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Title:
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Investigating diagnostic approaches to feline subclinical bacteriuria and urinary tract infections and responsible use of antimicrobials.

To Treat or Not to Treat: Investigating Diagnostic Approaches to Feline Subclinical Bacteriuria and Urinary Tract Infections and Responsible Use of Antimicrobials

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Science by Research in the Faculty of Health Sciences.

Bristol Veterinary School

November 2019

Word Count – 31,571

ABSTRACT

Subclinical bacteriuria is reported in up to 25-30% of cats, particularly those with predisposing factors such as chronic kidney disease, diabetes mellitus or hyperthyroidism. The diagnostic approach and treatment guidelines for feline subclinical bacteriuria are not always clear which can lead to the overuse of antimicrobial agents and thus the selection of multidrug-resistant bacteria.

This study reported the prevalence of subclinical bacteriuria in a population of 56 cats referred to Langford Vets Small Animal Hospital and described all cases of positive urine cultures as a series. Demographic data was collected for each cat and medical records were screened to identify any at-risk populations for subclinical bacteriuria or urinary tract infections. Routine urinalyses, including sediment examination and urine culture were performed on voided urine samples and cystocentesis samples. Proteomic techniques were utilised to identify a biomarker of infection or inflammation of the lower urinary tract. Finally, a method comparison study was conducted to investigate the effect of non-absorbent hydrophobic sand litter on the urine protein-to-creatinine ratio followed by a study comparing the effect of urine collection technique on common urinalysis parameters.

The prevalence of subclinical bacteriuria in this population was 4%. All affected cats were female and over 6 years old. Myeloperoxidase was identified in all cases of subclinical bacteriuria and urinary tract infection and so further studies exploring the utility of this enzyme as a biomarker seem justified. Non-absorbent hydrophobic sand litter did not interfere with urine protein-to-creatinine ratio measurements and agreement between the ratio determined from voided urine and cystocentesis samples appeared excellent for urine with an inactive sediment and urine protein-to-creatinine ratio <0.5. Bacterial growth was significantly more likely in voided samples compared to cystocentesis samples, $p<0.0005$, although this difference was not significant when accepted quantitative cut-off values for bacterial growth in urine were applied, $p=0.5$.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Christina Maunder and Marta Costa, for your endless guidance, encouragement and kindness over the past 12 months. You have both supported me more than you probably realise and for that I will always be grateful. I'd also like to thank Professor Matthew Avison for introducing me to field of proteomics and sharing your passion and enthusiasm for antimicrobial resistance.

Thank you to the entire team at the Langford Vets Diagnostic Laboratory for making me feel so welcome and for sharing your skills and expertise in your respective fields. Particularly the biochemistry and microbiology departments that aided with sample processing for cystocentesis samples, including urine culture and susceptibility testing. I must also thank the feline nursing team for all your help in collecting my samples.

I'd like to thank The Proteomics Facility at the University of Bristol for running the proteomic analysis of all urine samples and providing me with the datasets for analysis. To the Animal Welfare and Behaviour group at Langford, thank you for adopting me into your group, Wednesdays will not be the same without 11 o'clock coffee and cake. The past year would not have been the same without some of the friendships I am leaving here with today.

Thank you to BSAVA PetSavers for the funding of this project. The knowledge and skills I have gained will prove useful throughout my career.

Lastly, I cannot thank my friends and family enough for your continued love and support. You were there when I needed you most and you will never know how much that meant to me. The biggest thank you of all goes to my mum. You've fought my corner from day one, you're my best friend and I hope I've made you proud.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: J. KENNILS DATE: 12/11/19

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LIST OF ABBREVIATIONS

ACVIM – The American College of Veterinary Internal Medicine

AST – Antimicrobial susceptibility testing

CFU – Colony forming unit

CKD – Chronic kidney disease

CLED – Cystine-Lactose-Electrolyte-Deficient

DM – Diabetes mellitus

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

EQUC – Expanded quantitative urine culture

HMP – The Human Microbiome Project

HPF – High power field

IRIS – The International Renal Interest Society

ISCAID – The International Society for Companion Animal Infectious Diseases

LC – Liquid chromatography

MS – Mass spectrometry

NICE – National Institute for Health and Care Excellence

OD – Optical density

RAI – Radioactive iodine

RBC – Red blood cell

rRNA – Ribosomal ribonucleic acid

SAVSNET - Small Animal Veterinary Surveillance Network

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide electrophoresis

SUB – Subcutaneous ureteral bypass

UPC – Urine protein-to-creatinine ratio

USG – Urine specific gravity

UTI – Urinary tract infection

WBC – White blood cell

Chapter 1 INTRODUCTION

1.1 BACKGROUND

Clinical signs of lower urinary tract disease occur frequently in cats, yet urinary tract infections (UTI) confirmed by bacterial culture represent only a small percentage of cases presenting with such signs. In contrast, up to 30% of urine cultures from cats without any current or historical signs of lower urinary tract disease may be positive for bacterial growth (Litster *et al.*, 2009). These apparent contradictory findings can make antimicrobial prescribing challenging for veterinary practitioners. Clear evidence-based guidelines exist in human medicine outlining when antimicrobial therapy is warranted in cases of positive urine cultures (Nicolle *et al.*, 2005). Although guidelines do exist in veterinary medicine, the evidence base is somewhat lacking and many recommendations have been extrapolated from human data (Weese *et al.*, 2019).

Antimicrobial therapy is not a benign treatment and their unnecessary use has been associated with selection of multidrug-resistant bacteria, dysbiosis of intestinal flora leading to an increased risk of *Clostridium difficile* infections and a range of gastrointestinal side effects (Trautner, 2012). The close physical relationship between companion animals and their owners may play a role in the dissemination of antimicrobial resistance, impacting both human and animal health (Harrison *et al.*, 2014). In this era of One Health, antimicrobial stewardship is therefore of utmost importance in both human and veterinary medicine.

Urinary tract infections are a leading cause of antimicrobial prescription in human and veterinary medicine (Cordoba *et al.*, 2015). The current gold standard test for the diagnosis of UTI, urine culture, can take 48 hours or longer to obtain results (Bartges, 2004). Urine culture is not a perfect test and a need therefore exists for improved ways in which to diagnose UTI and subclinical bacteriuria to enable prudent use of antimicrobials in the future.

1.2 AIMS AND OBJECTIVES

This research project aimed to identify the prevalence of subclinical bacteriuria and UTI in a population of cats referred to the Small Animal Hospital of Langford Vets as well as identify any results of urinalyses that could discriminate between subclinical and clinical infections. Diagnostic tests were applied to voided urine samples and cystocentesis samples to determine whether results were valid for both methods of collection.

As a preliminary investigation, proteomic techniques were utilised to identify potential urinary biomarkers for feline subclinical bacteriuria and UTIs. Again, proteomic techniques were applied to voided urine samples and cystocentesis samples to compare differences in protein expression between methods of collection. Any potential biomarkers identified through proteomic analysis could be exploited for future research with a long-term aim of the development of a patient-side test for the diagnosis of subclinical bacteriuria or UTI.

During data collection it became clear the prevalence of subclinical bacteriuria and UTI within our population was insufficient to provide adequate power for statistical analyses to be performed and meaningful conclusions be drawn. Other research questions had been generated from the preliminary work of this project and the decision was made to explore these areas further alongside describing any cases of subclinical bacteriuria and UTI and highlighting differences between methods of urine sampling.

The four main themes that were investigated as part of this project and their intended outcomes were:

1) To investigate the diagnostic approach to feline subclinical bacteriuria and UTI:
    i) Determine the prevalence of subclinical bacteriuria and UTI within a referral population.
    ii) Describe all cases of subclinical bacteriuria and UTI highlighting any differences in urinalyses results between voided samples and cystocentesis samples.
    iii) Review the medical records of cases of subclinical bacteriuria to identify any at-risk populations.

