R.P. Jechorek, K.M. Kinneberg, S.M. Debol, S.L. Erlandsen. 1999. The isoflavone genistein inhibits internalization of enteric bacteria by cultured Caco-2 and HT-29 enterocytes. J Nutr. 129: 634-640
34. Wells, C.L., E.M. van de Westerlo, R.P. Jechorek, S.L. Erlandsen. 1996. Intracellular survival of enteric bacteria in cultured human enterocytes. Shock 6: 27-34

Additional comment

The invasion of epithelial cells and cellular stress may be involved in a number of cellular reactions which can enhance the production of mucins and thereby increase the effect of crystal adhesion. The effect on antibiotic resistance may play an important role in the persistence of an infection and requires a first step of bacterial adhesion in the process of invasion. How mucins are involved in this process will be further discussed in the next chapter.

Chapter 6

Mucin mediated cellular adhesion and crystal formation by Proteus mirabilis in an enterocystoplasty infection stone model.



(Submitted)

Abstract

Introduction:
Proteus mirabilis infection of the surgically augmented bladder is a well known event leading to stone formation. We evaluated how bacterial adhesion may be involved in stone formation using an enterocystoplasty tissue model.

Materials and methods:
Intestinal and bladder tissue cultures were infected to resemble an early *P.mirabilis* infection. Paraffin sections were double stained to evaluate bacterial, mucin and crystal colocalisation. Scanning Electron Microscopy (SEM) and Von Kossa staining evaluated phosphate salt precipitation on bacteria. Far-Western analysis of mucin adhesion to the *P.mirabilis* capsule was done for 8 augmented bladder strains and 8 normal urinary strains. Enterocystoplasty urine untreated, or treated with neuraminidase or periodate was used in Far Western analysis or preceded by a sialic acid, or MUC3 block.

Results:
Correlations between tissue type, bacterial adherence to MUC2 producing cells and crystal formation were found. Bacterial/mucin colocalisation could be clustered according to tissue type. All strains bound Human Gastric Mucin (HGM) and Human Colonic Mucin (HCM). Far-western analysis showed sialic acid dependent binding of HCM and HGM. Furthermore HCM adhesion depended on intact sugar O-rings. Von Kossa staining of sections and SEM showed phosphate salt precipitation on the bacterial surface in intestinal crypts.

Discussion:
Bacterial adhesion in enterocystoplasties is specifically mediated by HGM (mainly consisting of MUC5AC) and HCM (mainly consisting of MUC2) presence. *P.mirabilis* strains derived from enterocystoplasties prefer HCM instead of HGM binding. This may lead to an increased risk for bladder stone formation in enterocystoplasties. Bacterial adhesion can be blocked by other mucins and sialic acid.

Introduction

In recent years several attempts have been made to either confirm or reject the hypothesis that mucins are adhesion molecules for *Proteus mirabilis*. Some aimed at chemotaxis[22], others at the interaction between epithelial cells and *Proteus mirabilis* in general and enteric binding and endocystosis of bacteria[32,35,37,38,39,40], but most have focused on bacterial pili and fimbriae[29]. As *P.mirabilis* is the most important bacterial agent in the formation of infection stones, both in normal and augmented bladders[16,20,29], it is important to establish the presence or absence of this interaction as it relates to the production of certain mucin types by intestinal tissue. It has been suggested that not only urease[10] but also the bacterial capsule[9] could play an important role in the formation of stones by acting as a template for the crystallization of ammonium magnesium phosphate. Similar claims, that they can serve as crystallization templates have been made for several mucins[17, ,28,31,34]. This crystal/mucin interaction explained crystal agglomeration in a previous study[21]. A possible bacterial/mucin interaction may have similar agglomeration enhancing characteristics. GroEL, present on the bacterial capsule, may play a role in this interaction[13,12,15,]. This was previously described for other bacteria after exposure to salts or other stressors[5] and found in *P.mirabilis* in a previous study[19]. When the pH of urine rises, ammonium magnesium phosphate, calcium phosphate and calcium apatite crystals will form in vitro in normal human urine and calcium phosphate crystals in artificial urine above pH 7.3 [10,14,18,23] . For the formation of bladder calculi, often called infection stones or triple phosphate stones, it is necessary that the pH level reaches such high levels in order for these crystals to form and that these crystals remain in the bladder and mature into detectable bladder calculi. Bacterial adhesion and invasion of epithelial cells may be elemental in this crystallization and adhesion process. We have previously shown that bacterial adhesion and invasion may lead to a relative antibiotic resistance and to the formation of small crystals on and in the epithelial cells. Many of these crystals initially share the same size as *P.mirabilis* and could be the result of calcium precipitation on the bacterial capsule. Adhesion prevention may thus be a target in stone and infection prevention in enterocystoplasty patients; it may also enhance antibiotic treatment or even replace antibiotic prophylaxis strategies.

The incidence of symptomatic urinary tract infection correlates with the incidence of urinary stones in enterocystoplasties[20]. In complicated urinary tract infections, the number of unusual urinary pathogens increases. It is not clear however, why the incidence of induced stones is directly correlated with the relatively low incidence of *P.mirabilis* infections despite the fact that other (urease producing) pathogens cause the majority of infections. This would imply that *P.mirabilis* infections lead to stone formation more effectively compared to infections caused by other bacteria. Further study of the adhesion/ invasion process for *P.mirabilis* is therefore warranted. Most of the enterocystoplasty patients are burdened by bacteriuria and multiple urinary tract infections, mucus and stone formation. Thus, enterocystoplasties in children are well suited for this study by supplying a situation where infection stone formation is more frequent than in the general population and easily studied. In children the cystoplasties are usually constructed to eleveate small non-compliant bladders. They consist of an ileal or colonic pouch attached to the remaining bladder [8,16,20]. These epithelia differ in surface characteristics and are represented in our model systems. Here we used an *in-vitro* enterocystoplasty model, to study bacterial/epithelial interaction.

Materials and Methods

Bacterial culture and preparation

One *Proteus mirabilis* strain was obtained from the ATCC (49565), a O:6 serotype strain and stored in 15% glycerol at -80°C until needed. Bacteria where cultured in Luria broth 0.05% glycerol until late log-fase growth before use. Seven *P.mirabilis* strains were isolated from children with an enterocystoplasty (AB129, AB142, AB364, AB474, AB520, AB780, AB964) and identified using the API- system of identification. In addition seven strains were isolated from adult patients with normal bladders (U101, U103, U124, U238, U366, U530, U679) and stored in 15% glycerol supplemented Luria broth at -20°C until needed. One strain was obtained from a patient with a bladder stone in an enterocystoplasty (S468). The origin of the bacterial strains used including their biochemical profiles (API-codes) are given in **Table**.1.

Bacterial FITC labelling

Bacterial FITC labeling and incubation protocols were adapted from a previously published protocol for *H.pylori* [11]. All *P.mirabilis* strains were cultured overnight on bloodagar after which the bacterial cells were collected in Eppendorf tubes from both the swarming edge as well as the center of the colonies and suspended in sterile water, transferred to Eppendorf tubes and pelletted to approximately 1 gram pellets. The pellets were resuspended in 1ml of 0.15M NaCl/ 0.1M sodium carbonate pH 9.0 supplemented with 10 µl of a 10 mg/ml flourescein isothiocyanate (FITC, Sigma) solution and incubated for 1 hour at room temperature in the dark. The bacteria were recovered by centrifugation at *3000 x g* and washed for 5 times in PBS/ 0.05% Tween 20. The intensity of 10 µl spots of labeled bacterial suspensions was checked under a fluorescence microscope. Undiluted labeled bacterial strains were stored at -20°C as a stock solution. 50µl aliquots were diluted in 1 ml PBS before use and only refrozen once to serve as a working dilution.

Bacterial Capsule preparation

Bacterial capsules were isolated from an overnight culture of P.mirabilis grown in Luria broth at 37°C. Bacteria were collected by centrifugation at 1500x *g* for 10 minutes at 4°C in an Eppendorf 5804R centrifuge and transferred to 25 ml 1 M TRIS-HCl pH 7, for every 100 ml broth. The bacteria were sonicated for 3 minutes at a peak to peak amplitude of 9 micron in a Soniprep sonicator. After sonication, bacterial capsules were separated from intact bacteria by differential centrifugation at 500x *g* rpm for 20 minutes, removing the bacterial pellet, followed by centrifugation at 1700x *g* for 1 hour to isolate the bacterial capsules. The capsular rich material was resuspended in 1 ml PBS.

In-vitro enterocystoplasty infection model

Bladder and ileal tissues were taken peroperatively and frozen at -80°C until use. For each experiment tissue cultures from enterocystoplasty tissues were cultured on solidified 0.5% low melting point agar (Gibco) in DMEM supplemented with 20% FBS and overlaid with DMEM for 24-48 hours. Functionality of the tissues was indicated by the discoloration of the agar without microbiological overgrowth. Intestinal and bladder tissue cultures were thus prepared and subsequently infected with a 1:10 dilution of a 18 hour *Proteus mirabilis* (ATCC 49565) culture in Luria broth that was centrifuged and resuspended in centrifuged patient urine. Tissues

were thus incubated with bacteria in urine for 4 hours at 37°C. In this manner intestinal and bladder tissues from 7 and 7 patients respectively were infected with a single strain of *P.mirabilis* (ATCC49565). In addition, sections of ileal tissue from a single patient was likewise infected with all *P.mirabilis* strains. Following infection with *P.mirabilis* tissue cultures were fixed in 4% formaldehyde for 2 days and paraffin embedded.

Immunohistochemistry

Lipopolysaccharide (LPS) - Alcian Blue (AB) staining of infected ileal sections

Tissues were sectioned at 5 μm and deparaffinized in xylene and dehydrated in alcohol for 10 minutes before endogenic peroxidase blocking in methanol /3% H_2O_2 and antigen enhancement in 0.05 M pH 6.0 citrate buffer using a microwave at 700W for 15 minutes. Sections were washed in PBS for 5 minutes and blocked in 30 minutes in 1x Teng-T made from a 10x stock solution in warm water (100 mM Tris, 50 mM EDTA, 1.5M NaCl, 2.5% gelatin, 0.5% Tween 20) and adjusted to pH 8.0 . Blocked sections were rinsed 3 times in PBS/ 0.1% Tween 20 (PBST) and once in PBS. The primary polyclonal sheep antibody against the LPS core region (Nuclilab, Biogenesis, 5684-1000) was applied in 5% BSA / PBST for 1 hour at a 1:80 dilution and washed 3 times with PBST. A indirect immunostaining protocol was used to stain the core region of the bacterial LPS as a general bacterial staining method. A rabbit-anti-sheep-HRP antibody was applied for 30 minutes at a 1:50 dilution in 5% BSA/ PBST and washed 3 times with PBS. Sections were incubated for 15-20 minutes with AEC (3-amino-9-ethylcarbazole) as chromogen. Sections were washed 2 times in PBS and once in distilled water. Alcian Blue 8GX 1% in 3% acetic acid was applied for 20 seconds and rinsed with tap-water for 30 seconds. Sections were washed in 3% acetic acid for 10 minutes and 2 times in distilled water for 5 minutes, air-dried and mounted with pertex.

Proteus mirabilis –Mucin specific staining of infected ileal sections

Proteus mirabilis and mucin specific staining was used to evaluate and count specific interactions. Tissues were sectioned at 5 μm and deparaffinized in xylene and dehydrated in alcohol for 10 minutes before endogenic peroxidase blocking in methanol /3% H_2O_2 and antigen enhancement in 0.05 M pH 6.0 citrate buffer using a microwave at 700W for 15 minutes. An alkaline phosphate staining procedure was used to stain MUC2 or MUC5AC at 1:80 primary antibody dilution for 1 hour. Followed by three times 5 min. washing with PBS/0.1% Tween20 and application of an alkaline phosphatase secondary labeled antibody for 45 min. at a 1:50 dilution. Followed by three times washing with PBS and staining using Fast-Blue RR (Sigma-Aldrich) as a chromogen. Slides were blocked using goat-anti-mouse- immunoglobulins (DAKO A/S) at a 1:50 dilution for 30 minutes. A peroxidase-anti-peroxidase protocol was used to stain the *Proteus* bacteria using a 1:100 dilution of the first anti-*Proteus mirabilis*-antibody (Biogenesis, Nuclilab) overnight at 4˚C, an anti-mouse antibody (DAKO) at 1:50 dilution for 30 minutes, 1:300 dilution of the PAP complex (DAKO A/S) for 30 minutes and AEC (3-amino-9-ethylcarbazole) as a chromogen. Anti-MUC2 and anti-MUC5AC monoclonal antibodies were NCL-MUC-2 and NCL-MUC-5AC respectively (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and specific for the respective precursor protein. Correct staining was verified by staining positive control sections of stomach and colon and comparing staining patterns with a known MUC2 specific monoclonal antibody (We9) on control sections[28]. Washing steps in between antibodies were done using PBS/ 0.1% Tween 20 and a last washing step with PBS prior to staining with the AEC chromogen. Slides were air-dried and mounted with pertex. Slides

were evaluated at 400x and 1000x magnification with and without polarization filters. In each section the number of bacteria associated or unassociated with MUC2 and MUC5AC stained cells were counted in 10 high power fields (1000x). Similarly the number of bacteria in the mucosa and the submucosa were counted.

Von Kossa staining

Following in-vitro infection, infected ileal sections were stained according to an adaptation of the Von Kossa staining procedure for phosphate salts to show calcium and magnesium salt precipitation[26] as an antibody independent bacterial staining method. Deparaffinized 5µm sections were hydrated to distilled water and placed in a 5% silver nitrate solution. Sections were stained for 30-45 min. under a UV-light source. Sections were washed three times in distilled water and placed in a 5% sodium thiosulfate solution for 5 min. after which sections were washed in distilled water three times and counterstained with nuclear-fast Red for 5 minutes and washed in distilled water. Sections were dehydrated in an alcohol gradient and after clearing in xylene mounted with pertex.

Adherence of FITC-labeled bacteria on deparaffinized ileal tissue sections

Bacterial hybridization experiments were conducted according an abridged version of a method described for *Helicobacter pylori* by Falk e.a. in order to evaluate bacterial adhesion to undisturbed epithelial cells and the effect of sialic acid and MUC3 blocking on epithelial cell adhesion.

Uninfected fresh ileal tissue from patients was embedded in paraffin and sectioned at 5 µm on 3-aminopropyltriehoxysilane (APES) coated slides after which the sections were deparaffinized in xylene for 10 minutes and alcohol for 10 minutes. Slides were coated after cleaning the slides with detergent for 30 minutes and washing in tap-water for 30 minutes followed by two washing steps in distilled water and 2 washing steps in 95% alcohol of 5 minutes each. Slides were coated by immersion in a fresh 2% 3-aminopropyltriethoxysilane in dry acetone for 5 seconds. Slides were washed twice in distilled water and dried at 42 °C overnight in an incubator. Sections were rehydrated by serial incubation in 70%, 50% alcohol , distilled water and PBS for 5 minutes each. Sections were blocked for 15-30 minutes in 0.2 % Bovine Serum Albumin /0.05% Tween 20 in PBS. Sections were incubated for 1 hour with 200 µl labeled bacteria in a 1/20 working dilution, covered with parafilm slips in a humidified chamber at 37°C. The 200 µl FITC-labeled bacterial suspensions were either used directly or pre-incubated with a 1mg/ml solution of sialic acid (Sigma Aldrich, N-acetylneuraminic acid, A9290) or 10 mg/ml MUC3 for 1 hour and washed with PBS. After incubation the sections were washed in PBS for 6 times for 3-5 minutes and air-dried. Sections were checked for a FITC signal under a fluorescence microscope and washed another 2-4 times after which the sections were covered in Vectashield ® (Vector Laboratories, Inc., Brunschwig) mounting medium supplemented with 0.2 mg/ml 4 , 6-diamidino-2-phenylindole, dihydrochloride (DAPI) to visualize epithelial cell nuclei. Blocked sections were evaluated in comparison with unblocked sections.