2) Proteomic analysis of urine sediments and cultured bacteria:
    i) Identify a potential biomarker(s) that could be used to discriminate between subclinical bacteriuria and UTI in cats.
    ii) To determine whether any potential biomarker identified was present in voided samples and cystocentesis samples.
    iii) To determine whether proteomic techniques could identify the causative pathogen of any case of subclinical bacteriuria or UTI.

3) To investigate the effect of non-absorbent hydrophobic sand litter on urine protein-to-creatinine ratio (UPC) measurements:

   i) To evaluate whether a commonly used non-absorbent litter accounts for any preanalytical variability when determining the UPC in cats.

4) To investigate the effect of urine sample collection technique on common urinalysis parameters, particularly the UPC:
   i) To determine whether results of urinalyses, including sediment examination, and urine culture different significantly between voided samples and cystocentesis samples.
   ii) To assess whether the UPC determined from voided urine samples collecting using non-absorbent hydrophobic sand litter differed significantly from the UPC determined from cystocentesis samples.
   iii) To measure agreement between the UPC of voided urine samples and cystocentesis samples and assess whether this differed according to urine sediment status.

1.3 Potential Benefits of this Work

At present there is currently a lack of good evidence regarding the treatment of subclinical bacteriuria in cats. Little is also known about the prevalence of subclinical bacteriuria in the United Kingdom. In human medicine clear guidelines exist outlining when antimicrobial therapy is warranted in cases of asymptomatic bacteriuria yet up to one-third of patients with asymptomatic bacteriuria are prescribed antimicrobials unnecessarily (Kjolvmark *et al.*, 2016). A need therefore exists for a test to be able to differentiate between UTI and asymptomatic bacteriuria/subclinical bacteriuria in order to reduce the overuse of antimicrobials.

In veterinary medicine, cystocentesis is considered the gold standard means of urine collection when bacterial culture is required. This is an invasive procedure whereby urine is collected percutaneously from the bladder using a sterile needle and syringe. Sedation may be required to facilitate urine collection by cystocentesis which can lead to increased client costs, an increased duration of visit to the veterinary practice and increased patient stress, all of which could be mitigated if voided urine samples collected in the home environment were shown to be of diagnostic value.

Urine culture and susceptibility testing can take at least 48 hours to obtain results. Empiric antimicrobial therapy is often initiated in cases of suspected UTI and studies have shown that even upon receipt of negative culture results, veterinary surgeons do not always advise clients to discontinue antimicrobial therapy (Sorensen *et al.*, 2018). The use of fluoroquinolone and cephalosporin antimicrobials has been shown to select for methicillin-resistant *Staphylococcus aureus* and extended-spectrum β-lactamase producing-*Escherichia coli (E. coli)* in human medicine and so their overuse is of concern (Guardabassi and Prescott, 2015). Proteomic

techniques have the potential to identify a biomarker which could seed the development of a rapid patient-side test; this in turn would improve antimicrobial prescribing in veterinary practice and reduce the development of antimicrobial resistance.

The UPC is frequently determined in number of disease states including glomerulopathies, hypertension and chronic kidney disease (CKD) and it has both diagnostic and prognostic implications (Lees *et al.*, 2005, Syme, 2009). Correlation exists between UPCs determined from voided samples and cystocentesis samples in dogs and cats, but it is not known whether the use of non-absorbent hydrophobic sand litter interferes with UPC measurement (Beatrice *et al.*, 2010, Vilhena *et al.*, 2015). Should it be proved that the use of a non-absorbent litter does not affect UPC measurement, urine samples intended for this purpose could also be collected in the home environment. This work has the potential to increase the frequency of which the UPC is determined in feline patients enabling disease processes to be picked up, and therefore treatment plans be instigated, earlier.

1.4 Thesis Outline

This thesis begins with a review of the current literature concerning feline subclinical bacteriuria and UTI, urinary proteomics, the urinary microbiome and the UPC. This review concentrates on the aforementioned areas within feline medicine but where appropriate comparisons to other veterinary species and human medicine are made.

Following on from this are chapters describing the methodology and results for each study within the project. Any conclusions drawn and any potential limitations of each study are outlined in the discussion chapter. The final chapter of this thesis discusses opportunities for future research that have arisen as part of this project.

Full descriptions of proteins identified in each urine sample in Sections 4.2.2 and 4.2.3 are provided as supplementary information (S1-S14). Data are available at the University of Bristol data repository, data.bris, at http://doi.org/10.5523/bris.2k17yid6ng8hu24xboldyrkj2y (Maunder & Kennils, 2020).

Chapter 2 LITERATURE REVIEW

2.1 URINARY TRACT INFECTIONS AND SUBCLINICAL BACTERIURIA

2.1.1 Definitions

Bacterial UTI refers to bacterial colonisation of the urinary tract in which bacterial adhesion and invasion of host cells, replication and survival incites an inflammatory response and clinical signs of lower urinary tract disease (Teichmann-Knorrn *et al.*, 2018, Weese *et al.*, 2019).

Subclinical bacteriuria is defined as a positive bacterial urine culture in the absence of lower urinary tract clinical signs. The most recent guidelines by the International Society for Companion Animal Infectious Diseases (ISCAID) for the diagnosis and management of bacterial urinary tract infections in dogs and cats refined this definition to specify that urine samples should be 'properly collected' discouraging clinicians from using voided urine samples for bacterial culture (Weese *et al.*, 2019). Subclinical bacteriuria has previously been referred to as: asymptomatic bacteriuria, occult urinary tract infections and covert urinary tract infections (Litster *et al.*, 2009, Eggertsdottir *et al.*, 2011). Asymptomatic bacteriuria is the term used to describe the same phenomenon in human patients and has been well studied (Nicolle *et al.*, 2005).

2.1.2 Prevalence

The prevalence of UTI in cats presenting with lower urinary tract signs has typically been reported as <3% (O'Neill *et al.*, 2014, Dorsch *et al.*, 2015), however other studies across Europe suggest the prevalence may be as high as 12% (Gerber *et al.*, 2005, Saevik *et al.*, 2011). The reported prevalence of subclinical bacteriuria in cats varies widely from 0.9-29% (Litster *et al.*, 2009, Eggertsdottir *et al.*, 2011). More recent prospective studies report a more consistent prevalence of 6-13% (White *et al.*, 2016, Moberg *et al.*, 2019).

Over a five-year period, Litster *et al.* (2009) identified 38 out of 132 (29%) urine samples obtained by cystocentesis from cats with no prior history of lower urinary tract clinical signs were positive on culture. The median age of cats with a positive culture in this study was 14 years which was considerably higher than the median age of 4 years from the Eggertsdottir *et al.* (2011) study. Eggertsdottir *et al.* (2011) conducted a prospective study to identify the prevalence of subclinical bacteriuria in healthy cats. One hundred and eight urine samples were obtained by cystocentesis from cats deemed healthy based on owner history and the results of a physical examination, complete blood count and serum biochemistry profile. One

urine sample was positive on culture with >10^5 cfu/ml of *Enterococcus* species and *Staphylococcus* species documented.

The large discrepancy in reported prevalence data is likely multi-factorial. Increased age has been identified as a risk factor for UTI and subclinical bacteriuria in cats (Lekcharoensuk *et al.*, 2001, White *et al.*, 2016). The inclusion criteria of the Eggertsdottir et al. (2011) study was stricter and prevented the inclusion of cats with CKD, diabetes mellitus (DM) and hyperthyroidism in which subclinical bacteriuria is more prevalent (Mayer-Roenne *et al.*, 2007, Bailiff *et al.*, 2008, White *et al.*, 2016). Geographically these studies were conducted in Australia and Norway respectively which also may have contributed to the difference in prevalence reported.

2.1.3 Risk Factors

Sporadic UTI occur less commonly in cats than dogs (Weese *et al.*, 2019). Risk factors predisposing to UTI or subclinical bacteriuria are reported in up to 87% of cats with positive urine cultures (Dorsch *et al.*, 2019).

Disease states such as CKD, DM and hyperthyroidism have been associated with an increased risk of both UTI and subclinical bacteriuria (Mayer-Roenne *et al.*, 2007, Puchot *et al.*, 2017). Chronic kidney disease, DM and uncontrolled hyperthyroidism can lead to the production of poorly concentrated urine which may increase the risk of bacterial colonisation of the urinary tract (Sparkes *et al.*, 2016, Sparkes *et al.*, 2015, Carney *et al.*, 2016). The association between urine specific gravity (USG) and UTI has been evaluated in many studies with conflicting conclusions (Martinez-Ruzafa *et al.*, 2012, Bailiff *et al.*, 2006, Bailiff *et al.*, 2008, Lund *et al.*, 2013, Mayer-Roenne *et al.*, 2007).

The largest of which is the retrospective study by Bailiff *et al.* (2008) in which the medical records and urinalysis results of 614 cats with either CKD, DM, uncontrolled hyperthyroidism or lower urinary tract disease were evaluated in order to identify risk factors for positive urine cultures. Positive urine cultures were identified in 17%, 13%, 22% and 5% of each group respectively. The authors found no association between a reduced urine concentrating ability and a positive urine culture in any disease group. These findings were in agreement with an earlier study by the same author in which an association between USG and UTI was not established in cats with DM (Bailiff *et al.*, 2006). This study is limited by its retrospective nature in that urine culture was only performed as directed by the attending clinician, prevalence rates of UTI in these patient groups may therefore have been over- or underestimated. By using control cats from the same disease group but with a negative culture, it is possible the

study control population also had reduced urine concentrating ability making associations between USG and UTI more difficult to ascertain.

In a later retrospective study of 155 cats with UTI and 186 control cats, Martinez-Ruzafa *et al.* (2012) identified that a reduced urine concentrating ability was significantly associated with UTI using a multivariable logistic regression model, p=0.0055. This study partly agreed with Mayer-Roenne *et al.* (2007) who identified a significant association between a USG <1.020 and a positive urine culture in cats with DM but no association was identified between USG and UTI in cats with CKD or hyperthyroidism.

Finally, Lund *et al.* (2013) evaluated the urinalyses of 111 cats that presented to a first opinion practice with clinical signs compatible with lower urinary tract disease. Cats were classified as having either: idiopathic cystitis, urethral plugs, bacterial cystitis or urolithiasis. Although USG was excluded from the statistical modelling of this study due to many of the cats receiving intravenous fluid therapy, the authors commented that no significant difference in USG was identified between the different causes of lower urinary tract disease.

The contrasting results from these studies suggest that mechanisms beyond a reduced urine concentrating ability such as impaired host defences may contribute to the increased prevalence of UTI reported in these patient groups.

Surgical interventions such as perineal urethrostomy or renal decompressive surgeries are reported risk factors for both UTI and subclinical bacteriuria (Bass *et al.*, 2005, Kopecny *et al.*, 2019). Griffin and Gregory (1992) proposed that underlying compromised host defences contributed more to the increased prevalence of UTI seen in cats with perineal urethrostomies rather than the anatomic changes caused by the surgery. In their study, increased prevalence of recurrent UTI and subclinical bacteriuria was documented in cats undergoing perineal urethrostomy due persistent urethral obstruction compared to healthy cats undergoing the same surgery.

Urological implants such as ureteral stents or subcutaneous ureteral bypass (SUB) devices are indicated in cases of ureteral obstruction that are non-responsive to medical management (Berent *et al.*, 2018). The most common cause of ureteral obstruction in cats is ureterolithiasis, which itself appears to predispose to UTI (Wormser *et al.*, 2016). In a retrospective study of 43 cats having either a ureteral stent or SUB device placed surgically, Kopecny *et al.* (2019) reported post-operative positive urine cultures in 25% of cats. This study also reported that cats that received post-operative antimicrobials were significantly less likely to develop a positive urine culture. Uropathogens such as *E. coli* and *Enterococcus* spp. are able to form

biofilms on urological implants, and this has been a proposed mechanism for the increased risk of positive urine cultures in this population (Arias and Murray, 2012, Flores-Mireles *et al.*, 2015, Kopecny *et al.*, 2019).

Long-term glucocorticoid treatment and other immunosuppressive agents have been reported as a risk factor for UTI in dogs (Torres *et al.*, 2005), but no evidence exists supporting this increased risk in cats (Lockwood *et al.*, 2018).

2.1.4 Pathogens

Common pathogens isolated from cats with UTI are also frequently isolated in cases of subclinical bacteriuria (Puchot *et al.*, 2017, Teichmann-Knorrn *et al.*, 2018). *E. coli* is consistently reported as the most frequent pathogen isolated from the urinary tract of cats, followed by *Staphylococcus* spp. and *Enterococcus* spp. (Lund *et al.*, 2015, Marques *et al.*, 2018).

In a retrospective study evaluating antimicrobial resistance patterns of feline uropathogens in Germany, Teichmann-Knorrn *et al.* (2018) reported that *Enterococcus* spp. were significantly more likely to be isolated from cats with subclinical bacteriuria compared to cats with UTI. Enterococcal infections are also frequently reported in cats with urological implants although whether these infections are more likely to produce clinical UTIs or subclinical bacteriuria is currently unclear (Berent *et al.*, 2018, Kopecny *et al.*, 2019).

Most cases of UTI and subclinical bacteriuria involve only one pathogen, although polymicrobial infections have been reported in up to 25% of cases (Byron, 2018). Where polymicrobial infections involve pathogens that may typically be seen as low-pathogenic such as *Enterococcus* spp., KuKanich and Lubbers (2015) suggest targeting antimicrobial therapy towards the more pathogenic organisms present may be successful in eliminating all organisms.

2.1.5 Diagnosis

2.1.5.1 Clinical signs

Clinical signs of lower urinary tract disease may include haematuria, dysuria, stranguria, periuria or pollakiuria although no combination of lower urinary tract disease clinical signs are pathognomonic for UTI (Gunn-Moore, 2003). The presence of lower urinary tract clinical signs cannot be used to exclude a diagnosis of subclinical bacteriuria as cases of idiopathic cystitis with concurrent subclinical bacteriuria have been reported (Lund and Eggertsdottir, 2019).

2.1.5.2 Urinalysis

Fundamentally, quantitative bacterial culture of urine is required for diagnosis of UTI or subclinical bacteriuria although the indications for urine culture in cats without clinical signs of lower urinary tract disease are few (Weese *et al.*, 2019). Results of urinalyses, including sediment examination, may increase suspicion of UTI or subclinical bacteriuria (Piech and Wycislo, 2018).

2.1.5.2.a Colour and Turbidity

Pigmenturia is reported to be a leading cause for cat owners to seek veterinary advice and in one recent study of 77 cats evaluated for lower urinary tract disease, 55% of cats presented with haematuria. Of 5 cats diagnosed with a UTI in the study, 4 presented with gross haematuria (Reppas and Foster, 2016a, Gerber *et al.*, 2005). Blue-green discolouration of urine has rarely been reported in association with *Pseudomonas aeruginosa* UTI (Reppas and Foster, 2016a).

In a study investigating the association between lower urinary tract clinical signs and UTI in 424 dogs, gross haematuria was found to be a significant predictor of UTI. Of 198 dogs presenting with gross haematuria, 54% were diagnosed with a UTI (Sorensen *et al.*, 2019). A similar study in cats has yet to be conducted, although the % of cats presenting with haematuria diagnosed with UTI would likely be much lower due to the increased prevalence of feline specific disease processes that can cause haematuria, such as idiopathic cystitis (Sparkes, 2018).

2.1.5.2.b Urine Specific Gravity

Whether a reduced USG predisposes to UTI or subclinical bacteriuria is discussed in section 2.1.3. In a retrospective study of 500 cats, USG did not differ significantly between cats with subclinical bacteriuria and control cats (Puchot *et al.*, 2017).