Far-Western Analysis

Far Western analysis was used to evaluate specific bacterial capsule-mucin interactions, sialic acid and MUC3 blocking. The bacterial capsules were analyzed on a 4% stacking/ 10% separating SDS-PAGE by staining with Coomassie Brilliant Blue or transblotted. The gels were transblotted at 100 mV for 1 hour to a nitrocellulose transfer membrane (Protran® BA 85, Schleicher&Schuell), washed in PBS/0.1%Tween 20 three times and blocked for 1 hour in PBS/

0.1%Tween 20/ 2% skimmed milk. A second blocking step with 1mg/ml MUC3 (Sigma Aldrich M1778, partially purified powder) or 1mg/ml purified sialic acid (Sigma Aldrich, N-acetylneuraminic acid, A9290) for 45 minutes was used to test sialic acid specific binding in two separate series. The transfer membrane was overlaid for 45-60 minutes with 5 ml PBS/0.1 % Tween 20/ 2 % skimmed milk supplemented with 5 ml patient urine after centrifugation at 1500x *g* for 10 minutes. Urine was used either untreated or pre-treated with either 0.002u /ml neuraminidase (Sigma-Aldrich) or 1% periodate for 30 min. at 37˚C. After washing the nitrocellulose membrane 3 times for 10 minutes in PBS/0.1 %Tween 20/ 1 % skimmed milk the first antibody was applied. Rabbit polyclonal antibodies against HCM or HGM in PBS/0.1 %Tween 20/ 2% skimmed milk at a 1:1000 dilution were used and incubated for 1 hour. Anti-HCM and anti-HGM characteristics were previously described by Tytgat et.al.[33]. Anti-HCM (Human Colonic Mucin) is a rabbit polyclonal antibody raised against purified human colonic mucin. It recognizes the mature, fully glycosylated MUC2. Anti-HGM (Human Gastric Mucin) is a rabbit polyclonal antibody raised against purified human gastric mucin it recognizes mainly the mature, fully glycosylated MUC5AC. The first antibody must be able to recognize the mature protein, therefore anti-HCM was used to show MUC2, and anti-HGM was used to show MUC5AC. After that the membrane was washed 3 times in PBS/0.1 % Tween 20/ 1 % skimmed milk and the second HRP labeled anti-rabbit-antibody was applied in PBS/0.1 % Tween 20 / 2 % skimmed milk and incubated for 45-60 minutes. Horse radish peroxidase labeled antibodies were visualized by chemiluminescence using BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics GmbH, Mannheim) and exposure to film. The prestained Seeblue molecular weight standard (Invitrogen) provided a positive control to which both MUC2 and MUC5AC would adhere.

Western Blotting analysis of bacterial strains for GroEL

Western Blotting was used to evaluate GroEL (Cpn60) in different strains. Gel electrophoresis and transblotting and blocking was performed similar to Far-Western Blotting. Rabbit-anti-GroEL (Hsp60/ Cpn60) antibody (Sanbio BV, Stressgen) was used as a primary antibody in PBS/0.1 %Tween 20/ 2% skimmed milk at a 1:1500 dilution and incubated for 1 hour. After that the membrane was washed 3 times in PBS/0.1 % Tween 20/ 1 % skimmed milk and the second HRP labeled anti-rabbit-antibody was applied in PBS/0.1 % Tween 20 / 2 % skimmed milk and incubated for 45-60 minutes. Horse radish peroxidase labeled antibodies were visualized by chemiluminescence using BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics GmbH, Mannheim) and exposure to film.

Statistical Analysis

Statistical analysis was performed using SPSS 9 software. The association between bacteria, mucins and crystals was analyzed using Pearson and Spearmann correlation, and hierarchical cluster analysis was performed for the infected bladder and intestinal tissues using median clustering by squared Euclidean distance. Far-Westerns were evaluated by binomial tests and Pearson and Spearmann correlation, Wilcoxon signed rank, Mann-U-Whitney and Mc Nemar tests.

Results

Adherence of FITC-labeled bacteria on deparaffinized tissue sections

P.mirabilis adheres to goblet cells and to some extent to enterocytes. *P.mirabilis* does not adhere to the lamina propria. After sialic acid blocking specific adhesion to goblet cells disappeared but increased on enterocytes at the top of the intestinal villi. Bacterial adhesion to Paneth cells in the intestinal crypts also disappeared. MUC3 blocks all specific interactions with ileal epithelial cells. Results are presented in **Table** 1 and **Fig**.1.E, F, G, H..

Immunohistochemistry of in-vitro infected tissues

In **Fig**.1 A,B sections are doublestained for respectively MUC2 and MUC5AC respectively and *P.mirabilis*. Infected and damaged epithelia show bacterial adhesion/ invasion in the mucin producing epithelial cells. **Fig**. 1.C shows salt precipitation on bacteria in the intestinal crypts.

P.Mirabilis strain	Origin	API-code	Falk's bacterial-hybridization			Alcian Blue / LPS staining
			Unblocked	Sialic acid block	MUC-3 block	
AB129	Enterocystoplasty	0336000	-	-	-	+
AB142	Enterocystoplasty	0735000	+/-	-	-	+
AB364	Enterocystoplasty	0737000	+/-	-	-	+/-
AB474	Enterocystoplasty	0737000	+	-	-	+/-
AB520	Enterocystoplasty	0737000	+/-	-	-	+
AB780	Enterocystoplasty	0737000	+	+	-	+/-
AB964	Enterocystoplasty	0733000	+	-	-	+/-
S468	Enterocystoplasty Infection Stone	0736000	+/-	-	-	+
U101	Normal Bladder	0737000	+	-	-	+
U103	Normal Bladder	0334000	+	-	-	-
U124	Normal Bladder	0735000	+	-	-	+/-
U238	Normal Bladder	0337000	+/-	-	-	+
U366	Normal Bladder	0736000	-	-	-	-
U530	Normal Bladder	0737000	+	-	-	+
U679	Normal Bladder	0757000	+	-	-	+/-
ATCC	Normal Bladder Infection Stone	0737000	+	-	-	+

***Table** 1. P.Mirabilis origin of strains and Falk's bacterial-hybridization with fresh ileal tissue sections and immunological staining of infected ileal tissue sections. Sections were scored for association of Proteus mirabilis with goblet-cells in the ileal sections (+=strong colocalisation with goblet-cells, +/-= association with goblet-cells and some other cell types, -= no typical association with goblet-cells). Strong co-localization with enterocytes was also observed in all sections (especially after sialic acid blocking) except in the MUC-3 blocked hybridization experiments. LPS-Alcian Blue staining of infected tissue sections showed bacterial localization in the goblet-and enterocytes.*
In binomial tests the LPS-Alcian Blue staining and unblocked hybridization experiments were significantly positive (+ or +/-, p=0.004) and the sialic acid blocked (p=0.001) and MUC3 blocked (p<0.001) hybridization experiments were significantly negative for goblet-cell adhesion / infection

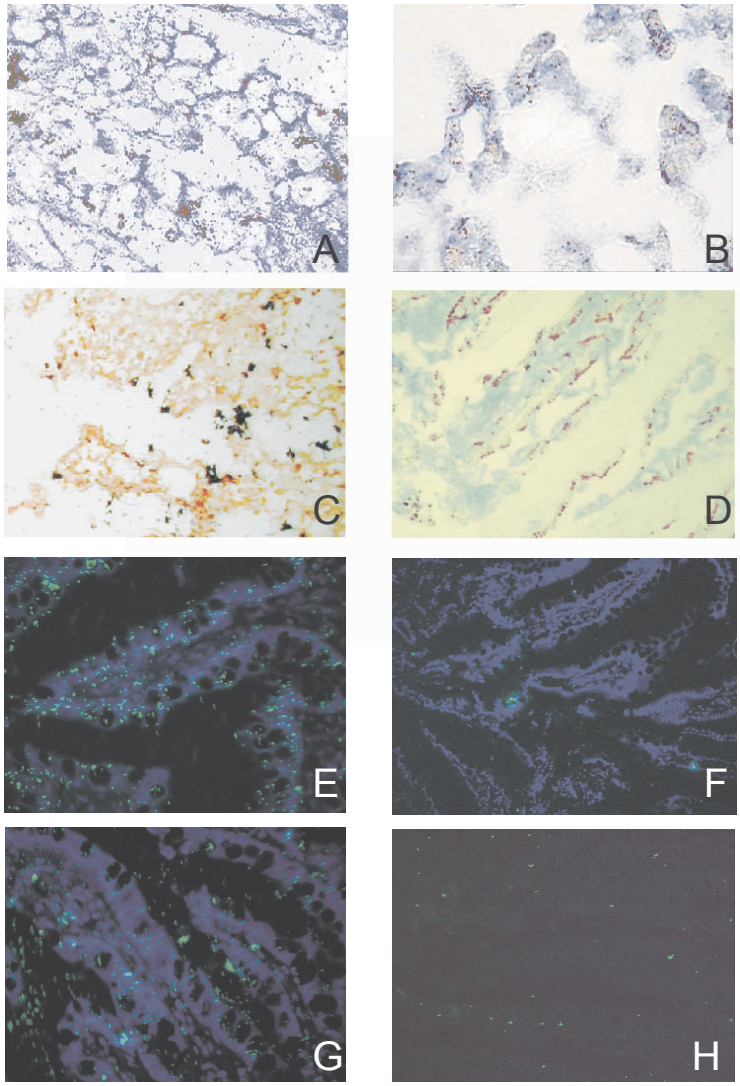


***Fig**.1. Histochemistry and Immunohistochemistry*
A) Immunohistochemical staining of ileum indicates bacterial colocalisation (orange-black) with MUC2 stained epithelial cells B) Immunohistochemical staining of ileum indicates bacterial colocalisation with MUC5AC producing cells. C) Bacterial localization between intestinal villi, bacteria stain positive for phosphate salts in Von Kóssa staining. D) Bacterial colocalisation with epithelial cells in ileal villi. red= bacterial LPS Proteus mirabilis, blue = sulfated mucins. Reduced from x 800
Bacterial Hybridization of tissue sections with FITC-labeled bacteria. E) DAPI/ FITC fluorescent microscopy image of hybridization with ileal tissue (800x), F) hybridization after Sialic acid blocking (400x), G) hybridization after Sialic acid blocking (800x), H) hybridization after MUC3 blocking (800x).

A) Intestinal and bladder tissue infection with P.mirabilis ATCC49565

P. mirabilis has a different adhesion pattern to MUC2 and MUC5AC producing epithelial cells in bladder and bowel tissues. The total number of bacteria influences the number of bacteria in the lamina propria and epithelium. The total number of bacteria correlates with the number of bacteria that are free from MUC2, which correlate with the number of bacteria in the lamina propria and epithelium. Pearson correlations are presented in **Table**.2A. Colocalisation of bacteria with MUC2 becomes clear in a group of sections with an equal amount of bladder and ileal sections where more MUC2 and bacteria are found in the ileal sections (**Table** 2.B). However bacteria with MUC5AC and without MUC2 are also correlated. The number of bacteria in the lamina propria correlates with the number of bacteria that are free from MUC2. The adhesion of *P.mirabilis* to MUC2 seems to be limited and dependent on MUC2 expression. Invasion and loss of the mucosal barrier function occurs with decreasing fraction of MUC2 adherent *P.mirabilis,* apparent with increasing number of bacteria.

			Pearson correlation	*p-value*
Total number of bacteria	vs.	Bacteria without MUC2	P = 0.801	*p = 0.0009*
Bacteria in epithelium	vs.	Total number of bacteria	P = 0.891	*p = 0.043*
Bacteria in epithelium	vs.	Bacteria without MUC2	P = 0.597	*p = 0.031*
Bacteria in epithelium	vs.	Bacteria in lamina propria	P = 0.559	*p = 0.047*
Bacteria in lamina propria	vs.	Total number of bacteria	P = 0.874	*p = 0.009*
Bacteria in lamina propria	vs.	Bacteria without MUC2	P = 0.827	*p = 0.0004*
Bacteria with MUC5AC	vs.	Bacteria without MUC2	P = 0.629	*p = 0.028*

***Table** 2A. Colocalisation in Immunohistochemical doublestaining for 13 infected tissue sections (7 bowel and 6 bladder). Only significant correlations are presented. 'Crystals with MUC5AC' were not presented since the significant correlations were similar to values for 'crystals without MUC5AC' . Correlations are significant at the p<0.05 level.*

B) Intestinal tissue with different P.mirabilis strains

In only ileal tissue sections bacterial adhesion to MUC2 producing cells was significant on the epithelial surface, but not in the lamina propria. The number of bacteria associated with MUC5AC producing cells and with non-MUC5AC producing cells were related and both were found preferentially in the epithelium. Correlations are presented in **Table**.2.C. LPS-Alcian blue staining showed colocalisation of bacteria with the basolateral part of goblet-cells and the apical part of enterocytes in the mucin filled inter-villous space, but not in the ileal crypts, the lamina propria or any other part of the ileal tissue. Results are presented in **Table** 1. and **Fig**.1D.

Far-Western Analysis

In Far-Western analysis a binding capability was shown for proteins in the bacterial capsule to MUC5AC and MUC2 in all cases. Adhesion was effective enough to deplete the enterocystoplasty urine from mucins. This capability disappeared after periodate treatment in the case of MUC2 and remained in the case of MUC5AC, destroying the sugar-O-rings in the glycosylated tandem repeats. Furthermore, sialidase treatment of enterocystoplasty urine used in the Far-Western showed a inverse relation between sialidase pre-treated MUC2 and MUC5AC. This procedure digests sialic acid in mucins to load the enterocystoplasty urine with a large amount of both sialic acid and a more accessible protein backbone. Most of the enterocystoplasty

strains of *P.mirabilis* showed the ability to adhere to MUC2 despite sialidase pre-treatment and lacked the ability to adhere to sialidase treated MUC5AC. The strains isolated from normal urinary bladders were able to bind to MUC5AC, but not MUC2 in sialidase treated enterocystoplasty urine. These (glyco)proteins involved in the binding of MUC2 and MUC5AC are approximately similar in size and can be separated on a non-reducing SDS-PAGE. (**Fig**.4.) MUC3, neuraminidase treated MUC3 and sialic acid solutions before the application of enterocystoplasty urine in Far-Western analysis was able to block the adhesion of HCM and HGM to the bacterial capsules, **Table**.3

	Group Statistics					t-test for Equality of Means						
		TISSUE	Mean	Sd	SEM	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
MUC2	Bacteria with MUC2	Bowel	30.71	14.49	5.48	4.36	8.60	0.002	26.55	6.09	12.67	40.42
		Bladder	4.17	6.52	2.66							
	Bacteria without MUC2	Bowel	12.71	5.82	2.20	-1.77	5.62	0.13	-16.12	9.09	-38.73	6.50
		Bladder	28.83	21.61	8.82							
	Bacteria in lamina propria	Bowel	16.14	12.38	4.68	-1.28	11.00	0.228	-10.86	8.51	-29.58	7.87
		Bladder	27.00	18.19	7.43							
	Bacteria in epithelium	Bowel	39.14	11.75	4.44	0.64	11.00	0.534	6.14	9.57	-14.91	27.20
		Bladder	33.00	22.02	8.99							
MUC5AC	Bacteria with MUC5AC	Bowel	12.67	8.96	3.66	-3.45	10.00	0.006	-23.50	6.81	-38.68	-8.32
		Bladder	36.17	14.08	5.75							
	Bacteria without MUC5AC	Bowel	12.50	4.81	1.96	-0.10	10.00	0.922	-0.33	3.34	-7.77	7.10
		Bladder	12.83	6.62	2.70							
	Bacteria in epithelium	Bowel	25.17	12.58	5.13	-2.67	10.00	0.023	-23.83	8.91	-43.69	-3.98
		Bladder	49.00	17.84	7.28							
	Bacteria in lamina propria	Bowel	29.67	20.39	8.33	1.16	10.00	0.273	18.67	16.09	-17.18	54.51
		Bladder	11.00	33.72	13.76							

***Table** 2.B. Bacterial colocalisation with mucins in ileal and bladder sections.*
T-test for mean number of bacteria in 10 high power fields. Shaded areas indicate significant differences between adhesion in bladder and ileal sections.