2.1.5.2.c pH

Urinary tract infections caused by urease-producing bacteria such as *Staphylococcus aureus*, *Staphylococcus felis*, *Proteus* spp., or *Klebsiella* spp. can lead to alkalinuria (Reppas and Foster, 2016a). In the study by Litster *et al.* (2009), in which 38 out of 132 urine samples obtained by cystocentesis from cats without clinical signs of lower urinary tract disease were positive on urine culture, a significantly higher urine pH was identified in culture positive samples, p=0.0002. The association between an increased urinary pH and subclinical bacteriuria however was not found in a more recent retrospective study (Puchot *et al.*, 2017). This disagreement may in part be due to the pathogens identified in each study, with a higher % of urease-producing bacteria cultured in the study by Litster *et al.* (2009).

2.1.5.2.d Dipstick Evaluation

Dipstick analysis provides a semiquantitative measure of the chemical properties of urine. Common parameters measured by standard dipsticks include specific gravity, pH, leukocytes, nitrites, protein, glucose, ketones, urobilinogen, bilirubin and blood/haemoglobin. Urine specific gravity, leukocytes, nitrite and urobilinogen are reported to be unreliable when analysing feline urine (Piech and Wycislo, 2018, Reppas and Foster, 2016a). Dipstick analysis can be performed manually, by visually comparing the colours of the reagent pad to a result chart, or by using an automatic dipstick reader. In environments where multiple personnel perform urinalysis, automated readers have been shown to provide superior precision compared to manual reading of results for glucose, ketones, bilirubin, blood, protein and pH. The most frequent error observed when performing manual readings is failing to observe waiting times prior to reading a result which is overcome by the use of an automated reader (Ferreira *et al.*, 2018).

In one study evaluating the dipstick leukocyte pad for the detection of pyuria in feline urine, Holan *et al.* (1997) compared the dipstick result to that obtained from manual microscopy for 213 urine samples. The authors reported a sensitivity and specificity of 77% and 34% respectively and a positive and negative predictive value of 14% and 91% respectively. The test performance reported is poor when compared to human medicine, in which sensitivities and specificities of 69-96% and 69-98% are reported. The authors concluded that the leukocyte pad of standard urine dipsticks is not reliable for the analysis of feline urine and that sediment examination should be utilised to confirm the presence of pyuria.

An increased prevalence of UTI and asymptomatic or subclinical bacteriuria has been reported in diabetic human, canine and feline populations although the specific mechanisms for increased risk of infection have yet to be properly defined (McGuire *et al.*, 2002, Bailiff *et al.*, 2006, Flores-Mireles *et al.*, 2015). The presence of glucosuria has been proposed as one mechanism of increased susceptibility to UTI by permitting increased bacterial growth although no direct association between glucosuria and UTI risk has been identified in human diabetic patients or diabetic dogs (Bailiff *et al.*, 2006, Chen *et al.*, 2009). Other proposed mechanisms of increased susceptibility to UTI in diabetic populations include: an altered host immune response to infectious agents, diabetic associated neuropathies altering micturition patterns and enhanced binding between uropathogens and uroepithelial cells (Bailiff *et al.*, 2006, Chen *et al.*, 2009).

One retrospective prevalence study of UTIs in diabetic cats did identify a significant association between glucosuria and risk of UTI development (Mayer-Roenne *et al.*, 2007). This is however in disagreement with a larger retrospective study evaluating urinalyses of diabetic cats with and without UTI in which no significant association between glucosuria and UTI was established (Bailiff *et al.*, 2006). Zeugswetter *et al.* (2019) recently demonstrated that very low concentrations of glucose were present in a population of 132 healthy cats. These concentrations were below the limit of detection for standard urinary dipsticks. Enrolled cats were considered to be euglycaemic based on blood glucose testing although a delay between blood sampling and glucose testing of up to 5 hours occurred in some cases. This delay could have allowed the inclusion of cats with a blood glucose concentration above the renal threshold of 14mmol/L as delayed sample processing without the addition of an anti-glycoltic agent can cause an artefactual reduction in blood glucose measurements. Further prospective studies are needed to enhance the understanding between glucosuria and UTI pathogenesis although the increased prevalence of UTI in diabetic populations is likely multi-factorial.

Haematuria is a non-specific finding in UTIs and may also been seen in other disease processes such as neoplasia, coagulopathies, inflammatory processes and idiopathic disorders. Iatrogenic haemorrhage can be caused when obtaining urine samples via manual compression, cystocentesis or catheterisation (Manfredi *et al.*, 2018). The sensitivity of the blood pad is reported to be between 5-50 intact RBC/µL of urine whereas gross haematuria occurs with approximately 2500 RBC/µL. The strongest reaction of the blood pad (+++) occurs with >200 RBC/µL (Sink and Weinstein, 2012, Reppas and Foster, 2016a). A positive reaction for blood on dipstick evaluation should be confirmed with sediment examination (Piech and Wycislo, 2018).

2.1.5.2.e Sediment Examination

Urine sediment examination should be performed in a standardised manor and a minimum starting volume of 5mL is suggested. Relative centrifugation forces should not exceed 400rcf (where rcf = $1.12 \times \text{Radius} \times (\text{rpm}/1000)^2$) so as not to damage any casts or cells. The supernatant should be decanted gently, leaving approximately 1mL of urine in which the sediment can be resuspended prior to microscopic examination (Sink and Weinstein, 2012).

The correct identification of bacteria on urine sediment examination is multifactorial and can depend on operator experience, technique used and the number and type of bacteria present. It is generally reported that $>10^4$ rods/mL of urine and $>10^5$ cocci/mL must be present before positive identification on unstained sediment examination can be made (Reppas and Foster, 2016b).

Swenson *et al.* (2011) compared the microscopic examination of an unstained wet preparation of urine sediment against a modified Wright's stained air-dried preparation for the detection of bacteriuria in 472 feline urine samples obtained by cystocentesis. Twenty-nine of the urine samples were positive on urine culture. A superior sensitivity and specificity of 82.8% and 98.7% respectively were obtained with the stained air-dried technique versus a sensitivity and specificity of 75.9% and 56.7% respectively for the unstained wet preparation examination. The stained air-dried technique also improved morphological identification of bacteria which may aid a clinician's choice of empiric antimicrobial treatment. This study is limited by having seven laboratory technicians of varying experience performing the wet preparation sediment examination with agreement between individual technicians ranging from 20-69.2%. Quantitative urine culture was used as the gold standard in this study which too is prone to false negative and false positive results (Reppas and Foster, 2016b, Rowlands *et al.*, 2011).

In a similar study, O'Neil *et al.* (2013) compared the microscopic examination of an unstained wet preparation of urine sediment against a modified Wright's stained air-dried preparation and a Gram-stained preparation for the detection of bacteriuria in 111 canine and 79 feline urine samples obtained by cystocentesis. Eleven canine and seven feline samples had a positive urine culture result. In this study the authors found that Wright's staining and Gram staining performed equally well in terms of sensitivity and specificity. For feline samples the sensitivity of all methods was equal at 85.7%. The specificity for the unstained wet preparation was 88.8% which increased to 98.6% by using either a Wright's stain or Gram stain on an air-dried sample. This study used only three laboratory technicians, which may in part explain the increased specificity reported for the unstained wet preparation sediment examination.

Pyuria is generally defined a >5WBC/HPF although the definition does vary between studies evaluating urine sediment examinations. Pyuria may be seen with inflammatory or infectious processes occurring within the urinary tract and contamination from the genital tract should be considered if voided urine samples are being examined (Piech and Wycislo, 2018). Multiple studies have identified associations between pyuria and positive urine cultures but pyuria is not always documented in subclinical bacteriuria (Weese *et al.*, 2019).

In the study by Litster *et al.* (2009), pyuria was significantly more likely to be documented in cats with subclinical bacteriuria compared to culture negative controls, $p<0.0001$, suggesting the presence of bacteria still evoke an inflammatory response despite a lack of lower urinary tract clinical signs. Swenson *et al.* (2011) also identified a strong association between pyuria identified on unstained sediment examination and a positive urine culture, although the

presence or absence or lower urinary tract signs were not recorded in this study. Despite this association, pyuria was not documented in 66% of culture positive samples suggesting pyuria is an insensitive predictor of a positive culture. The authors concluded that bacteriuria should be confirmed by quantitative urine culture and that a lack of pyuria should not be interpreted as a lack of infection.

The retrospective study by Puchot *et al.* (2017) agreed with this finding by reporting a strong association between the presence of pyuria (defined as >3WBC/HPF in this study) and subclinical bacteriuria, $p<0.0001$. Pyuria was however not recorded in 11 of 31 culture positive samples further demonstrating that pyuria is not always documented in cases of subclinical bacteriuria.

Identification of erythrocytes can confirm that a positive result on the dipstick blood pad is due to haematuria rather than haemoglobinuria or myoglobinuria. Up to 5 RBC/HPF can be found in healthy cats and >5RBC/HPF is considered abnormal (Reppas and Foster, 2016b). There is currently conflicting literature regarding associations between the presence of microscopic haematuria and a positive urine culture.

A retrospective study evaluating urine sediment examinations in cats presenting with lower urinary tract clinical signs or diagnosed with CKD, DM or uncontrolled hyperthyroidism found a significant association between the presence of haematuria and a positive urine culture. This finding was established for the study population as a whole and for each individual disease state (Bailiff *et al.*, 2008). Litster *et al.* (2009) also identified a significant association between the presence of haematuria and a positive urine culture in a study of 132 cats without lower urinary tract clinical signs, $p=0.013$.

These findings contrast with that of Puchot *et al.* (2017) who failed to find a significant association between haematuria as an individual variable and subclinical bacteriuria. Puchot *et al.* (2017) did however conclude that classifying the presence of one or more of the variables of haematuria, pyuria or bacteriuria as an active sediment, greatly improved the negative predictive value of sediment examination.

In a prospective study of 111 cats presenting to primary care practices with clinical signs of lower urinary tract disease Lund *et al.* (2013) identified the presence of haematuria as a significant risk factor for lower urinary tract disease versus non-lower urinary tract disease. However, no significant differences between the various diagnoses of lower urinary tract disease were established. O'Neil *et al.* (2013) reported haematuria was not a useful predictor of positive urine culture with haematuria identified in 29% of cats with a positive culture and

28% with a negative culture in a study evaluating urine sediment examination in 81 cats. The inconsistent reports in the literature at present suggest that haematuria based on sediment examination should not be used to predict positive urine cultures. Interestingly, in the study by O'Neil *et al.* (2013), higher rates of false identification of bacteria occurred when haematuria was present.

Struvite urolithiasis and crystalluria occurs frequently in dogs with UTI caused by urease-producing bacteria (Dear *et al.*, 2019). Struvite urolithiasis is often not associated with UTI in cats (Lulich *et al.*, 2016), and O'Neil *et al.* (2013) found no association between crystalluria and a positive urine culture result. In the same study O'Neil *et al.* (2013) did identify a negative association between lipiduria and bacteriuria, $p<0.01$. This suggests that the presence of lipid particles on sediment examination, which is seen more commonly in cats than it is dogs, may obscure the accurate identification of bacteria.

2.1.5.3 Quantitative Urine Culture

Quantitative urine culture is considered the gold standard for diagnosing UTI and urine samples should be obtained for culture prior to any antimicrobial administration (Lulich and Osborne, 2004). Cystocentesis is the preferred method of collection for bacterial culture as this minimises the risk of contamination from the skin or distal genital tract flora (Rowlands *et al.*, 2011). Cystocentesis may not be always be possible and is contraindicated in patients with coagulopathies, therefore urinary catheter-obtained or voided samples may sometimes be submitted for urine culture (Vilhena *et al.*, 2015).

Urine culture should be performed within 24 hours of sample collection to reduce the risk of false positive results (Patterson *et al.*, 2016). Urine samples not being submitted for immediate processing should be refrigerated to retard bacterial growth (Acierno *et al.*, 2018).

For canine urine samples, the use of the preservative boric acid is no longer recommended when samples are posted to external laboratories for urine culture. Rowlands *et al.* (2011) demonstrated false negative results were significantly more likely in samples that had been preserved with boric acid. Too few cats were included in this study to know whether the same effect is true for feline urine samples.

In a retrospective study of 134 cats presented to a primary care practice with lower urinary tract clinical signs in Norway, Eggertsdottir *et al.* (2007) reported a 33% prevalence rate of UTI. In this study, 26% of positive urine cultures were obtained from voided urine samples. Whilst this % was not significantly different from the 23% of samples obtained by cystocentesis, other studies have demonstrated voided urine samples can produce bacterial growths of sufficient

magnitude such that genuine infection, rather than contamination is diagnosed (Lees *et al.*, 1984, van Duijkeren *et al.*, 2004). The current ISCAID guidelines for the diagnosis and management of bacterial UTI in dogs and cats stress that cystocentesis is the preferred method of sample collection for urine culture (Weese *et al.*, 2019).

Although considered the gold standard diagnostic test, quantitative urine culture is not without its limitations. For example, infection with a fastidious organism may not produce a positive result using standard laboratory culture techniques (Reppas and Foster, 2016b). In human medicine, this limitation has been somewhat addressed by the introduction of Expanded Quantitative Urine Culture (EQUC) whereby an increased volume of urine is cultured using an extended range of media. The addition of EQUC in human patients with clinical signs of cystitis but negative standard urine cultures identified a 90% false-negative rate for standard culture techniques (Hilt *et al.*, 2014, Tang, 2017).

2.1.6 Treatment

Urinary tract infections should be treated based on antimicrobial susceptibility testing (AST) (Lulich and Osborne, 2004). Given that the prevalence of UTI in cats in considered to be low, particularly amongst young male cats presenting with lower urinary tract clinical signs, empirical antimicrobial therapy is rarely indicated (Gerber *et al.*, 2005, Sparkes, 2018, Dorsch *et al.*, 2019).

Given the current lack of prospective studies regarding subclinical bacteriuria in cats, the recommendations regarding treatment have largely been extrapolated from human studies (Weese *et al.*, 2019). A systematic review and meta-analysis by Koves *et al.* (2017) identified surgical patients undergoing urological procedures in which the mucosal layer may bleed and pregnant women as the two populations in which the treatment of asymptomatic bacteriuria was of benefit. The review did however conclude that the evidence base for the screening and treatment of asymptomatic bacteriuria in pregnant woman was low and that further randomised controlled trials evaluating the benefit of treatment for both mother and developing foetus are needed. The National Institute for Health and Care Excellence (NICE) in the United Kingdom state pregnant women should be screened and treated for asymptomatic bacteriuria due to the risk of development of pyelonephritis and an increased risk of preterm delivery in untreated cases (NICE, 2018). For all other populations, the screening for, and treatment of, asymptomatic bacteriuria is discouraged due to the risk of antimicrobial therapy selecting for multidrug resistance (Cai *et al.*, 2017).

In a prospective longitudinal study conducted over three and a half years, White *et al.* (2016) obtained urine samples by cystocentesis from a sample of 67 non-azotaemic, non-pregnant cats that did not display any lower urinary tract clinical signs. The population of cats belonged to a university breeding colony primarily used for nutritional research. In total, 28 positive urine cultures were obtained from 11 cats. Antimicrobials were withheld from cats diagnosed with subclinical bacteriuria and no significant association between subclinical bacteriuria and survival was identified. Due to the study population belonging to a colony of genetically related cats receiving identical standards of care and husbandry, it is difficult to extrapolate the results from this study to a wider population. Colony policies dictated any cat with chronic weight loss due to a disease process other than hyperthyroidism were euthanised, preventing any associations being made between subclinical bacteriuria and other pathology. The authors concluded that antimicrobial therapy for subclinical bacteriuria in non-azotaemic cats may not be indicated but longer studies in alternative populations of cats are needed. Data is currently lacking to support or discourage the use of antimicrobials for subclinical bacteriuria in cats with CKD (Dorsch *et al.*, 2019). Current ISCAID guidelines state treatment 'may be considered if there is a particularly high risk of ascending or systemic infection,' although no good data exist defining high-risk populations (Weese *et al.*, 2019).