Western Blotting analysis of strains for GroEL

All strains showed GroEL expression on Western Blot, which correspond in size with the interacting protein in Far Western Blot, (**Fig**. 5.)

Von Kóssa staining of sections

Von Kóssa staining showed a precipitation of phosphate salts, mainly on bacteria. Bacteria were found in the intestinal crypts, mainly at the top of the crypts (**Fig**.1.C.). In these parts of the crypts the epithelium mainly stained positive for MUC2 and less for MUC5AC. Agglomerations of these precipitates were observed, which almost blocked the crypts. Where crypts were blocked, large deposits of bacteria accumulated at the luminal side of the crypts. Precipitation was observed in all samples, and could be well distinguished from background staining. Bacteria in crypts, without invasion of epithelial cells stained positive in Von Kóssa staining and negative with routine immunostaining and enabled an evaluation of the bacterial load as homogenous and diffuse over the entire crypt.

			Pearson correlation	*p-value*
Bacteria in epithelium	vs.	Bacteria with MUC2	P = 0.964	*p < 0.001*
Bacteria in epithelium	vs.	Bacteria in lamina propria	P = 0.525	*p = 0.037*
Bacteria with MUC5AC	vs.	Bacteria without MUC5AC	P = 0.671	*p = 0.004*
Bacteria in epithelium	vs.	Bacteria with MUC5AC	P = 0.989	*p < 0.001*
Bacteria in epithelium	vs.	Bacteria without MUC5AC	P = 0.773	*p < 0.001*

***Table** 2.C. Colocalisation in Immunohistochemical doublestaining for 1 infected bowel tissue section, infected with 16 P.mirabilis strains. Only significant correlations are presented. Correlations are significant at the p<0.05 level.*

	MUC2 (p-value)	MUC5AC *(p-value)*	*MUC2 vs. MUC5AC (p-value)*[a]
Patient urine	16(<0.01) †	16(<0.01) †	-
Patient urine + periodate	0(<0.01) †	16(<0.01) †	≤0.001*
Patient urine + neuraminidase	10(<0.45)	5(<0.21)	0.302
Patient urine + MUC3 block	0(<0.01) †	14(<0.01) †	≤0.001*
Patient urine + hydrolyzed MUC3 block	1(<0.01) †	10(<0.45)	0.004*
Patient urine + sialic acid block	0(<0.01) †	16(<0.01) †	≤0.001*
Normal urine	0(<0.01) †	16(<0.01) †	≤0.001*

***Table** 3. Far-Western analysis of 16 P.mirabilis strains.*
Positive staining lanes with the mucin binding capsule protein bands were counted
[a] p-values from McNemar test
†=Significant p-values by binomial distribution within groups of 16 strains (p<0.01)
**=Differences between MUC2 and MUC5AC interaction are significant in Wilcoxon Signed Ranks and McNemar test (p≤ 0.005), and significant in Mann-U-Whitney test (p≤0.001)*
Competitive inhibition of sialic acid with MUC5AC and MUC2, achieved by neuraminidase treatment of patient urine showed a Pearson correlation of –0.595 (p=0.021) between MUC5AC and MUC2 interaction. Differences between the AB and U strains were significant for the competitive sialic acid block in a Mann-U-Whitney test (p=0.046)
Strains isolated from enterocystoplasties preferentially bind MUC2 compared to MUC5AC (p<0.001)

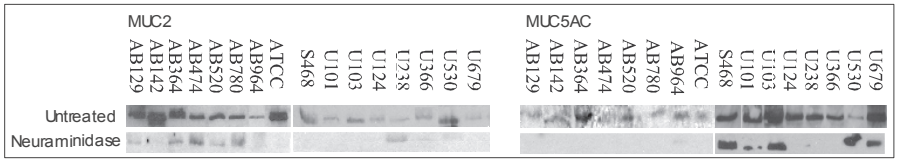


***Fig**.2. Far-Western Analysis of 16 P.mirabilis. Top)Transblotted bacterial capsules interacted with MUC2 and MUC5AC from untreated enterocystoplasty urine. Bottom) Transblotted bacterial capsules overlaid with neuraminidase treated enterocystoplasty urine interacted with both the mucin and the free sialic acid, and shows a preference for either MUC2 or MUC5AC in the presence of sialic acid and the mucin residue.*

***Fig**. 3 . Western Blotting analysis of bacterial strains for GroEL (Cpn60). 1 = AB129, 2= AB142, 3= AB364, 4= AB474, 5= AB520, 6= AB780, 7= AB964, 8= S468. Strains from normal urinary bladders showed similar expressions (not shown)*

Discussion

Interactions between crystals and the bacterial capsule[9] and between crystals and mucins[21] have been described before. Here we describe the interaction between bacteria and mucins in relation to the stone formation process.

It seems bacterial adhesion to the epithelial surface is tissue type specific and that different tissue types have different capabilities to adhere bacteria and crystals. *Proteus mirabilis* in between the intestinal villi gathers phosphate salts on the bacterial capsule. These bacteria are found in the intervillous space, filled with sulphated mucins from where they infect the epithelial surface. An affinity for goblet cells and enterocytes is expressed. The affinity for goblet cells and MUC2 and MUC5AC changes after sialic acid blocking to a enterocyte preference. The high sialic acid concentration is necessary to achieve a blocking effect, which could be attributed to an osmolyte function of sialic acid in the interaction of GroEL with a protein substrate[5,36]

Affinity for goblet cells and enterocytes disappears after MUC3 blocking. This can be explained by the application of competitive substrate for GroEL.

After degradation of mucins by sialidase, sialic acid is released to enhance the GroEL⇔ substrate interaction in small amounts of sialic acid and a large free section of the protein core with α-β transitions. Interaction between GroEL, MUC2 and MUC 5AC changes in certain strains after sialidase (neuraminidase) treatment of enterocystoplasty urine in a similar manner. Common urinary proteins are less glycosylated and become better substrates for GroEL. The mucins lose sialic acid as well and GroEL either prefers MUC2 or MUC5AC as a substrate. The osmolyte function of saccharides would enhance the blocking capability of urinary proteins and mask some available α-β transitions in MUC2. MUC2 interaction is decreased and remaining interactions may be explained by remaining free GroEL due to high expression of GroEL. MUC5AC binding is increased for certain strains, which were blocked more effectively for MUC2. Increased band intensity can be explained by the osmolyte function of sialic acid. In some strains interactions disappear for MUC2, but become stronger for MUC5AC, due to a preferred interaction with the freed α-β transition in MUC5AC. Von Willibrand factor domains could be the general interaction sites with GroEL on the mucin molecules. The repeat domains

with large amounts of sialic acid could contain the freed α-β transitions involved in the interaction with GroEL.

Bacteria available for antibodies must be free from any significant precipitation that would interfere with a bacterial-host interaction. This preferentially occurs in the epithelial cell and demonstrates bacterial invasion and infection. Precipitation accumulates after a 3-4 hours period at 37˚C in artificial urine when bacterial growth has long reached its lag-phase. Bowel tissue seems to be more capable of adhering *P.mirabilis*. Furthermore, these bacteria seem to be located mostly in the epithelial part, where the mucins are located and at the center or upper half of the villi where the are produced. This indicates a bacterial binding capability of mucin producing cells. When the bacterial load exceeds the bacterial binding capability, the mucosal barrier is breeched.

Human Colonic Mucin is the mature form of MUC2 and is secreted into the urine of enterocystoplasty patient in relatively large amount. HGM is the mature form of the secreted MUC5AC and is also produced by the veru montanum of the normal urogenital tract in boys[4]. In enterocystoplasties in children the bladder is usually enlarged to relieve a compromised kidney and the bladder trigonum remains. The normal bladder urine was acquired from healthy male subjects and it is therefore not very surprising that MUC5AC appears in the normal urine Far-Western. MUC5AC and other urinary (glyco)proteins may interact with the epithelial surface of the pouch and for infection of sections enterocystoplasty urine was used to allow for these interactions to take place.

It seems the interaction between mucins and *P.mirabilis* is not a random event induced by the "stickiness" of the sialated mucins, but is a specific interaction between host and bacteria, which can be blocked by high concentrations of sialic acid and aspecifically by MUC3. This specific interaction shifts in preference in the presence of neuraminidase. MUC2, the most prominent secretory mucin type in large bowel[33] , important in enterocystoplasties plays an important barrier and adhesion role for *P.mirabilis*. In the presence of other bacteria, producing neuraminidase, the interaction preference can change to involve other mucins as well.

Other sugar residues besides sialic acid and encapsulation of the bacteria by the mucin molecule may play a role in interaction inhibition. Similarly *Streptococci* have also been observed to bind to sialic acid residues on mucins[30]. The differences in binding characteristics of the different *P.mirabilis* strains may be more dependent on conformation or glycosylation then actual amino acid differences. Neuraminidase is also known as sialidase and is very effective at low concentrations. It has been known that bacterial pathogens target sialic acid residues[23], which may be conformation dependent.

Maybe we should therefore compare the interaction with sialic acid residues we found for *P.mirabilis* with the interactions seen in other urease producing bacteria such as *H.pylori*, which also shows a strain specific interaction with sialic acid[3] and interacts with MUC2 and MUC5AC[35]. In MUC2 intact sugar O-rings and therefore glycosylation seems to be important for the adhesion of *P.mirabilis*. The disappearance of interaction with GroEL can be explained by a masking effect of the available interaction site.

The idea that the formation of infection stones is related to the production of urinary urokinase and sialidase has been proposed[6,7]. However sialidase was thought to enhance the formation of a mineral matrix from uromucoid. Uromucoid and probably uromodulin[27] are identical to Tamm-

Horsfall protein which can be found in collecting tubule casts present in normal urine and which can trap Type I fimbriated *E.Coli*[25] . It now seems that the sialidase pre-treatment of mucins also changes the targeting capabilities of the interaction between bacteria and host. Bacteria which do not produce urease, but do produce neuraminidase such as *E.Coli* and *Staphylococcus* could therefore influence the bacterial adhesion of *P.mirabilis* and therefore contribute directly to bacterial strain selection on the basis of MUC2 adhesion and the formation of an infection stone, besides influencing urinary protein excretion.

It seems that mucin production inhibitors can decrease the number of epithelial internalized bacteria[37], which may play a role in *P.mirabilis* infection[2,24]. Alkalisation[18] and urease[10] have been involved in the formation of stones and since usually both of these events are related the knowledge that intermittent catheterizing patient who rinse their bladder still produce stones[1] presents a therapeutical possibility. Sialic acid, MUC3 or similarly acting substances may prove valuable additives for bladder rinsing solutions.

Acknowledgement
We would like to acknowledge Dr. Kees Verduin for urinary strains isolated from normal bladder urine samples.

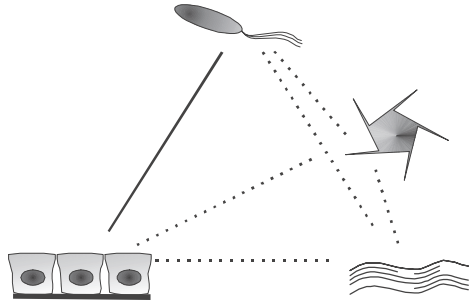
References

1. Barroso U. Jr., R . Jednak, P. Fleming, J.S. Barthold, R. Gonzalez, 2000. Bladder calculi in children who perform clean intermittent catheterization. BJU Int. 85: 879-884.
2. Braude A.I., J. Siemienski, 1960 Role of bacterial urease in experimental pyelonephritis. J. Bact. 80:171-179
3. Chmiela M., M. Lawnik, E. Czkwianianc, T. Rechcinski, I. Planeta-Malecka, T. Wadstrom, W. Rudnicka, 1997. Attachment of Helicobacter pylori strains to human epithelial cells. J Physiol Pharmacol. 48(3):393-404
4. Daher N, J. Gonzales, R. Gautier, J. Bara, 1990. Evidence of Mucin M1 Antigens in Seminal Plasma and Normal Cells of Human Prostatic Urethra in relation to embryonic development and tumors. The Prostate 16: 57-69
5. Diamant S, Eliahu N, Rosenthal D, Goloubinoff P. 2001. Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. J Biol Chem. 276(43): 39586-39591
6. du Toit P.J., C.H. van Aswegen, J.A. Nel, P.L. Steyn, A.J. Ligthelm, D.J. du Plessis., 1995. In vivo effects of urease-producing bacteria involved with the pathogenesis of infection-induced urolithiasis on renal urokinase and sialidase activity. Urol Res 23(5):335-338
7. du Toit P.J., C.H. van Aswegen, , P.L. Steyn, A . Pols, D.J. du Plessis., 1992. Effects of bacteria involved with the pathogenesis of infection-induced urolithiasis on the urokinase and (neuraminidase) activity. Urol Res 20(6):393-397
8. Duel B.P., R. Gonzalez, J.S. Barthold, 1998. alternative techniques for augmentation cystoplasty. J. Urol. 159(3): 998-1005
9. Dumanski A.J., H. Hedelin, A. Edin-Liljegren, D. Beauchemin, R.J.C. Mc Lean, 1994. Unique ability of the Proteus mirabilis capsule to enhance mineral growth in infectious urinary calculi. Infect Immun. 62(7) : 2998-3003
10. Ebisuno S., T. Komura, K.Yamagiwa, T. Ohkawa, 1997. Urease induced crystallizations of calcium phosphate and magnesium ammonium phosphate in synthetic urine and human urine. Urol Res. 25: 263-267
11. Falk P., R.A Roth, B. Thomas, U. Westblom, J.I. Gordaon, S. Normark, 1993. An in vitro adherence assay reveals that Helicobacter pylori exhibits cell lineage-specific tropism in the human gastric epithelium. Proc. Natl. Acad. Sci. 90: 2035-2039
12. Frisk A, Ison C.A., Lagergard T. 1998. GroEl Heat Shock protein of Haemophilus ducreyi:association with cell surface and capacity to bind eukaryotic cells. Infect. Immun. 66(3):1252-1257
13. Garduno R.A., Garduno E., Hoffman P.S., 1998. Surface –associated Hsp60 Chaperonin of Legionella pneumophilia mediates invasion an a HeLa Cell Model. Infect, Immun. 66(10): 4602-4610
14. Griffith D.P., 1978. Struvite stones. Kidney Int. 13: 372-382
15. Hennequin C., Pocheray F., Waligora-Dupriet, A.J., Collignon A., Barc, M.A, Bourlioux P., Karjalainen T. 2001. GroEl (Hsp60) of Clostridium difficile is involved in cell adherence. Microbiology 147:87-96
16. Kaefer M., M.S. Tobin, W.H. Hendren, SB. Bauer, C.A. Peters, A. Atala, A.H. Colodny, J. Mandell, A.B. Retik, 1997. Continent urinary diversion: the Children's Hospital experience. J Urol 157(4):1394-1399
17. Ko C.W., S.P. Lee., 1999.Gallstone formation. Local factors. Gastroenterol Clin North Am. 28(1):99-115
18. Kok D.J., J. Poindexter, C.Y.C. Pak, 1993. Calculation of titratable acidity from urinary stone risk factors. Kidney Int. 44: 120-126
19. Mathoera R.B., Hoogenboezem T., de Jong A.P.J.M, Einerhand A.W.C., Verduin C.M., Nijman R.J.M. The identification of GroEL as MUC2 and MUC5AC adhesion molecule in *P.mirabilis.* (*Submitted)*
20. Mathoera R.B., D.J. Kok, R.J.M. Nijman. 2000. Bladder calculi in augmentation cystoplasty in children. Urology 56(3);482-487
21. Mathoera R.B., D.J. Kok, W.J. Visser, C.M. Verduin, R.J.M. Nijman., 2001. Cellular membrane associated mucins in artificial urine as mediators of crystal adhesion: an in vitro enterocystoplasty model., J Urol. 166(6):2329-2336