Novel treatment modalities for UTI such as the establishment of asymptomatic bacteriuria or subclinical bacteriuria have been the subject of clinical trials in humans and dogs respectively over recent years. In a double-blind placebo-controlled cross-over trial of 20 patients with incomplete bladder emptying, Sunden *et al.* (2010) demonstrated both a reduced frequency of UTI, and longer symptom free periods, by establishing asymptomatic bacteriuria with *E. coli* 83972. This protective effect was also characterised in patients with spinal cord injury by Darouiche *et al.* (2005).

Single and multi-dose protocols of establishing *E. coli* 83972 subclinical bacteriuria have been described in dogs with little difference in the duration of colonisation identified between protocols (Thompson *et al.*, 2011, Thompson *et al.*, 2012). Clinical trials evaluating the use of *E. coli* 83972 as an alternative to antimicrobial therapy for the treatment of UTI in dogs or cats have yet to be reported.

In dogs, Segev *et al.* (2018) recently evaluated the use of an intravesicular infusion of an asymptomatic bacteriuria strain of *E. coli* 2-12 as an alternative to antimicrobial therapy for the treatment of recurrent UTI. *E. coli* strain 2-12 has been shown to have superior analgesic qualities when compared to ciprofloxacin in murine models, hence its choice over *E. coli* 83972

for this trial (Rudick *et al.*, 2014). An initial safety study was conducted using 6 purpose-bred research Beagle bitches. Two infusions of *E. coli* were carried out on day 0 and day 8 of the experiment and further urine samples were obtained for culture and urinalysis by cystocentesis on days 1, 3, 8, 9, 11, 16 and 30. No major adverse events were reported during the infusion process and no dogs demonstrated any lower urinary tract clinical signs during the study. One dog had a positive urine culture on day 8, prior to the second infusion, of a *Streptococcus* sp. of $3x10^2$ CFU/mL but all subsequent urine cultures were negative. Positive cultures of the asymptomatic bacteriuria *E. coli* 2-12 were observed in 4 of the 6 dogs at various time points but all dogs were culture negative by day 16.

The safety study was followed by a clinical efficacy study in 9 client-owned dogs. All dogs displayed lower urinary tract clinical tract signs and had infections documented with the following bacteria: *E. coli* (4), *Proteus* spp. (3), *Staphylococcus* spp. (1) and co-infection of *Proteus* spp. and *Staphylococcus* spp. (1). Intravesicular infusion of asymptomatic bacteriuria *E. coli* 2-12 was performed on day 0 with further urine samples obtained for culture and urinalysis by cystocentesis on days 1, 7 and 14. Outcomes measured were clinical signs and bacterial growth. Microbiological cure was defined as no growth or growth of only the asymptomatic bacteriuria *E. coli* 2-12. Resolution of clinical signs was achieved in 4 dogs, 3 of which had microbiological cure. Microbiological cure was also documented in 2 other dogs on day 1, but subsequent cultures documented a reinfection with the original pathogen.

This was the first trial evaluating the use of asymptomatic bacteriuria *E. coli* 2-12 for the treatment of recurrent UTI in dogs. Segev *et al.* (2018) concluded that a single intravesicular infusion of *E. coli* 2-12 was safe and that further studies exploring alternative dosing intervals are warranted given the promising results of this trial. This study is limited by its small sample size of 9 dogs and its short term follow up of 14 days in the clinical efficacy phase and so future work should look to address these limitations.

2.2 URINARY PROTEOMICS

2.2.1 Background

The field of proteomics encompasses research techniques that serve to identify and quantify the proteome of a cell, tissue or entire organism (Aslam *et al.*, 2017). The proteome of a cell is defined as the total protein content expressed by the genetic material at any particular time (Ceciliani *et al.*, 2014). The actual proteome can differ markedly from that predicted from the genome of a cell due to post-translational mechanisms and so the fields of proteomics and genomics are considered complementary (Reusz, 2019). Proteomic techniques have provided advances in both diagnostics and in understanding the pathogenesis of a number of diseases, including urinary tract infections, by identifying novel biomarkers and identifying changes in host protein expression associated with diseased states respectively (Katsafadou *et al.*, 2016, Floyd *et al.*, 2015).

2.2.2 The Urinary Proteome and Its Applications

The canine urinary proteome was first characterised by Brandt *et al.* (2014). Over 500 unique proteins were identified from pooled urine samples of 11 healthy dogs. Three hundred and thirty-eight of these proteins overlapped with the human urinary proteome characterised by Adachi *et al.* (2006). Brandt *et al.* (2014) concluded that canine urinary proteomics may be utilised as a model for human disease studies in future research as many of the proteins common to both proteomes have been associated with specific disease states in humans. Despite all dogs enrolled in this study having a full physical examination, complete blood count, serum biochemistry profile, urinalysis and UPC measurement, the pooling of all urine samples is a limitation to this study as the authors were not able to determine how many urinary proteins were common to each dog.

To date, feline urinary proteomic studies have largely focussed on identifying biomarkers for disease states such as CKD, idiopathic cystitis, UTI and urolithiasis (Ferlizza *et al.*, 2015, Jepson *et al.*, 2013, Lemberger *et al.*, 2011, Treutlein *et al.*, 2013).

Ferlizza *et al.* (2015) compared the urinary proteome of 23 healthy cats to that of 17 cats with proteinuric (UPC > 0.2) CKD. Thirteen proteins were identified that differed in abundance between the two groups including retinol-binding protein (RBP), beta-2-glycoprotein 1 and cauxin, all of which have been associated with renal disease in either humans or cats (Pallet *et al.*, 2014, van Hoek *et al.*, 2009). Whilst the authors concluded that the potential biomarkers identified may prove useful in the diagnosis and monitoring of cats with CKD in the future, given that the majority of cats with CKD have a UPC <0.2, further work should investigate if

differences in urinary protein profiles exist between healthy cats and cats with non-proteinuric CKD (Bartges, 2012).

In a prospective study, Jepson *et al.* (2013) used proteomic techniques to evaluate urinary proteins as potential biomarkers for the development of azotaemia in 20 cats. Over a 12-month period, 10 of 20 cats developed azotaemia and eight potential urinary biomarkers were identified from these cats. The potential biomarkers were of low molecular weight and further study could permit their identification and evaluation of their function and utility as a biomarker for the prediction of azotaemia.

Lemberger *et al.* (2011) used proteomic techniques to compare the nature and abundance of urinary proteins present in cats without lower urinary tract clinical signs and cats with idiopathic cystitis, UTI and urolithiasis. The authors concluded that fibronectin was significantly more abundant in the urine of cats with idiopathic cystitis compared to all other groups. Follow up histopathological examination, including immunohistochemical analysis, of two bladder biopsies from two cats with idiopathic cystitis identified reduced fluorescence for fibronectin compared to control cats. These findings suggest damage to bladder urothelium may permit leakage of fibronectin into the bladder lumen thus allowing fibronectin to serve as a potential biomarker for idiopathic cystitis. However, a follow up study by Treutlein *et al.* (2013) failed to find consistently elevated urinary fibronectin concentrations in a population of 27 cats with obstructive idiopathic cystitis, questioning the utility of urinary fibronectin as a biomarker for this disease.

2.3 THE URINARY MICROBIOME

2.3.1 Background

In recent years it has been established that complex microbial communities exist within many different body sites. These communities have been termed the microbiota, whilst the genetic material of these microbes is referred to as the microbiome (Brubaker and Wolfe, 2016). The Human Microbiome Project (HMP) was established in 2008 and has started to characterise the microbiome of the gastrointestinal tract, the mouth, the vagina, the skin and the nasal cavity (HMP, 2019). Urine has traditionally been considered sterile and so the bladder was not an initial site of interest for the HMP (Thomas-White *et al.*, 2016).

The canine urinary microbiome was characterised by Burton *et al.* (2017) in a study designed to both define the urinary microbiome but also compare this to the gastrointestinal and genital microbiomes of the same individual. Nine male neutered, one male entire and ten female neutered dogs were included in the study. Exclusion criteria included: administration of antimicrobials, probiotics or corticosteroids within the last 30 days, administration of intravenous fluid therapy within the last 24 hours, clinical signs suggestive of systemic infection, any history of lower urinary tract clinical signs or a positive urine culture. Urine samples were obtained by cystocentesis. Following DNA extraction, the hypervariable region V4 of the 16S rRNA gene was amplified and sequenced. Analysis of only one hypervariable region limits taxonomic classification to the level of family or genus (Thomas-White *et al.*, 2016). Genera identified at >1% relative abundance included *Pseudomonas* spp., *Sphingobium* spp. and *Acinetobacter* spp.. No significant difference was found between the urinary microbiome of male or female dogs. The authors concluded that although some overlap between the three sites evaluated exists, the urinary microbiome is largely unique compared to that of the gastrointestinal tract and genital tract. One limitation in the study population selection is the lack of complete blood count, serum biochemical profile and urinalysis evaluation to define a healthy subject for the study. Dogs included may have suffered from subclinical disease, such as early stage CKD, that may influence, or be influenced by the urinary microbiome.

To date, one abstract has been presented at the American College of Veterinary Internal Medicine (ACVIM) annual forum describing a feline urinary microbiome. Vester Boler *et al.* (2018) extracted bacterial DNA in urine samples obtained by cystocentesis from 15 healthy adult cats. The hypervariable region V4 of the 16S rRNA gene was amplified and sequenced. Taxonomic phyla identified in the samples included Proteobacteria, Firmicutes, Actinobacteria,

Bacteroidetes and Cyanobacteria. Genera identified varied between cats but *Staphylococcus* spp. were identified in all samples. Other genera identified included: *Enterobacter* spp., *Acinetobacter* spp., *Streptococcus* spp., *and Shigella* spp..

2.3.2 Applications of Understanding the Urinary Microbiome

The role of the urinary microbiome in diseases such as urgency urinary incontinence, neoplasia of the prostate or bladder wall, urolithiasis and UTI is an area of active research (Li *et al.*, 2019).

The diversity of the female urinary microbiome has been shown to vary according to hormone status. The urinary microbiome of pre-menopausal women tends to be dominated by *Lactobacillus* spp. whereas the urinary microbiome of post-menopausal women tends to be more diverse (Brubaker and Wolfe, 2016). A more diverse urinary microbiome has been associated with recurrent UTI in elderly patients and urgency urinary incontinence (Thomas-White *et al.*, 2017, Tang, 2017). It has been suggested that a predominance of *Lactobacillus* spp. may protect against UTI as products of their metabolism have been shown to impair *E. coli* adhesion to uroepithelial cells (Tang, 2017). Raz *et al.* (2003) showed that the use of estriol-containing vaginal pessaries reduced the frequency of clinical episodes of cystitis in post-menopausal women with recurrent UTI although the mechanism of this effect has yet to be fully elucidated.

Clinical trials exploring *Lactobacillus* spp. probiotics as an alternative to antimicrobial therapy for UTI have yet to demonstrate acceptable efficacy, although research is likely to continue in this area as some benefit for prevention of recurrent UTI has been reported (Beerepoot *et al.*, 2012).

2.4 THE URINE PROTEIN-TO-CREATININE RATIO

2.4.1 Background

The ACVIM has defined the term proteinuria as the 'detection of an abnormal amount of protein in the urine' (Lees *et al.*, 2005). Urine from healthy cats typically contains very little protein, often <1mg/dL of albumin (Grauer, 2011). Albumin is the most abundant protein found in the urine of the majority of proteinuric patients, but other proteins such as globulins and Bence-Jones proteins may also be identified due to a number of physiological or pathological processes (Lyon et al., 2010). A single UPC has been shown to correlate well with 24-hour urinary protein loss in the cat which is considered the gold standard for proteinuria quantification (Monroe *et al.*, 1989). The UPC therefore provides a practical and quantitative assessment of proteinuria for practitioners.

2.4.2 Effects of Urine Sampling Technique

To date, three studies have evaluated the effect the method of urine sample collection has on UPC measurement. Beatrice *et al.* (2010) and Marynissen *et al.* (2017), compared the UPC in samples obtained by cystocentesis with the UPC obtained from voided urine samples in 81 and 74 dogs respectively. Both reported no significant difference between the UPCs obtained by the two methods of collection and significantly strong correlation was also identified.

Vilhena *et al.* (2015) evaluated the difference in UPC obtained by cystocentesis and urine obtained from manual compression of the bladder in 43 cats. Significant strong correlation was reported between both methods of collection in both the study population as a whole, ρ=0.987, and for males and females separately, Pearson r^2 = 0.987 and Pearson r^2 = 0.987 respectively. There are two major limitations of this study. Firstly, mean or median differences in UPCs obtained via different methods of collection were not calculated. Correlation alone does not infer agreement nor that two methods of obtaining urine for UPC measurements are interchangeable. Further statistical analysis of these data would be required to demonstrate agreement. Secondly, urine was obtained by manual compression of the bladder which may not always be tolerated by cats and rarely can cause damage resulting in haematuria (Balakrishnan and Drobatz, 2013). Manual compression of the bladder has also been associated with ureteral reflux, which in cases of UTI can lead to upper urinary tract bacterial colonisation and so this method of urine sample is not recommended (Schaeffer, 2001, Vilhena *et al.*, 2015).

Voided urine samples in the UK are often obtained by means of a non-absorbent cat litter substrate such as hydrophobic sand or polypropylene beads (Delport and Fourie, 2005). It has

been demonstrated that polypropylene beads do not interfere with the measurement of pH, protein concentrations or the sediment evaluation of red and white blood cells in human urine (Pastoor *et al.*, 1990). Hydrophobic sand has only been evaluated in laboratory animals to date, with no interference reported in the measurement of urine specific gravity, urinary dipstick parameters of: leukocytes, nitrites, urobilinogen, protein, pH, blood, USG, ketones, bilirubin and glucose and urinary creatinine and corticosterone concentrations in rats when comparing urine obtained from standard metabolic cages to urine collected from hydrophobic sand (Hoffman *et al.*, 2018).

Due to the day-to-day variation in urinary protein excretion, serial UPC measures are required to demonstrate persistence in proteinuria (Hoskins *et al.*, 1991, Lees *et al.*, 2005). To overcome the need to individually measure UPCs on serial samples, the use of 3 pooled urine samples has been shown to be acceptable in most situations (LeVine *et al.*, 2010, Shropshire *et al.*, 2018). Where UPC values are close to clinical decision thresholds, repeated measures of serial urine samples are suggested (LeVine *et al.*, 2010).

2.4.3 The Effect of Haematuria and Pyuria on Urine Protein-to-Creatinine Ratios

The effect that pyuria, haematuria or bacteriuria has on interpretation of UPC measurement is currently of debate. Studies evaluating the effect of blood contamination of urine samples vary in methodology and statistical analysis making direct comparisons difficult.

In one study, Vaden *et al.* (2004) reported that canine urine samples with pyuria were still likely to have a UPC of <0.5. No correlation was evident between the degree of pyuria and either urinary albumin concentrations or UPC. Concurrent haematuria or bacteriuria was shown to increase urinary albumin concentrations but not significantly increase the UPC. When urine samples were contaminated to the extent that urine turned red, UPCs did not exceed 0.5 in this study. The authors did however conclude that given the increase in urinary albumin concentrations observed in visibly red urine samples, haematuria should be considered as a differential diagnosis for an elevated UPC in these cases.