22. Mouricout M., 1997. Interactions between the enteric pathogen and the host. An assortment of bacterial lectins and a set of glycoconjugate receptors., Adv Exp Med Biol. 412: 109-123
23. Nelson J.W., M.W. Tredgett, J.K. Sheehan, D.J. Thornton, D. Notman, J.R. Govan., 1990. Mucinophilic and chemotactic properties of Pseudomonas aeruginosa in relation to pulmonary colonization in cystic fibrosis. Infect Immun. 58(6):1489-1495
24. Oelschlaeger T.A., B.D. Tall. 1996. Uptake pathways of clinical isolates of Proteus mirabilis into human epithelial cell lines. Microb Pathog. 21(1):1-16
25. Orskov I, Ferencz A, Orskov F. 1980. Tamm-Horsfall protein or uromucoid is the normal urinary slime that traps type 1 fimbriated Escherichia coli. Lancet. 1(8173): 887.
26. Pearse A.G.E., 1972,Substitution methods for calcium p1138-1140 *In* A.G.E. Pearse, Histochemistry Theoretical and applied 3rd ed. Vol.2 Churchill Livingstone, Edinburgh and London.
27. Pennica D, Kohr WJ, Kuang WJ, Glaister D, Aggarwal BB, Chen EY, Goeddel DV. 1987. Identification of human uromodulin as the Tamm-Horsfall urinary glycoprotein. Science 236(4797) :83-8
28. Qiu S.M., G.Wen, J. Wen, R.D. Soloway, R.S. Crowther. 1995. Interaction of human gallbladder mucin with calcium hydroxyapatite: binding studies and the effect on hydroxyapatite formation. Hepatology. 21(6):1618-1624
29. Rozalski A., Z. Sidorczyk, K. Kotelko. 1997. Potential virulence factors of Proteus bacilli. Microbiol Mol Biol Rev. 61(1):65-89
30. Ryan P.A., V. Pancholi, V.A. Fischetti. 2001. Group A streptococci bind to mucin and human pharyngeal cells through sialic acid-containing receptors. Infect Immun 69(12):7402-7412
31. Schwartz B.F., M.L. Stoller, 2000. The vesical calculus.Urol. Clin. North Am. 27(2):333-346
32. Thomas V.L., B.A. Sanford., M.A. Ramsay., 1993. Calcium- and mucin-binding proteins of staphylococci. J Gen Microbiol 139 (Pt 3):623-629
33. Tytgat K.M., L.W. Klomp, F.J. Bovelander, F.J. Opdam, A. Van der Wurff, A.W. Einerhand, H.A. Buller, G.J. Strous, J. Dekker, 1995.Preparation of anti-mucin polypeptide antisera to study mucin biosynthesis. Anal Biochem. 226(2):331-341.
34. van den Berg A.A., J.D. van Buul, G.N. Tytgat, A.K. Groen, J.D. Ostrow., 1998. Mucins and calcium phosphate precipitates additively stimulate cholesterol crystallization. J Lipid Res. 39(9):1744-1751
35. Van den Brink G.R., K.M. Tytgat, R.W. Van der Hulst, C.M. Van der Loos, A.W. Einerhand, H.A. Buller, J. Dekker, 2000. H.pylori colocalises with MUC5AC in the human stomach. Gut. 46(5):601-607
36. Voziyan PA, Jadhav L, Fisher MT. 2000. Refolding a glutamine synthetase truncation mutant in vitro: identifying superior conditions using a combination of chaperonins and osmolytes. J Pharm Sci. 89(8):1036-10345
37. Wells C.L., R.P. Jechorek, S.L. Erlandsen. 1995. Inhibitory effect of bile on bacterial invasion of enterocytes: possible mechanism for increased translocation associated with obstructive jaundice. Crit Care Med. 23(2):301-307
38. Wells C.L., Jechorek R.P., Kinneberg K.M., Debol S.M., Erlandsen S.L., 1999.The isoflavone genistein inhibits internalization of enteric bacteria by cultured Caco-2 and HT-29 enterocytes. J Nutr. 129(3):634-640
39. Wells C.L., E.M. van de Westerlo, R.P. Jechorek, S.L. Erlandsen. 1995. Exposure of the lateral enterocyte membrane by dissociation of calcium-dependent junctional complex augments endocytosis of enteric bacteria. Shock . 4(3):204-210
40. Wells C.L., E.M. van de Westerlo, R.P. Jechorek, H.M. Haines, S.L. Erlandsen. 1998. Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. Infect Immun. 66(6):2410-2419

Chapter 7

The identification of GroEL as MUC2 and MUC5AC adhesion molecule in P.mirabilis.



(Submitted)

Abstract

Introduction:
Proteus mirabilis is a known bacterial agent in the pathogenesis of urinary infection stones. Previously we demonstrated that *P.mirabilis* colocalizes with mucins MUC2 or MUC5AC. These mucins are abundant surface molecules at the cellular surface of enterocystoplasty epithelial cells. Here we prove that *P.mirabilis* interacts with mucins, MUC2 and MUC5AC, and identify the bacterial counterpart in the interaction.

Materials and methods:
Two previously described *P.mirabilis* strains (AB780, AB474) were evaluated by sequential Far-Western analysis of bacterial lysates and fractionated bacterial lysates. Urine from enterocystoplasty patients were used in Far-Western analysis to provide fresh mucins. Two dimensional gel electrophoresis, ms-ms mass spectrometry, electron microscopy and Western blotting analysis were carried out to identify the bacterial adhesin.

Results:
Bacterial lysates and fractionated samples derived from bacterial lysates contained a MUC2 and MUC5AC interacting molecule by Far Western analysis, which was identified by ms-ms mass spectrometry as GroEL after separating the samples of interest from contaminants and co-migrating molecules in 2D-gel electrophoresis. Electron microscopy showed GroEL localization at the bacterial surface. Furthermore western blot analysis of outer membrane fractions, also demonstrated the presence of GroEL at the bacterial surface. Using purified GroEL interaction with MUC2 and MUC5AC was confirmed in Far-Western analysis.

Discussion:
GroEL from *P.mirabilis* interacts with MUC2 and MUC5AC present in urine derived from enterocystoplasties**.** These interactions may be of importance during enterocystoplasty infection with *P. mirabilis* in stressed conditions such as antimicrobial treatment of a hostile urine environment.

Introduction

Proteus mirabilis has been designated the most important bacterial agent in the formation of infection stones, both in normal and augmented bladders[1-3]. Enterocystoplasties have been an adequate surgical intervention for almost 50 years[4]. In children, the intention is to enlarge small non-compliant bladders with an intestinal pouch to create a continent urinary diversion, usually an ileal reservoir. Nevertheless, in the last few decades, several adaptations of the augmentation method were necessary to achieve a satisfactory result[4]. At this time there are still morbidity risks related to enterocystoplasties. Complications and morbidity can often be related to mucus production, infection and catheterization[1].

Stone formation is one of the important complications in children. In the period following augmentation of the bladder the pH rises and often stones are formed consisting of ammonium magnesium phosphate, calcium phosphate and calcium apatite. The formation of these stones has in most cases been attributed to the rise in pH as a result of the size of the incorporated intestinal segment and urea splitting by urease [5,6]. When the pH of urine rises crystals will form in vitro in normal human urine as well as *in-vitro* in artificial urine above pH 7.3[7,8] .

Urease producing organisms and other bacteria encounter a pivotal step in symptomatic infection. *P.mirabilis* requires adhesion to the cellular surface of the augmented bladder as the first step of infection of the enterocystoplasty.

Some adhesion factors have been described for *P.mirabilis,* such as Non-agglutinating fimbriae (NAF)[9], mannose resistant pili (MR/P and MR/K)[10] and urothelial cell adhesions (UCA)[11]. These adhesion molecules are thought to interact with the bladder epithelium. In the enterocystoplasty, a large portion of the augmented bladder consists of intestinal epithelium, secreting intestinal mucins of the MUC family[12]. To date there are approximately 16 mucin types (MUC1-13, and MUC16-18) of which 14 have been approved by the Human Genome Organization Gene Nomenclature Committee (http:/www.hugo-international.org/hugo/)[13]. MUC2 is the most abundant mucin type in the small intestine and colon[14], whereas MUC5AC can be found preferentially in gastric mucus[15], but also in the duodenum[16]. MUC5AC is also produced by the veru montanum of the bladder trigonum[17], which often remains in the augmented bladder. These mucins are secreted by the intestinal epithelium and appear in the enterocystoplasty urine. Although *P.mirabilis* may adhere to mucins such as MUC2 and MUC5AC[18], it is not clear which bacterial molecule is involved in this interaction. To identify the bacterial adhesin used a procedure of Far-Western interaction studies, 2D electrophoresis, and ms/ms mass-spectrometry[19] and finally with Transmission electron microscopy. In this order this method can identify the unknown interacting molecule of one cell with the known molecule of another.

Materials and Methods

The methods described here are adaptations of a previously described method for the evaluation of protein-protein interactions by Pasquali et al.[19] using Far-Western blotting, 2D-electrophoresis and mass-spectrometry for the identification of interacting molecules.

Bacterial Strains

Two previously described *Proteus mirabilis* strains AB474 and AB780[18] were isolated from children with an enterocystoplasty and identified using the API- system (BioMerieux, France)

and Gram staining. Bacteria were stored in 15% glycerol at -80°C until needed and cultured in Luria broth 0.05% glycerol until late log-fase growth before use.

Bacterial Capsule preparation

Bacterial capsules were isolated from an overnight culture of P.mirabilis and grown in Luria broth at 37°C until late log-phase (3-4hours). Bacteria were collected by centrifugation at 3000x *g* for 10 minutes at 4˚C in an Eppendorf 5804R centrifuge and transferred to 25 ml 1 M TRIS-HCl pH 8.5, for every 100 ml broth. The bacteria were sonicated for 3 minutes at a peak to peak amplitude of 9 micron in a Soniprep sonicator. After sonication, bacterial capsules were separated from intact bacteria by differential centrifugation at 900x *g* rpm for 20 minutes, removing the bacterial pellet, followed by centrifugation at 1700x *g* for 1 hour to isolate the bacterial capsules. The capsular rich material was resuspended in 1 ml PBS. This crude capsular material was used for Far-Western blotting and for further fractionation over a sucrose gradient before Western and Far-Western analysis.

Fractionation of bacterial capsular material

The samples were prepared according to an abridged version of a method previously described by R.A. de Maagd e.a.[20] Shortly described the method is as follows. Cells were grown overnight in Luria broth as described above and harvested. After being harvested cells were resuspended into 50 mM Tris (pH8.5) – 20% sucrose – 0.2 mM dithiotreitol (Sigma) supplemented with 0.2 mg. RNAse T1 (Boehringer Mannheim, GmbH, Germany) per ml. The cells were broken by sonication in a Soniprep sonicator at a wave to wave amplitude of 9 microns for 5 minutes. After sonication whole cells were separated from broken cells by centrifugation at 900 x g for 20 minutes. The supernatant fluid was transferred to a new tube and supplemented with KCl to a final concentration of 0.2 M and centrifuged for 2 hours at 100 000 x g. The pellet was resuspended in 0.5 to 1.0 ml of 20% (wt/wt) sucrose- 5 mM EDTA – 0.2 mM dithiotreitol (pH7.5) and layer on top of a discontinuous density gradient of 60% to 25% sucrose in 5 mM EDTA in 5% increments of 0.5 ml each. The gradient was centrifuged for 17 hours at 58 000 x g in a Beckman SW50 rotor. The fractions were collected in fractions of 0.5 ml and the small residue was resuspended in 20% sucrose 5mM EDTA. Malate Dehydrogenase activity in the separate fractions was determined according to Kitto to distinguish intracellular from outer membrane fractions.[21] The fractions were used for Western and Far-Western Blotting.

Western Blotting analysis of fractionated samples

Western Blotting was used to evaluate GroEL (Cpn60) in different fractions. Gel electrophoresis, transblotting and blocking was performed similar to Far-Western Blotting. Anti-GroEL (Hsp60/ Cpn60) antibody (Sanbio BV, Stressgen) was used as a primary antibody in PBS/0.1 %Tween 20/ 2% skimmed milk at a 1:1500 dilution and incubated for 1 hour. After that the membrane was washed 3 times in PBS/0.1 % Tween 20/ 1 % skimmed milk and the second HRP labeled anti-rabbit-antibody was applied in PBS/0.1 % Tween 20 / 2 % skimmed milk and incubated for 45-60 minutes. Horse radish peroxidase labeled antibodies were visualized by chemiluminescence using BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics GmbH, Mannheim) and exposure to film.

Far-Western Analysis

Far Western analysis was used to evaluate specific bacterial capsule-mucin interactions. Protein solutions were diluted to concentrations of approximately 750 µg/ml. Bacterial capsule proteins were solubilized in Laemli sample buffer without dithiotreitol (DTT) or mercaptoethanol. The bacterial capsule proteins were separated on a 35 cm 4% stacking/ 10% separating SDS-PAGE. A small strip was stained with Coomassie Brilliant Blue. The major part remained unstained. Another small strip of gel was transblotted at 100 mV for 1 hour to a nitrocellulose transfer membrane (Protran® BA 85, Schleicher&Schuell), washed in PBS/0.1%Tween 20 three times and blocked for 1 hour in PBS/ 0.1%Tween 20/ 2% skimmed milk. The transfer membrane was overlaid for 45-60 minutes with 5 ml PBS/0.1 % Tween 20/ 2 % skimmed milk supplemented with 5 ml urine from enterocystoplasty patients after centrifugation at 1500x *g* for 10 minutes. After washing the nitrocellulose membrane 3 times for 10 minutes in PBS/0.1 %Tween 20/ 1 % skimmed milk the first antibody was applied. Rabbit polyclonal antibodies against Human Colonic Mucin or Human Gastric Mucin in PBS/0.1 %Tween 20/ 2% skimmed milk at a 1:1000 dilution were used and incubated for 1 hour. Anti-HCM and anti-HGM characteristics were previously described by Tytgat et.al.[22]. Anti-HCM (Human Colonic Mucin) is a rabbit polyclonal antibody raised against purified human colonic mucin. It recognizes the mature, fully glycosylated MUC2. Anti-HGM (Human Gastric Mucin) is a rabbit polyclonal antibody raised against purified human gastric mucin it recognizes mainly the mature, fully glycosylated MUC5AC. The first antibody must be able to recognize the mature protein, therefore anti-HCM was used to show MUC2, and anti-HGM was used to show MUC5AC. After that the membrane was washed 3 times in PBS/0.1 % Tween 20/ 1 % skimmed milk and the second HRP labeled anti-rabbit-antibody was applied in PBS/0.1 % Tween 20 / 2 % skimmed milk and incubated for 45-60 minutes. Horse radish peroxidase labeled antibodies were visualized by chemiluminescence using BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics GmbH, Mannheim) and exposure to film.
To induce gel shift of GroEL in Far-Western analysis, residual fractions were incubated for 30 minutes with polyclonal rabbit-anti-GroEL before application to 4% stacking/ 10% separating SDS-PAGE. Purified recombinant GroEL (Sanbio BV, Stressgen) was used for Far-Western analysis.

Sample elution from 1D gel

Gels were aligned to identify the section of the Coomassie Brilliant Blue stained gel containing the positive band in Far-Western analysis. The unstained gel was cut out to isolate the appropriate region of the positive band.
The unstained gel fragment was sliced to 1mm^3 pieces and excess 100 mM NH_4HCO_3 was added. Elution of proteins was allowed overnight at 4°C. The supernatant was collected and the elution was repeated. Pooled supernatants were freeze-dried and redissolved in solubilization fluid: 9M Urea, 100mM DTT, 0.5% (v/v) Pharmalytes pH 3-10 (Amersham), 5% (v/v) Triton X-100, 5mg Pefabloc (Merck) per 25 ml.

2D Gel electrophoresis of eluted samples.

First dimension: IPG-strips pH 4-7, 17 cm (Bio-Rad) were rehydrated with 250 µl solubilization fluid with sample at 16°C overnight. Rehydrated strips were focused for 3 hours at 500 V and overnight at 3000 V.
Second dimension: Focused strips were applied to a 12-20% acrylamide gradient gel and a prestained molecular weight marker was added. Gels were run at 118 V 300 mA overnight. Gels were fixated in 4:1:5 methanol/ acetic acid/ water for 10 minutes and stained in Cypro Orange (Bio-Rad) for 3 hours. Spots were detected on a UV transluminator. Abundant spots were cut out and processed for Nanoscale LC-MS.

Mass spectrometric analysis

Spots of interest were punched out from the gel and sliced in 1 mm^3 pieces.
Slices were then washed three times with water, shrunk with acetonitril, swollen again with 100 mM ammonium bicarbonate and dried under vacuum.
The proteins in the slices were digested with modified trypsin (Promega) at a temperature of 37 ^oC for 15h. The resulting peptides were extracted with 5% formic acid and subsequently analyzed by nano-HPLC-MS/MS[23].
The resulting spectra were interpreted and compared to derived peptide patterns of the Swissprott database. (http://www.matrixscience.com) [Electrophoresis, **20(18)** 3551-67 (1999)]

Transmission Electron Microscopy of P.mirabilis

Bacterial strains were cultured overnight, grown in Luria broth and suspended in PBS similar to the bacterial capsule preparation. Bacterial strains were washed three times in PBS and resuspended in 0.5% BSA/ PBS/ 0.1%Tween 20. Bacteria were divided in two equal batches and centrifuged at 1500 *g* x 10 minutes at 4°C and resuspended in PBS/ 0.1%Tween 20 supplemented with rabbit-anti-GroEL (Hsp60) antibody (Sanbio BV, Stressgen) at a 1:160 dilution or without a primary antibody and incubated for 1 hour. Bacteria were washed in PBS/ 0.1%Tween 20 twice and incubated with a secondary gold-labeled anti-mouse antibody for 1 hour. Gold-labeled bacteria suspensions were applied to the grids and air-dried. Bacteria were viewed under a Philips Morgagni ™ 268 transmission electron microscope connected to a CCD camera (Mega view II).

Results

SDS-PAGE and Western analysis of fractions

Western analysis for GroEL showed a constant band between 50 and 64 kDa, present in residual fractions and more in low sucrose fractions then in high sucrose fractions. Most of the GroEL was present in the residual fraction and in the fractions of 30-45% sucrose (**Fig**1A). Most of the proteins were found in the residual fractions and in the fractions of 30-35% sucrose (**Fig**.2.). The latter showed low levels of Malate dehydrogenase (MDH) activity whereas the residual fractions

showed high levels of MDH activity (**Fig**.3.). We interpret the levels of MDH activity as the presence of intracellular material in the residual fraction and the 30-45% sucrose fractions as fractionated capsular material.

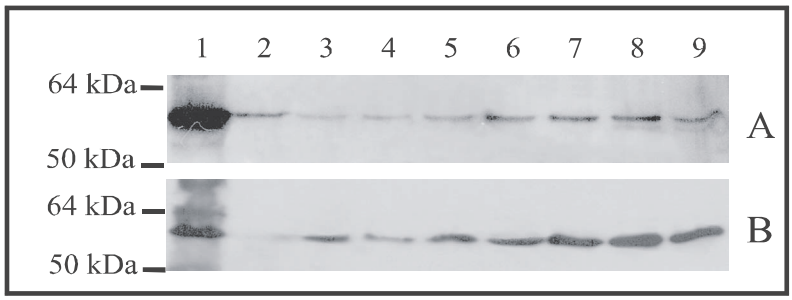


***Fig**1.A. AB780: Western blot of fractionated samples showing GroEL in both the residue and the fractions with lower sucrose concentration, but less in the 60- 50% sucrose fraction. B, Far Western analysis of fractionated samples showing an identical profile with the GroEL Western Blot. A similar profile was found for AB474 (not shown). 1, residue, 2, 60% sucrose, 3, 55% sucrose, 4, 50% sucrose, 5, 45% sucrose, 6, 40% sucrose, 7, 35% sucrose, 8, 30% sucrose, 9, 25 % sucrose.*

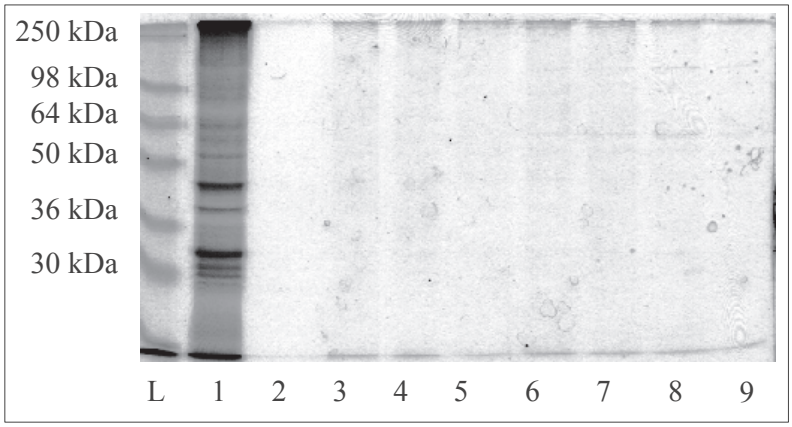


***Fig**2. AB780: Coomassie stained SDS-PAGE of fractionated samples by discontinuous sucrose gradients*

Far-Western Analysis

Far-Western analysis of fractionated samples showed interaction of bands corresponding with GroEL in Western analysis. Most of the interactions were present in the residual fraction and in the fractions of 30-45% sucrose (**Fig**1.B.). Purified GroEL interacts with MUC2 (**Fig**.4.B.) and MUC5AC (**Fig**.4.A.). This band is also present in the residual fractions and in the 35% and 40% sucrose fractions. Gel shift of GroEL in the residual fractions could be induced in Far-Western analysis and interacted with both MUC2 and MUC5AC.

Far-Western analysis reveals a constant band at approximately 56 kDa that interacts with both MUC2 and MUC5AC. Several other weak bands are visible as a smear **Fig**.5. A stronger signal can be found for MUC5AC adhesion then in MUC2 adhesion. The section indicated by an asterisk in **Fig**.5. was used for elution of the interacting protein band and 2D electrophoresis.

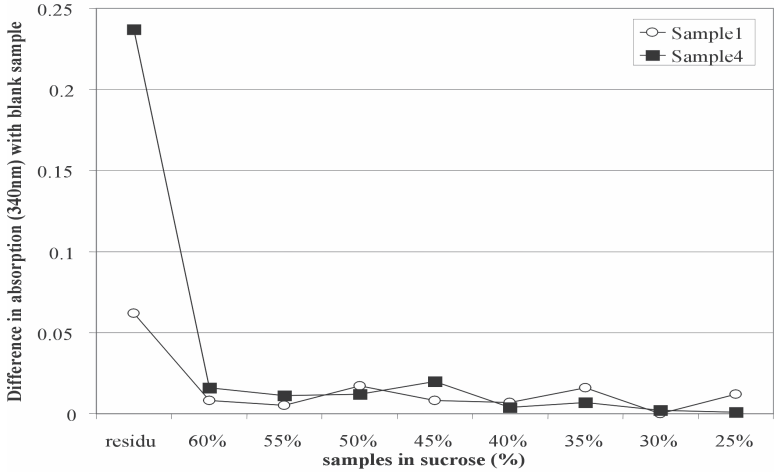


***Fig**3. Malate dehydrogenase activity in sucrose fractions and residue as a measure of intercellular contamination. Sample 1 = AB780, Sample4= AB474*

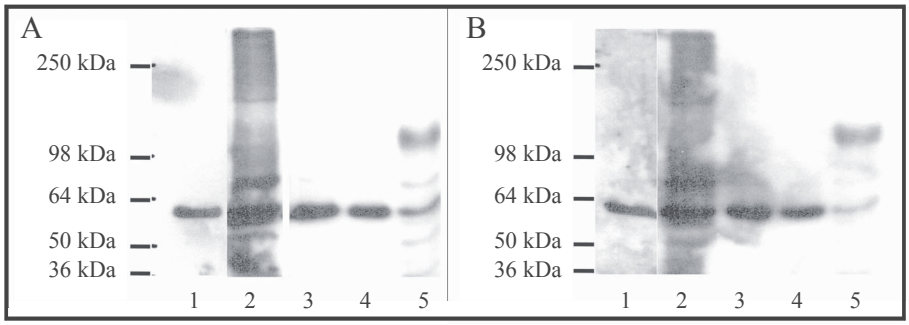


***Fig**4.A. Far-Western Blot experiments. MUC5AC interaction B. Far-Western Blot experiments. MUC2 interaction. 1, purified recombinant GroEL, 2, residue in 20% sucrose, 3, 40% sucrose fraction, 4, 35% sucrose fraction, 5, gel-shift of GroEL in diluted residue by rabbit-anti-GroEL, the original band disappears and shifts upwards.*

2D Gel electrophoresis of eluted samples.

Many spots were visible in 2 D-electrophoresis after reduction of the isolated sample. The most intense spots were approximately 56 kDa. The pattern of the spots was very similar between the 2D electrophoresis gels of proteins that comigrate with the Far-Western MUC2 and MUC5AC positive bands **Fig**.6. Many contaminants were separated from the predominant protein mixture. The 10 most intense spots are indicated with approximately similar mass and pH (1-5) from either gel and were evaluated in mass spectrometry.

Mass spectrometric analysis

Most of the 10 processed samples were identified as a Heat Shock Protein. Half of the samples could be identified as GroEL (Hsp60/ Cpn60). GroEL (Hsp60/ Cpn60) also fits the molecular weight found on gel in contrast to other identifications. Identifications are presented in **Table** 1. Almost all the identifications which correspond with the molecular weight found on gel correspond with GroEL (Cpn60). Trigger Factor was not regarded as a suitable adhesion molecule option since it does not occur in samples derived from both MUC2 and MUC5AC adhesive bands, but only in samples from MUC5AC adhesive bands. Furthermore, the mascot score and percentage of matched peptides is lower then scores and percentages found for other proteins. Identifications of samples 6-10 of both 2D-gels revealed mostly enolase contamination with relatively low molecular weights. The identified amino acid sequences used for a database search are presented in **Fig**.7. MUC2 and MUC5AC adhesion molecules in *P.mirabilis* seem to be identical in mass spectrometry.

Transmission Electron Microscopy

To confirm the identification of GroEL as the adhesion molecule for MUC2 and MUC5AC and the expression or presence of GroEL on the bacterial surface Transmission electron microscopy imaging was used. GroEL could be determined on *P.mirabilis*. Many of the bacteria were negative. Positive bacteria were found both as clusters and single bacteria. Negative controls without a primary antibody showed no staining **Fig**.8.

Sample code	*Mol. weigh On gel (kD*	*Mol. weight Found(kDa)*	*Result MASCOT search*	*Score MASCOT*	*Matched peptides*
MUC2-1	44-77	57.5	(NC_000907) heat shock protein (GroEL)	**414**	**16%**
MUC2-2	44-77	61.1	30S ribosomal protein S1 (P02349)	**638**	**20%**
MUC2-3	44-77	68.9	Chaperone protein dnaK (Heat shock protein 70) (Heat shock 70 kDa protein)(Q8ZIM7)	**420**	**11%**
MUC2-4	44-77		Only keratin		
MUC2-5	44-77	56.3	60 kDa chaperonin (Protein Cpn60) (GroEL protein) (O66214)	**603**	**19%**
MUC5AC-1	44-77	57.5	60 kDa chaperonin (Protein Cpn60) (GroEL protein (P43733)	**337**	**19%**
MUC5AC-2	44-77	56.3	60 kDa chaperonin (Protein Cpn60) (GroEL protein) (O66198)	**434**	**15%**
MUC5AC-3	44-77	56.3	60 kDa chaperonin (Protein Cpn60) (GroEL protein)	**583**	**17%**
MUC5AC-4	44-77	43.1	Elongation factor Tu (EF-Tu) (P-43)	**368**	**21%**
MUC5AC-5	44-77	56.3	Trigger factor (TF) (P22257)	**267**	**9%**

***Table** 1. Results of MASCOT search with ms-ms results. Five samples with highest intensity were analyzed. Best samples according to molecular weight on both Far-Western and 2D-gel are shaded grey.*

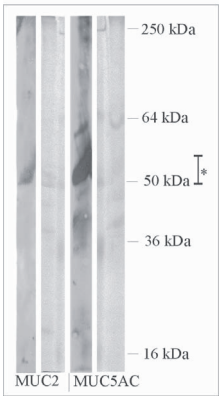


Fig*5.Far-western blot for MUC2 (left) and corresponding Coomassie stained gel, Far-western blot for MUC5AC (right) and corresponding Coomassie stained gel. Bar and asterisk indicate the section used for sample elution and 2D electrophoresis.*

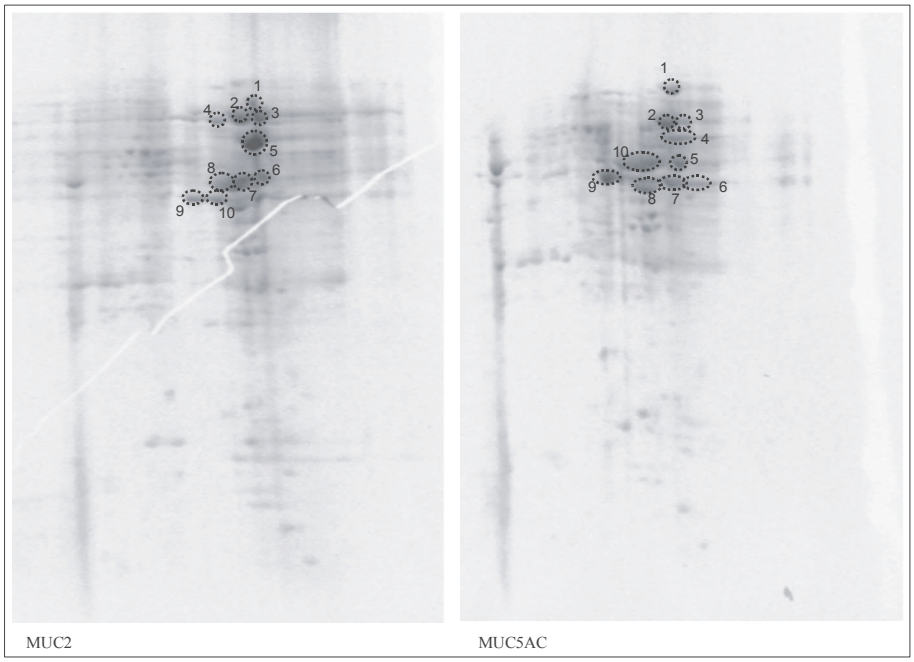


Fig*.6. 2D-electrophoresis. Spots 1-10 were analyzed. 1-5 were considered to be of importance as the possible MUC2 and MUC5AC adhesion molecule, 6-10 were considered contaminants and comigrating molecules, and were excluded based on molecular weight. The most intense spots were chosen. MUC2 indicates the 2D gel from the eluted sample of the positive band in MUC2 Far-Western analysis. MUC5AC indicates the 2D gel from the eluted sample of the positive band in MUC2 Far-Western analysis.*

H.influenza Cpn60 (GroEL)

1	MAAKDVK**FGN**	**DAR**VK**MLKGV**	**NVLADAVKVT**	**LGPK**GRHVIL	DK**SFGAPTIT**
51	**KDGVSVAREI**	**ELEDK**FENMG	AQMVKEVASK	ANDAAGDGTT	TATVLAQAIV
101	NEGLKAVAAG	MNPMDLKR**GI**	**DK**AVSAVVSE	LKNLSKPCET	AKEIEQVGTI
151	SANSDSIVGQ	LISQAMEKVG	KEGVITVEDG	TGLEDELDVV	EGMQFDRGYL
201	SPYFINKPET	ATVELDNPYL	LLVDKK**ISNI**	**RELLPVLEGV**	**AKA**GKPLLII
251	AEDVEGEALA	TLVVNKMR**GI**	**VKVAAV**KAP	**FGDRR**KAMLQ	DIAILTAGTV
301	ISBEIGMELE	K**ATLEDLGQA**	**K**RVVINK**DNT**	**TIIDGIGDEA**	**QIKGR**VAQIR
351	**QQIEESTS**DY	DKEKLQERVA	K**LAGGVAVIK**	VGAATEVEMK	EKKDR**VDDAL**
401	**HAT**RAAVBEG	IVAGGGVALV	RAAAKVAASL	KGDNEEQNVG	IKLALRAMEA
451	PLRQIVTNAG	EEASVVASAV	KNGEGNFGYN	AGTEQYGDMI	EMGILDPTKV
501	TRSALQFAAS	VAGLMITTEC	MVTDLPKDDK	ADLGAAGMGG	MGGMGGMM

Bold = peptide fragments from ms/ms
Gray = peptide fragments different from *P.mirabilis* GroEL
Black = peptide fragments identical to *P.mirabilis* GroEL
Underlined = Identified in mascot search as GroEL from *R.ornitholytica,*
otherwise the peptides where identified as GroEL from *Haemophilus Influenza*

P.mirabilis Cpn60 (GroEL)

1	ATVLAQAIIA	EGLKAVAAGM	NPMDLKR**GID**	**K**AVVGAVEEL	KKLSVPCSDT
51	KAIAQVGTIS	ANSDETVGTL	IAQAMEKVGK	EGVITVEEGT	GLEDELDVVE
101	GMQFDRGYLS	PYFINKPETG	TAELENPFIL	LVDKK**VSNIR**	**ELLPVLEGVA**
151	**K**ANKPLLIIA	EDVEGEALAT	LVVNNMR**GIV**	**KVAAV**	

Underlined = Identified in mascot search as GroEL, corresponding with proposed *P.mirabilis* GroEL

***Fig**.7. Sequence homology between GroEL from Haemophilus influenza and Proteus mirabilis. The similarity between GroEL from Haemophilus Influenza and Proteus mirabilis is very high. The known sequence of Proteus mirabilis GroEL corresponds for 85% with Haemophilus Influenza.*

Discussion

Bacterial adhesion by uropathogens in the bladder depends on several adhesion molecules. Some of these molecules have been described as NAF[9], MR/P[10], MR/K, or UCA[11], but involve adhesion to urothelium. In enterocystoplasties the most prominent surface molecules are intestinal mucins. MUC2 is the most abundant mucin type secreted by the intestinal pouch[14]. Here we showed that GroEL is involved in the adhesion of *P.mirabilis* to mucins from enterocystoplasties.

GroEL has been extensively described as an intracellular or membrane bound protein involved in protein folding[24,25]. As most heat shock proteins, GroEL is expressed increasingly in stress conditions to help maintain the secondary structure of proteins[26]. Enterocystoplasties as a continent urinary diversion under antibiotic prophylaxis can be considered a stressful environment for uropathogens. GroEL has also been described as an extracellular protein, associated with the bacterial outer membrane. Examples of GroEL on the bacterial outer membrane are found in *Legionella pneumophila*[27], *Haemophilus ducreyi*[28] and *Clostridium difficile*[29]. In *Legionella pneumophila* invasion is associated with the expression of GroEL. *P.mirabilis* is also capable of invading epithelial cells[18,30], but has so far not been associated with GroEL.

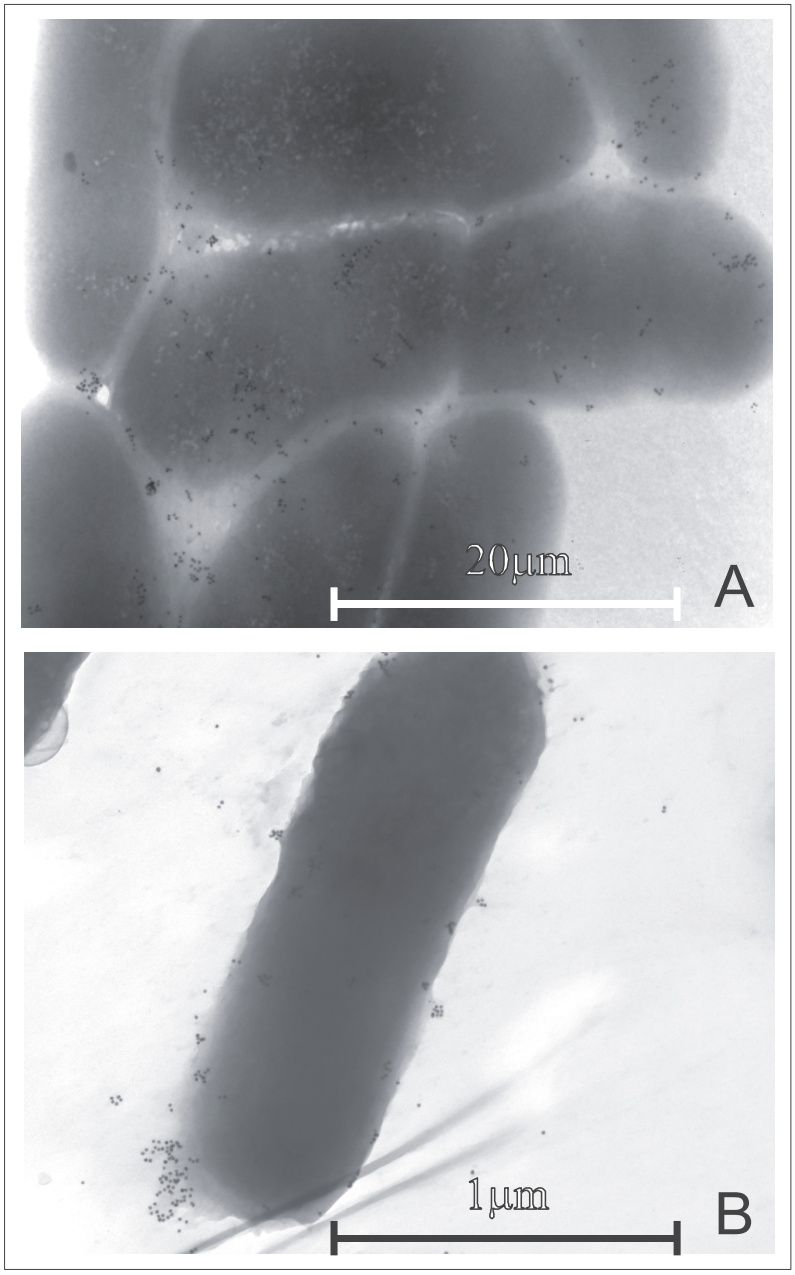


***Fig**. 8. Electron Microscopy images of AB780: immunostained with gold-labels to identify GroEL on the bacterial exterior. A clustered bacteria, positive for GroEL in the center, with a negative P.mirabilis in the top left. B. single P.mirabilis positive for GroEL.*

It is clear that GroEL interacts with MUC2 and MUC5AC from Far-Western experiments. This becomes evident with purified GroEL and by gel-shift experiments with GroEL from *P.mirabilis*.

Salts and osmolytes present in enterocystoplasty urine may play a role in the interaction between mucins and GroEL in enterocystoplasties and thereby enhance adhesion or if present in very high concentrations inhibit interaction[32,31]. High concentrations of osmolytes may mask the protein backbone structure of protein precursors or denaturated proteins.

Often GroEL is associated with other protein fragments or GroES[24,25]. This may explain a smear in separation of proteins on a SDS-PAGE gel. In retrospect The Far-Western analysis of samples resembles Western blots of GroEL which have been reported previously[33] and correspond with our results. GroEL adheres preferentially to the α-β transition in the protein structure[24]. By folding protein precursors or destabilized proteins to their proper 3D structure, GroEL can enhance the bacterial survival by increasing the durance of essential proteins. This 3D structure is available in mucins such as MUC2 and MUC5AC, but twice as much in MUC5AC[13]. The α-β transition is determined to great extent by the glycosylation of the mucin backbone, creating a rigid structure. Where glycosylation is absent or less profound, flexibility of the 3D structure occurs and GroEL adhesion can be expected. Some sections in the MUC2 and MUC5AC structure such as the Von Willibrand factor like domains and the cysteine rich domains are suitable candidates and could explain strong staining for MUC5AC and MUC2 adhesion in Far Western analysis.

In *Actinobacillus actinomycetemcomitans* GroEL was demonstrated to be present on the bacterial surface, but most likely as an extracellular component[34]. The GroEL-like protein present on the bacterial surface has been suggested to play a role in disease initiation. It can not be excluded that GroEL expression in *P.mirabilis* is present on the bacterial surface due to intracellular material from lysed cells, which will adhere to the surface of other cells. Altruistic (auto)lysis of single cells to enhance the survival of other bacteria in the colony is a possibility in a hostile environment such as enterocystoplasties. Expression is nevertheless retained after culturing in Luria broth in low concentration and may be increased by environmental factors. Whether GroEL is part of an extracellular component or is part of the outer membrane is not clear. The possibility remains that GroEL adheres to the bacterial capsule and protein after lysis. Nevertheless, *P.mirabilis* GroEL may be a part of the outer membrane as is determined by the method described for outer membrane isolation and may be an integral part of the bacterial capsule.

Conclusion

GroEL is an adhesion molecule for MUC2 and MUC5AC for *P.mirabilis.* In enterocystoplasties, producing several mucin types, the most likely interacting molecules are MUC2 and MUC5AC. *P.mirabilis* probably adheres to enterocystoplasties by GroEL. The expression of GroEL may be dependent on an external stressor. Far-Western blotting and mass spectrometry to identify protein- protein interactions, can be applied for research on protein-protein interactions extracellular in combination with transmission electron microscopy.

Acknowledgement

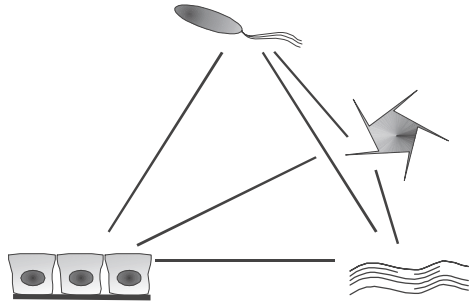
We would like to acknowledge J.H. ten Hove for his expert assistance and contribution to this paper as technical assistant in ms-ms mass spectrometry by nano-HPLC-MS/MS and Antonius W. de Jong for his excellent assistance in Transmission Electron Microscopy.

References

1. R.B. Mathoera, D.J. Kok, R.J.M. Nijman, Bladder calculi in augmentation cystoplasty in children. Urology 56(3); 482-487;2000
2. Schwartz BF, Stoller ML , The vesical calculus.Urol. Clin. North Am. 27(2):333-46; 2000
3. Kaefer M, Tobin MS, Hendren WH, Bauer SB, Peters CA, Atala A, Colodny AH, Mandell J, Retik AB, Continent urinary diversion: the Children's Hospital experience. J Urol 157(4):1394-9; 1997
4. Perez L.M., Webster G.D., History of urinary diversion techniques. In: Webster G.D., Goldwasser B. (eds.), *Urinary diversion, Scientific Foundations and Clinical Practice.*, 1st ed. pp. 2-22. Isis Medical Media Ltd., Oxford,UK
5. Lockhart JL, Davies R, Persky L, Figueroa TE, Ramirez G., Acid-base changes following urinary tract reconstruction for continent diversion and orthotopic bladder replacement. J Urol 152(2 Pt 1):338-42; 1994
6. Griffith D.P., Struvite stones. Kidney Int. 13: 372-382; 1978
7. Kok D.J., Poindexter J., Pak C.Y.C., Calculation of titratable acidity from urinary stone risk factors. Kidney Int. 44: 120-126; 1993
8. Ebisuno S., Komura T., Yamagiwa K., Ohkawa T., Urease induced crystallizations of calcium phosphate and magnesium ammonium phosphate in synthetic urine and human urine. Urol Res. 25: 263-267; 1997
9. Tolson DL, Harrison BA, Latta RK, Lee KK, Altman E. The expression of nonagglutinating fimbriae and its role in Proteus mirabilis adherence to epithelial cells. Can J Microbiol. 43(8):709-17; 1997
10. R K.Latta, A Grondin, H. C Jarrell, RG. Nicholls, L R. Bérubé1. Differential expression of nonagglutinating fimbriae and MR/P pili in swarming colonies of Proteus mirabilis. J Bacteriol 181(10):3220-3225; 1999
11. Wray SK, Hull SI, Cook RG, Barrish J, Hull RA.,Identification and characterization of a uroepithelial cell adhesin from a uropathogenic isolate of Proteus mirabilis. Infect Immun. 54(1):43-9;1986
12. N'Dow J, Pearson JP, Bennett MK, Neal DE, Robson CN., Mucin gene expression in human urothelium and in intestinal segments transposed into the urinary tract. J Urol 164(4):1398-404; 2000
13. Dekker J., Rossen J.W.A., Büller H.A., Einerhand A.W.C., The mucin family: an obituary. Trends Bioch. Sci.27(3):126-131; 2002
14. Tytgat K.M., Buller H.A., Opdam F.J., Kim Y.S., Einerhand A.W.C., Dekker J., Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology. 107(5):1352-1363; 1994
15. Nordman H, Davies JR, Lindell G, De Bolos C, Real F, Carlstedt I. Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. Biochem J. 364(Pt 1):191-200; 2002
16. van de Bovenkamp J.H., Hau C.M., Strous G.J., Buller H.A., Dekker J., Einerhand A.W.C. Molecular cloning of human gastric mucin MUC5AC reveals conserved cysteine-rich D-domains and a putative leucine zipper motif. Biochem Biophys Res Commun. 245(3):853-859; 1998
17. Daher N, J. Gonzales, R. Gautier, J. Bara,. Evidence of Mucin M1 Antigens in Seminal Plasma and Normal Cells of Human Prostatic Urethra in relation to embryonic development and tumors. The Prostate 16: 57-69; 1990
18. Mathoera, R.B., D.J. Kok., C.M., Verduin, R.J.M., Nijman.. Pathological and therapeutical significance of cellular invasion by Proteus mirabilis in an enterocystoplasty infection stone model. Infect. Immun. 70(12): 7022-7032; 2002
19. Pasquali C, F. Vilbois , M.L. Curchod , R. Hooft van Huijsduijnen , F. Arigoni . , Mapping and identification of protein-protein interactions by two-dimensional far-Western immunoblotting. Electrophoresis 21 :3357-3368; 2000
20. De Maagd, R.A., Lugtenberg B., Fractionation of Rhizobacterium leguminosarum cells into outer membrane, cytoplasmic membrane, periplasmic and cytoplasmic components. J. Bact. 167(3): 1083-1085
21. Kitto G.B., Intra- and extramitochondrial malate dehydrogeneases from chicken and tuna heart. Methods Enzymol. 13: 106-116; 1969

22. Tytgat K.M., L.W. Klomp, F.J. Bovelander, F.J. Opdam, A. Van der Wurff, A.W. Einerhand, H.A. Buller, G.J. Strous, J. Dekker, 1995.Preparation of anti-mucin polypeptide antisera to study mucin biosynthesis. Anal Biochem. 226(2):331-341
23. Meiring H.D., van der Heeft E., ten Hove G.J.,. de Jong A.P.J.M. Nanoscale LC–MS(n): technical design and applications to peptide and protein analysis. J. Sep Sci. 25 : 557-568 ; 2002
24. Feltham J.L., Gierash L.M., GroEL-substrate interactions : Molding the fold or folding the mold ? Cell 100: 193-196; 2000
25. Carrascosa J.L., Llorca O., Valpuesta J.M., Structural comparison of prokaryotic and eukaryotic chaperonins. Micron 32: 43-50; 2001
26. Flynn G.C., T.G. Campbell, Rothman J.E., Peptide binding and release by proteins implicated as catalysts of protein assembly. Science 245: 385-390; 1989
27. Garduno R.A., Garduno E., Hoffman P.S., Surface –associated Hsp60 Chaperonin of Legionella pneumophilia mediates invasion an a HeLa Cell Model. Infect, Immun. 66(10): 4602-4610; 1998
28. Frisk A, Ison C.A., Lagergard T., GroEl Heat Shock protein of Haemophilus ducreyi:association with cell surface and capacity to bind eukaryotic cells. Infect. Immun. 66(3):1252-1257; 1998
29. Hennequin C., Pocheray F., Waligora-Dupriet, A.J., Collignon A., Barc, M.A, Bourlioux P., Karjalainen T., GroEl (Hsp60) of Clostridium difficile is involved in cell adherence. Microbiology 147 :87-96 ;2001
30. Oelschlaeger TA, Tall BD. Uptake pathways of clinical isolates of Proteus mirabilis into human epithelial cell lines. Microb Pathog. 21(1):1-16; 1996
31. Diamant S, Eliahu N, Rosenthal D, Goloubinoff P., Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. J Biol Chem. 276(43):39586-39591; 2001
32. Voziyan PA, Jadhav L, Fisher MT. Refolding a glutamine synthetase truncation mutant in vitro: identifying superior conditions using a combination of chaperonins and osmolytes. J Pharm Sci. 89(8):1036-10345; 2000
33. Zhang Y., Ohashi N., Lee E.H., Tamura A., Rikihisa Y., FEMS Immun. Med. Micob. 18: 39-46; 1997
34. Goulhen F., Hafezi A., Uitto V.J., Hinode D., Nakamur R., Grenier D., Mayrand D., Subcellular localization and cytotoxic activity of the GroEL-like protein isolated from *Actinobacillus actinomycetemcomitans.* Infect. Immun. 66: 5307-5313 ;1998

General discussion



Risks and Resistance

The definition of a symptomatic infection "The multiplication and maintenance of pathogenic bacteria, fungi, parasites or viruses after contamination, causing local inflammation or systemic disease of the host" was evaluated in as many aspects as possible in relation to stone formation in enterocystoplasties by *P. mirabilis*. It seems clear that the differences between a normal bladder and an augmented bladder are such that stone formation is enhanced in certain conditions. Most prevalent risk factor dominating and connecting all the other risk factors seems to be the presence of symptomatic infections (chapter 2). However, prophylactic use of antibiotics seems to have little or no effect on the prevention of stone formation. Children with a cloacal malformation or a vaginal reconstruction (related to the occurrence of a congenital urinary malformation) appear to be at risk for stone formation. It is clear that concomitant surgical procedures at the time of augmentation or at a later stage are important risk factors, especially continence enhancing procedures to the bladder neck. These children are often treated extensively with antibiotics and are often hospitalised. The ambulant patients often encounter infections which are treated with antibiotics by general practitioners. The antibiotics that are prescribed are usually effective in a normal bladder but in patients who have been exposed to antibiotics to such an extent as patients with an enterocystoplasties the effectiveness may be less. How this effects the usefulness of existing antimicrobial options and stone formation were discussed in the chapter 3.
From the findings in the comparative study on bacteriological infections of the enterocystoplasty and bacteriuria, it becomes clear that community acquired infections should be checked for the correct bacteriological strain and treated specifically if symptoms occur instead of the empirical treatment, often used in general practice. The use of broad spectrum antibiotics and the preferential use of certain antibiotics such as cotrimoxazol and amoxicillin to treat the urinary tract infections seen in enterocystoplasties have a tendency to transform the characteristics of a community acquired infection into a nosocomial infection due to the increased resistance among bacterial strains (chapter 3). Furthermore, the high degree of mixed cultures and low frequency of detected *P.mirabilis* infections may be explained by intracellular infection and increased resistance may thus explain the formation of stones; this was discussed in chapter 5.
In chapter 4 the interaction between the enterocystoplasty and the urine content is discussed in relation to bacterial/crystal adhesion and crystal formation as it seems that infection of the enterocystoplasty increases the pH level of the urine content conditioned by the enterocystoplasty, which correlates with stone formation The high number of mixed cultures may mask a number of *P.mirabilis* infections. Further research has to be done whether a specific search for urease producing bacterial strains among patients with an enterocystoplasty will benefit stone prevention by more specific antibiotic therapy of these strains. At this point we can not advocate a specific search for urease producing bacteria with current laboratory testing to identify them in mixed cultures, but clearly identification of uropathogens should preceed specific antibiotic treatment of symptomatic urinary tract infections in enterocystoplasties.

Mucins and bladder stones
Mucins have been implicated with stone formation due to their ability to act as a framework for crystal growth. Furthermore, several compounds such as glycosaminoglycans have been correlated with crystal and bacterial evacuation by wrapping particles in the soluble polymer. It seems that some mucins are able to act in a similar manner. Many of the mucins secreted by the intestinal cells in the enterocystoplasty will remain in the bladder at the surface of the cells. Since these molecules are able to agglomerate crystals, they must be able to bind crystals. This is described in chapter 3. These mucins are not secreted by P.mirabilis. It seems that P.mirabilis

invades the epithelial cells. This phenomenon and its implication on the therapeutic effect of antibiotics was discussed in chapter 5. By invading the epithelial cells a safe haven was created where the bacteria could retreat and survive in case of antibiotic therapy. The safe haven was provided by the double membranes of the cell and a cytoplasmatic layer. The cytoplasmic colonies remain at the edge of the cell underneath the double membrane until the epithelial cell bursts under the burden of it's bacterial load. It seems that mucins MUC2 and MUC5AC play a role in the formation of crystal agglomerates which may bind to the epithelial cells on MUC2 and MUC5AC. How these mucins are involved in the adhesion of bacteria was discussed in chapter 6. The invasion of epithelial cells and cellular stress are probably involved in a number of cellular reactions which may enhance the production of mucins and thereby increase the effect of crystal adhesion.
The effect on antibiotic resistance may play an important role in the persistence of an infection but requires a first step of bacterial adhesion in the process of invasion. Mucins seem to have certain characteristics that enhance bacterial adhesion. Sialic acid seems to play an important role in this process. Adhesion to mucins could be demonstrated for both MUC2 and MUC5AC and could also be blocked by other mucin types. Many bacterial strains use mucins as a target for cellular infection. Thus mucins and sugar residues may be useful in the prevention and therapy of bacterial infections in the augmented bladder.
P.mirabilis adheres to mucins on the cellular surface and invades epithelial cells after which crystals are formed in the epithelial cells. This process can be blocked by MUC3, sialic acid and other methods to disable the interaction with certain mucin substructures. The approximately 50kDa molecule attached to the bacterial capsule still needs to be identified to have a clear idea of all the molecules and interactions that play a role in bacterial and crystal adhesion to the epithelial surface. In chapter 7 we attempt to identify the bacterial molecule responsible for MUC2 and MUC5AC binding.

Closing the circle of interactions
If GroEL (Hsp60) is indeed the bacterial molecule interacting with mucins, an interaction between the different molecules can be described. First, the crystals can agglomerate and adhere to mucins (MUC2) on the epithelial cells. Second, crystals can precipitate on the bacterial capsule, thus transforming the bacterial surface into a crystal surface. Furthermore, the bacterial surface by means of GroEL can interact with mucins (MUC2 and MUC5AC) at the epithelial surface. At the epithelial surface a docking process takes place which can preceed cellular invasion which enhances bacterial survival. In the epithelial cells crystals can be formed, which are small and protected from wash out by micturation. Of course bacterial urease elevates the pH of urine which enhances precipitation of crystals. By the combination of these processes an infectious bladder stone can be formed which is relatively resistant to antibiotic therapy and is securely fixed to the bladder wall, while providing the conditions for the growth of the bladder stone until it has reached a diameter that exceeds the diameter of the urethra or catheterisation channel. A diagram of the interactions as described above is presented in **Fig**.1. Crystals agglomerate mediated by mucins, crystals are formed by pH elevation due to urease activity from bacteria, while the bacteria find a safe haven inside the cell and fortify their safe haven by the precipitation of crystals. These interactions between GroEL and epithelial mucins can be inhibited by the addition of MUC3.

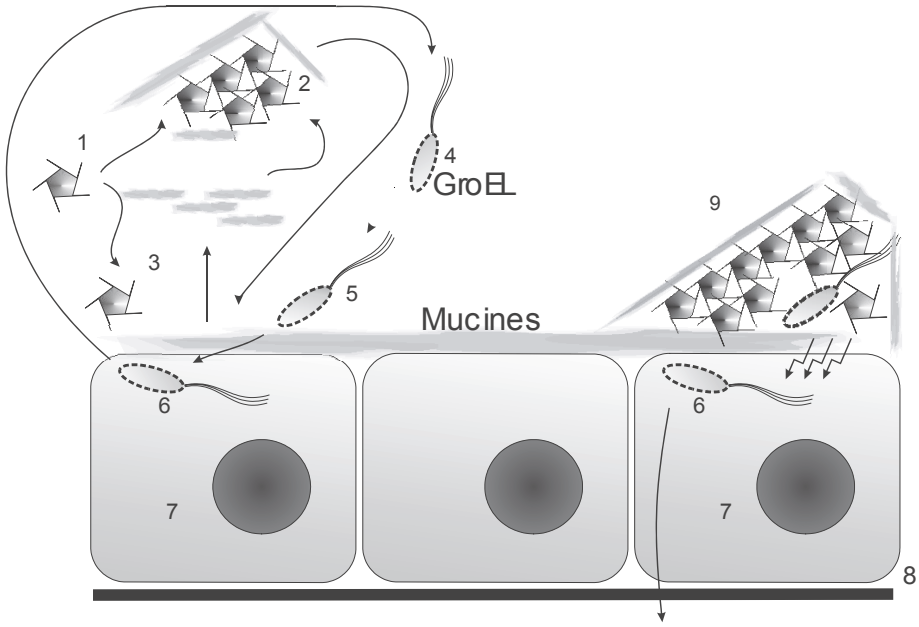


***Fig**.1. Interactions between bacteria, mucins and crystals in enterocystoplasties. 1= free crystal, 2= agglomeration of crystals mediated by mucins secreted by the epithelial surface, 3= adherent crystals to the epithelium, 4= free P.mirabilis, 5= adherent P.mirabilis, mediated by GroEL ⇔ mucin interaction, 6= invaded bacteria, safe from antibiotics, with the possibility to pass the lamina propria or restart the cycle after cell lysis, 7=epithelial cell, 8= lamina propria, 9= adherent agglomerate of crystals , mucins and bacteria, initiating the formation of an infection stone.*

Summary

Samenvatting

In dit proefschrift wordt de vorming van infectiestenen geëvalueerd in enterocystoplastieken onder invloed van *P.mirabilis* en mucines om de interactie tussen cellen, bacteriën en kristallen in dit proces op te helderen.
Er was bij het begin van dit onderzoek weinig bekend van *Proteus mirabilis* hechting in enterocystoplastieken en hun rol bij steenvorming in enterocystoplastieken werd vrijwel uitsluitend toegeschreven aan de aanwezigheid van urease. Er werd hierbij veelal voorbijgegaan aan de definitie van een infectie: "Het zich na besmetting handhaven en vermenigvuldigen in weefsels van ziekteverwekkende parasieten, schimmels, bacteriën of virussen waardoor plaatselijke ensteking of ziekte van het gehele organisme wordt veroorzaakt". Men richtte zich voornamelijk op het symptoom "bacteriurie" i.p.v. de ziekte "symptomatic infection/ cystitis" die gecorreleerd was met steenvorming in de condities van een enterocystoplastiek en een interactie met cellen verondersteld. Hierin is bacteriële hechting de eerste stap is gevolgd door een cellulaire reactie en in relatie staat tot een groot aantal cellulaire, urinaire en bacteriologische factoren. De sterke correlatie tussen het symptoom, bacteriurie, en de ziekte, cystitis, in de anatomisch normale blaas leidt tot misinterpretaties van gegevens uit de enterocystoplastiek. Het onderzoek zou zich gaan ontwikkelen in de richting van bacterie/ crystal/ cel interacties in enterocystoplastieken. In enterocystoplastieken lijken voornamelijk mucines als belangrijk bestanddeel van mucus en met name gesulfateerde mucines en hun suikerresidu's een belangrijke rol te spelen. Zij zijn een risicofactor in de hechting aan het epitheel van de verschillende componenten die samen met urine en intra-epitheliale componenten een therapie resistente blaassteen vormen.

Risico factoren en Resistentie

De belangrijkste risicofactor voor infectiesteenvorming blijkt een symptomatische infectie te zijn. Verder blijken ook bijkomende heelkundige ingrepen tijdens of na de blaasaugmentatie van invloed te zijn op de kans op steenvorming en infectie (Hoofdstuk 2). Mogelijk geldt dit ook voor kinderen met een vaginale reconstructie of cloacale malformatie. Deze en andere aandoeningen vereisen in sommige gevallen ingrepen in de blaas die een hogere kans geven op zowel infectie als blaassteenvorming (Hoofdstuk 3). Het voorkomen van deze infecties in combinatie met profylaxis d.m.v. gangbare antibiotica heeft een toenemend effect op resistentievorming en is minder effectief als bescherming tegen steenvorming. De mogelijkheid in cellen te penetreren biedt de bacteriën de kans zich terug te trekken uit de vijandige omgeving van het blaaslumen en geeft, in dit geval, *P.mirabilis* de kans om resistent te worden tegen de antibiotica in het lumen (Hoofdstuk 5).

De rol van mucines

Mucines zijn een belangrijk onderdeel van de epitheliale barrière en beschermen het epitheel tegen mechanische en chemische beschadiging. Het is echter ook bekend dat sommige mucines kunnen functioneren als een belangrijke hechtingsplaats voor zowel pathogene als niet pathogene bacteriën. Verder kunnen mucines de vorming van kristallen induceren of bevorderen. Dat mucines ook voor agglomeratie van kristallen kunnen zorgen –een veel sneller proces-, is beschreven in hoofdstuk 4. Deze mucines, die in een enterocystoplastiek voornamelijk door de intestinale pouch worden gesecreteerd in de urine kunnen zowel een beschermende rol hebben als een hechtingsplaats bieden voor bacteriën (Hoofdstuk 6 en 7). Het lijkt erop dat een bacterieel proteïne dat gevormd wordt onder invloed van stress, GroEL, een rol speelt in de hechting van *P.mirabilis,* aan het MUC2 en MUC5AC geproduceerd door het epitheel. Deze

hechting is weer de eerste stap van de infectie en de bescherming van de bacterie tegen het antibioticum. Het is mogelijk deze hechting te inhiberen en daarmee de cyclus te onderbreken.

Het sluiten van de cyclus.
Als GroEL (Hsp60/Cpn60) inderdaad de bacteriële tegenpool is in de interactie met MUC2 en MUC5AC in de enterocystoplastiek, dan wordt een volgorde van interacties duidelijk die kan leiden tot de vorming van een infectie steen. In deze interacties spelen mucines een verbindende rol tussen de interacties van bacteriën, cellen en kristallen. Er blijft wat betreft kristalvorming tot op zekere hoogte een scheiding tussen het intra- en extracellulaire milieu. Kristallen in suspensie hebben de mogelijkheid zich te vormen bij het stijgen van de urinaire pH en om te agglomereren onder invloed van mucines. Intracellulair kunnen eveneens kristallen worden gevormd op een gelijkaardige manier, maar vereist dat de cel niet meer in staat is haar eigen pH te regelen.
De gevormde kristallen kunnen worden gekoloniseerd door bacteriën. Dit kan direct op het kristaloppervlak maar ook indirect door tussenkomst van mucines. De mogelijkheid van kristallen om een interactie aan te gaan met mucines betekent tevens dat de mogelijkheid bestaat dat kristallen zich direct hechten aan het oppervlak van de epitheelcel die deze mucines produceert.
Proteus mirabilis kan door zich te hechten aan het epitheel en zich terug te trekken in de epitheelcel een veilige haven creëren tegen een vijandig milieu. Deze bacteriën kunnen door de productie van urease kristallen in stand houden. Tegelijkertijd bezorgen ze de cel veel schade en kan cellysis de intracellulaire bacteriën (en kristallen) weer doen vrijkomen in het lumen, wat aanleiding kan geven tot meer resistentie tegen veel gebruikte antibiotica. Een lang in stand gehouden infectie kan aanleiding geven tot een slecht behandelbare infectiesteen. De eerste stap van de infectie is weer de hechting van de bacterie aan het epitheel, waarin mucines MUC2, MUC5AC en GroEL waarschijnlijk een belangrijke rol spelen

Summary

In this thesis the formation of infection stones by *P.mirabilis* and mucins in enterocystoplasties is evaluated to clarify the interaction between cells, bacteria and crystals in the stone formation process

Little was known about *Proteus mirabilis* adhesion to enterocystoplasties at the start of this research project and stone formation in enterocystoplasties was almost exclusively contributed to the presence of urease. Most often this notion ignored the definition of a symptomatic infection: "The multiplication and maintenance of pathogenic bacteria, fungi, parasites or viruses after contamination, causing local inflammation or systemic disease of the host". Researchers primarily focused their attention to the symptom " bacteriuria" rather then the disease "symptomatic infection/ cystitis", which correlated with stone formation in enterocystoplasties and suggests an interaction with cells. Bacterial adhesion is the first step in this interaction, followed by a cellular reaction which is related to a great number of cellular, urinary and bacteriological factors. The strong correlation between the symptom, bacteriuria, and the disease cystitis in the anatomically normal bladder leads to misinterpretations of data acquired from enterocystoplasties. Research would evolve towards bacterial/ crystal and cellular interactions in enterocystoplasties. In enterocystoplasties, mostly mucins seem to play an important role as an important component of mucus and in particular the sulfated mucins and their carbohydrate residues. They are a risk factor in the adhesion of the several components that in conjunction with urine and intra-epithelial components act to form a therapy resistant bladder stone.

Risk factors and Resistance

The most important risk factor for infection stone formation seems to be a symptomatic infection. Furthermore, concomitant surgical procedures during or after bladder augmentation appear to influence the risk of stone formation and infection (*Chapter 2)*. Possibly this may also apply to children with a vaginal reconstruction or cloacal malformation. These and other conditions may sometimes require bladder surgery, which increases the risk for both infection and bladder calculi *(Chapter 3)*. The occurrence of these infections combined with the most common antibiotic prophylaxis increases resistance and supplies less protection against stone formation. The potential to invade cells, provides the bacteria with the opportunity to retreat from the hostile bladder lumen into the cells and thus gives *P.mirabilis* a chance to acquire resistance to the antibiotics in the lumen *(Chapter 5)*.

The role of mucins

Mucins are an important component of the epithelial barrier and protect the epithelium from mechanical and chemical erosion. Nevertheless, mucins have been known to act as important adhesion molecules for both pathogenic and non-pathogenic bacteria. Furthermore, mucins may induce or enhance the formation of crystals. That mucins may cause agglomeration of crystals, a much faster process, has been described in *Chapter 4*. These mucins, which are mostly secreted in urine by the intestinal pouch in enterocystoplasties may have both a protective role while providing the bacteria with a adhesion site *(Chapter 6* and *7)* It seems that a bacterial protein, expressed under stress, GroEL, contributes to the adhesion of *P.mirabilis* to the MUC2 and MUC5AC, produced by the epithelium. This adhesion is the first step of infection and the bacterial protection from the antibiotic. This adhesion can be inhibited and break the cycle.

Closing the cycle

If GroEL (Hsp 60/Cpn60) is indeed the bacterial counterpart in the interaction with MUC2 and MUC5AC in enterocystoplasties, a sequence of interactions appears that may lead to the formation of an infection stone. In these interactions mucins play a connecting role between the interactions of bacteria, cells and crystals. In regard to crystal formation to some extent a separation between the intra and extracellular environment remains. Crystals in suspension have the opportunity to form due to a rise in the urinary pH level and agglomerate mediated by mucins. Crystals may also form intracellular in a similar fashion, but requires a loss of cellular pH regulation.

The crystals may be colonized by bacteria. This may occur directly on the crystal surface or indirectly by the mediation of mucins. The capability of crystals to interact with mucins implies the possibility for crystals to adhere directly to the epithelial surface, producing these mucins.

Proteus mirabilis may create a safe haven from a hostile environment by epithelial adhesion and invasion. These bacteria can maintain crystals by the production of urease. At the same time they exert a lot of damage to the cell and cause cellular lysis liberating intracellular bacteria (and crystals), which may lead to an increased resistance to common antibiotics. A persisting infection can give rise to therapy resistant infection stones. Again the first step of an infection is adhesion of the bacteria to the epithelium with a probably important role for the mucins MUC2, MUC5AC and GroEL

Acknowledgement, Publications and CV

Dankwoord

Velen zijn gekomen en weer gegaan in de periode die dit proefschrift beslaat. Dit proefschrift is het gevolg van de prettige samenwerking met en de inzet van vele mensen en verschillende afdelingen. Velen die terloops een helpende hand hebben toegestoken, logistieke en morele steun hebben gegeven zou ik hier willen vermelden. Ik zou hier iedereen willen bedanken die direct of indirect een bijdrage heeft geleverd aan dit proefschrift. Met het gevaar toch namen over te slaan zou echter een aantal mensen met name willen noemen:

In de eerste plaats natuurlijk mijn directe begeleider en co-promotor Dik Kok. De nonchalante, relaxte manier waarmee jij onderzoekers begeleidde was onterecht een doorn in het oog van velen. Zelfs ik begreep in het begin niet wat ik met jou aan moest tot Dr. Rien Nijman mij op het hart drukte iets meer relaxed te zijn, en hij had gelijk. Ik heb veel gehad aan jouw steun en inzet. De echte waardering kwam toen wij het onderzoeksvoorstel voor de Nierstichting schreven en we in 3 maanden tijd een groot deel van het onderzoek moesten verwoorden om het op de valreep in te kunnen dienen. De waardering groeide voor je geduld, de manier waarop je het beste uit mensen naar boven wist te halen en de enthousiaste manier waarop je nieuwe ideeën benaderde . Ik heb nog steeds zeer goede herinneringen aan de congressen waar we samen heen zijn geweest en de discussies die we hebben gevoerd. Je stond altijd open voor een nieuw idee en gaf mij de vrijheid ze uit te werken. Drie hoofdstukken waren eigenlijk het resultaat van een middag in de zomerzon op een terras een wandeling langs Lake Michigan en een parkbankje in Chicago wachtend op het begin van de laatste lezing.

Dr.Nijman, beste Rien, jij bent jarenlang de stille, maar altijd aanwezige kracht geweest achter de schermen en de directe verbinding naar de kliniek. Als student ben ik bij jou begonnen en heb jij mij wegwijs gemaakt op de afdeling, voorzien van de nodige morele steun of advies en werd de kans geboden het onderzoek op te zetten, waarvoor nog altijd dank. Toen was het nog Dr. Nijman, na een paar jaar werd het langzaam Rien en sinds kort professor Nijman. Het onmisbare klinisch oordeel dat u gaf vond zij weerslag in het gehele proefschrift.

Verder wil ik m'n promotoren Prof. Verbrugh en in het bijzonder Prof. Schröder bedanken voor hun persoonlijke inzet bij de totstandkoming van dit proefschrift en de nodige correcties.De Stichting Urologisch Wetenschappelijk Onderzoek en de afdeling Klinische Microbiologie en Infectieziekten wil ik bij deze bedanken voor de jarenlange financiële ondersteuning zonder welke dit onderzoek niet mogelijk was geweest.

Beste Kees, ook jouw invloed is duidelijk aanwezig in dit proefschrift. Ik heb nog steeds moeite om zonder verdere test met enkel de koloniemorfologie, de geur en verkleuring van de plaat te bepalen welke bacterie is gekweekt, maar dat komt misschien nog wel. Je hebt me altijd terzijde gestaan met je opgewekte persoonlijkheid, logistieke en morele steun en een kritische blik voor methoden en interpretaties van bacteriologische gegevens. Meestal was de manier om een kritisch oordeel op een manuscript uit te lokken de vermelding: " Laatste versie". Hierop volgde veelal mijn verongelijkte antwoord "Dat weet jij toch ook?". Duidelijk is het mede dankzij jou geworden.

Lieve Sandra en Ingrid, jullie waren met de kennis over mucines en met name MUC2 en MUC5AC een belangrijke vraagbaak bij de afwezigheid van Jan Dekker. Sandra, het inzicht dat jij wist te verschaffen en de open manier waarop je met onderzoeksvragen omgaat maakte dat het laboratorium kindergeneeskunde uitnodigde tot meer. Er zouden meer vrouwelijke UHD's moeten komen en meer zoals jou. Lieve Ingrid, AJAX Rules! Theo bedankt voor de prettige sfeer en plek waar ik altijd heen kon met een idee. Ik wil jou bedanken voor je inzet, gewiekste opmerkingen en je onmisbare kennis op het gebied van gelelectrophoresis.

Pim, Ton en Piet, jullie EM foto's waren essentieel voor m'n onderzoek. Het was erg moeilijk kiezen uit alle mooie foto's, het is mij niet gelukt, vandaar het kaftje. Ik hoop dat jullie het toch mooi vinden.

Dear Mazen, you were the last one to leave the laboratory every evening to finish your work on Wilm's tumors. The long discussions and your inspiring drive to reach for the best have led to many joint manuscripts and publications and I'm very happy to hear about your upcoming promotion. I wish you the best of luck and hope to stay in touch.

Ad de Jong, Hugo Meijring en Jan ten Hove, bij deze wil ik jullie bedanken voor de gastvrijheid en de identificatie van GroEL tussen de bedrijven door. Jullie kennis van massaspectrometrie was van grote waarde en zonder de trips naar Bilthoven was dit proefschrift nooit zo mooi geworden.

Ik zou verder iedereen van het laboratorium Klinische Microbiologie en BAClab willen bedanken voor hun steun, advies, geduld en de prettige sfeer; met name Anton, Mariëlle, Marieke, Wim, Diana, Roel, Will, Alex, en bovenal Cindy van der Schee voor alle hulp op het moleculair biologisch vlak. Rood haar blijft mooi.

Iedereen van het Experimenteel Urologisch Lab, de Steengroep en de Kinderurologie en de afdeling Pathologie van het Josephine Nefkens Instituut zou ik bij deze willen bedanken. Met name Hans Romijn, Wytske van Weerden, Guido Jenster, Carl Verkoelen en Paul Verhagen, Ellen Zwarthoff, Winand Dinjens, Robert Kraaij, Gert-Jan Steenbrugge, Katja Wolffenbuttel, Ellen van den Berg en Brigitte van de Graaf, maar ook Arnold Hekman, Burt van der Boom, Niels-Peter Buchholz en Saima, Ruud de Water en Cindy, Karel van Wely, Nico en alle anderen die reeds vertrokken zijn. Alle analisten van het lab wil ik bij deze bedanken, in het bijzonder Joke en Laura, Monique Oomen en Corrina, Wilma, Sigrun (het zonnetje), en Angelique, alsook de AIO's, en de vele studenten die op het lab hebben gewerkt. Marieke en Marino, succes met jullie promotie.

Bij deze wil ik mijn paranimfen, mijn broer Anil en m'n goede vriend Reinout Haverkamp, bedanken voor hun steun, hun luisterend oor en hun bereidheid mij ook nu terzijde te staan.
M'n ouders en broer wil ik bij deze bedanken voor hun vertrouwen, steun en advies wanneer het even tegen zat.
Papa, mama, het boekje is af. Ik hoop dat jullie het mooi en de moeite waard vinden. Nog 2 jaar studeren en dan ben ik klaar

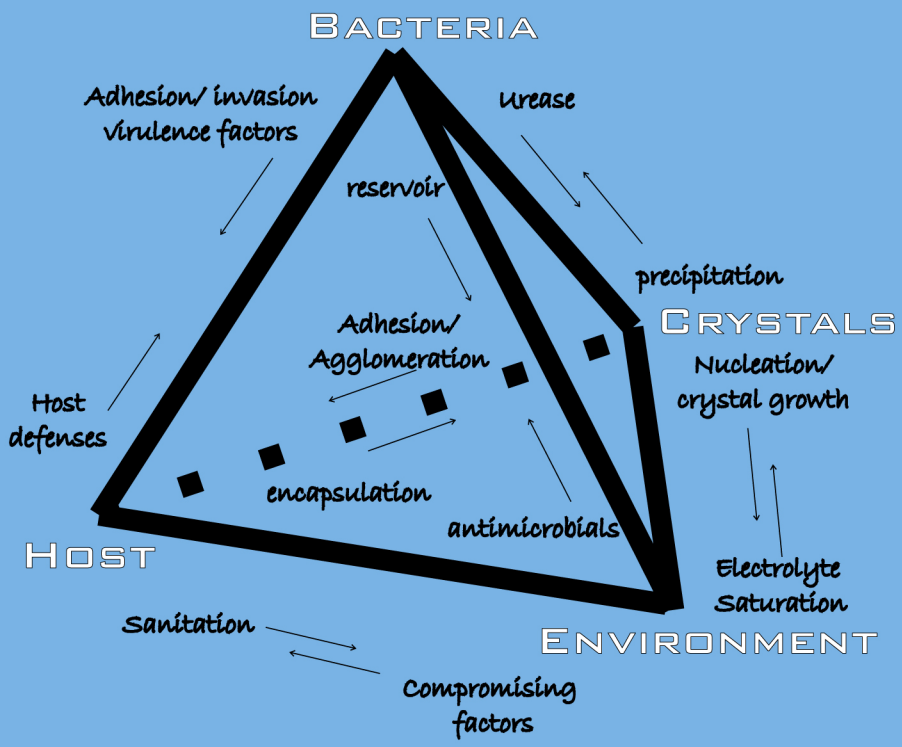
Rejiv

List of publications

1. Mathoera, R.B., D.J. Kok, R.J.M. Nijman. 2000. Bladder calculi in augmentation cystoplasty in children. Urology 56: 482-487 (*Chapter2*)
2. Mathoera, R.B., D.J. Kok, W.J. Visser, C.M. Verduin, R.J.M. Nijman. 2001. Cellular membrane associated mucins in artificial urine as mediators of crystal adhesion: an in vitro enterocystoplasty model. J Urol. 166: 2329-2336 (*Chapter4*)
3. Mathoera, R.B., D.J. Kok., C.M., Verduin, R.J.M., Nijman. 2002. Pathological and therapeutical significance of cellular invasion by Proteus mirabilis in an enterocystoplasty infection stone model. Infect. Immun. 70(12): 7022-7032 (*Chapter5*)
4. Mathoera, R.B., D.J. Kok, R.J.M. Nijman. 1999. Stone formation and risk factors in augmentation cystoplasty. Proceedings of the 8th Symposium on Urolithiasis p. 593-595
5. Mathoera, R.B., C.M. Verduin, D.J. Kok., R.J.M. Nijman. 2001. Urinary tract infections:A cellular perspective Proceedings of the 9th Symposium on Urolithiasis p. 163-165
6. Ghanem M.A., van der Kwast Th.H., Den Hollander J.C., Sudaryo M.K., Mathoera R.B., Van den Heuvel M., Noordzij M.A., Nijman R.J.M., van Steenbrugge G.J., Expression and prognostic value of epidermal growth factor receptor, transforming growth factor alpha and c-erbB-2 in nephroblastoma. Cancer, 92:3120-3129, 2001
7. Ghanem M.A., Van Steenbrugge G.J., Sudaryo M.K., Mathoera R.B., Nijman J.M., Van der Kwast Th..H. Expression and prognostic value of vascular endothelial growth factor (VEGF) and its receptor (Flt-1) in nephroblastom. Accepted J of Clinical Pathology, *in press*

Curriculum vitae

The author of this thesis was born on the 3rd of February in Oranjestad Aruba 1973 and immigrated to The Netherlands in 1977. He attended the Rotterdamsch Lyceum, where he finished the Gymnasium B in 1991 and started to study medicine in Antwerp, Belgium and finished his medical candidature in 1995 and first doctoral year at the Catholic University of Leuven. The author moved to Rotterdam to finish his medical studies in 1997 and started this research project in 1998 at the pediatric urology division of the Erasmus University. He finished his doctoral fase in 1999 during this research project. In 2003 the author started his internship at the Erasmus MC.


BACTERIA
Adhesion/ invasion
virulence factors
Urease
reservoir
precipitation
CRYSTALS
Adhesion/
Agglomeration
Nucleation/
crystal growth
Host
defenses
encapsulation
antimicrobials
HOST
Electrolyte
Saturation
Sanitation
ENVIRONMENT
Compromising
factors