This contrasts with another study where 18 urine samples obtained from dogs were contaminated with blood from the same individual. In this study, contamination or urine with blood resulting in red discolouration resulted in up to 94% of samples that were initially non-proteinuric being misclassified as either borderline proteinuric or proteinuric (Jillings *et al.*, 2019).

Vientos-Plotts *et al.* (2018) pooled urine samples obtained from 120 cats and contaminated these with feline blood submitted to their laboratory. The authors reported significant

increases in UPC and up to 76% of samples changed category for proteinuria as defined by the International Renal Interest Society (IRIS) when blood contamination resulted in a colour change to dark yellow.

The colour grading system in all three studies varied, which may in part explain the contrasting results reported. For example, in the study by Jillings *et al.* (2019), the authors concluded that if haematuria resulted in an orange urine sample, a clinically significant increase in UPC may be observed. In the study by Vientos-Plotts *et al.* (2018), orange was not part of their colour score and it is unclear whether samples considered orange by Jillings *et al.* (2019) would be considered dark yellow or light pink by Vientos-Plotts *et al.* (2018). The data from all studies suggest that caution should be exercised when interpreting UPCs from urine samples with evidence of haematuria, and to a lesser extent pyuria, and that an elevated UPC may not solely be attributable to post-renal causes in these cases.

2.4.4 The Link Between Proteinuria and Urinary Tract Infections

Current guidelines for human medicine state that due to the association between the presence of proteinuria and UTIs, any patient with a positive result for urinary total protein from either semi-quantitative or quantitative methods should have a UTI excluded by means of a urine culture (Carter *et al.*, 2006).

Associations between proteinuria and symptomatic UTIs and asymptomatic UTIs vary. Multiple studies have found associations between proteinuria and asymptomatic UTI in certain human populations, such as female type-1 diabetics and patients with nephrotic syndrome. Although these authors concluded that the presence of proteinuria may render these populations more susceptible to UTIs, causal relationships could not be established. Larger scale studies of healthy children and young adults failed to find any association between the presence of proteinuria and asymptomatic UTIs, with all authors concluding that both conditions frequently occur independently (Carter *et al.*, 2006, Bharara *et al.*, 2017).

On the other hand, proteinuria is a common finding in patients with symptomatic UTIs. A recent study investigating the ability to predict a positive urine culture based on the presence of proteinuria in humans concluded that proteinuria alone was not a strong predictor of UTI. However, the negative predictive value of proteinuria was reported as 87.8%, suggesting assessment for proteinuria may be a useful screening test for UTI (Bharara *et al.*, 2017).

Several studies in cats evaluating risk factors for both feline lower urinary tract disease and specifically subclinical bacteriuria have failed to identify the presence of proteinuria as a risk factor for both diseases (Lund *et al.*, 2013, Puchot *et al.*, 2017). These studies used semi-

quantitative assessments of proteinuria by use of a dipstick and sulfosalicylic acid test and it has yet to be evaluated whether a UPC above a certain value is associated with a UTI in cats.

Meindl *et al.* (2019) evaluated the relationship between UPC in dogs with and without positive urine cultures and demonstrated variable changes in UPC with positive cultures. The authors concluded that an active urine sediment may not fully explain an elevated UPC, and that repeat UPC measures should be obtained upon clearance of any infection to investigate for other causes of proteinuria. No significant difference in UPC was found between dogs with UTI or subclinical bacteriuria. Interestingly in this study, 19 of 482 urine samples analysed were positive for bacterial growth yet had a UPC of <0.5 demonstrating that an active sediment due to bacteriuria can occur without overt proteinuria.

Chapter 3 MATERIALS AND METHODS

3.1 THE DIAGNOSTIC APPROACH TO FELINE SUBCLINICAL BACTERIURIA AND URINARY TRACT INFECTIONS

3.1.1 Study Design

A prospective observational cohort study of cats referred to the Langford Vets Small Animal Hospital was conducted. Aerobic urine culture, AST, urinalysis including sediment examination, urine protein quantification by means of UPC measurement, cytological examination and proteomic analysis were performed on urine samples obtained by cystocentesis or aspiration from a SUB device and free catch methods from the same cat and results compared and analysed. The study design was reviewed and approved by The University of Bristol Research Ethics Committee (VIN/17/037).

3.1.2 Study Population

Cats referred to the Small Animal Hospital between November 2018 and June 2019 that had cystocentesis performed as requested by the attending clinician were included in the study. Collection of both a voided urine sample and cystocentesis sample were required for inclusion. Cats that had received antimicrobial therapy within the last 3 days were excluded from analysis. Where the same cat had repeat urine samples analysed, only the first sample was included for statistical analyses. Recruitment into the study was aided by the use of the client information leaflet provided in Appendix A.

3.1.3 Data Recording

Descriptive data collected from each cat included age, breed, sex, neuter status, body weight, any clinical history and nature of lower urinary tract signs, presenting clinical signs, current medications, specifically any recent antimicrobial therapy and any co-morbidities. The collection technique for obtaining each urine sample was recorded in addition to the volume analysed and the results of a complete urinalysis including sediment examination, UPC measurement, urine culture and cytological examination. The identification of each organism cultured from a cystocentesis sample and its AST profile were recorded. Results from descriptive data and all tests performed were recorded in a spreadsheet.

3.1.4 Sample Collection

A minimum of 5mL of urine were required to perform urine culture, urinalysis including sediment examination, UPC measurement and cytological examination. Any excess volume of urine was retained for proteomic analysis (see section 3.2).

3.1.4.1 Cystocentesis Samples

All cats were either sedated or anaesthetised for cystocentesis. Cystocentesis samples were collected by Veterinary Specialists in Diagnostic Imaging using ultrasound guidance. Half a millilitre of urine was collected in to an ethylenediaminetetraacetic acid (EDTA)-containing tube for cytological examination. The remaining volume was collected into a sterile plastic universal container. All samples were refrigerated at 4°C if not being analysed immediately. All cystocentesis samples were sent to Langford Vets Diagnostic Laboratories and processed within 18 hours of collection.

3.1.4.2 Subcutaneous Ureteral Bypass Samples

Urine samples were obtained by aspirating from the SUB device prior to routine flushing of the system by a Veterinary Specialists in Diagnostic Imaging using ultrasound guidance. Volumes outlined in section 3.1.4.1 were used for analysis.

3.1.4.3 Voided Samples

Voided samples were collected by lining a litter tray with Medicat® non-absorbent hydrophobic sand (GlobalTech International Ltd., London, United Kingdom). Half a millilitre of urine was pipetted from the surface of the non-absorbent litter in to an EDTA-containing tube. The remaining volume was collected into a plastic conical container provided by the litter manufacturer. All samples were refrigerated at 4°C if not being analysed immediately. Where a voided sample was not collected during hospitalisation, owners were asked to collect a sample at home within 2 days of being discharged using the same litter and refrigerated where possible prior to being posted to the Langford Vets Diagnostic Laboratory.

3.1.5 Urine Culture

3.1.5.1 Cystocentesis Samples

Cystocentesis samples were processed by Langford Vets Diagnostic Laboratory technicians. Any refrigerated samples were brought to room temperature before being cultured. Samples were gently mixed by inversion prior to inoculation. Using a sterilised wire loop, 5µL of urine were inoculated onto Columbia agar + 5% sheep blood (Oxoid Ltd., Hampshire, United Kingdom) and onto Cystine-Lactose-Electrolyte-Deficient (CLED) agar (Oxoid Ltd., Hampshire,

United Kingdom). Blood agar plates were incubated at 37°C in 5% CO_2 and CLED plates at 37°C in an aerobic atmosphere. Plates were examined at 24 and 48 hours post incubation. Quantification of bacterial growth was calculated by multiplying the number of bacterial colonies present on an agar plate by 200. Bacterial growth of $>10^3$ cfu/mL were deemed significant as per current guidelines (Weese *et al.*, 2011). Any cultured organism was identified by laboratory technicians using a combination of colony morphology and standard microbiological techniques outlined in Table 3.1, in accordance with Langford Vets Diagnostic Laboratories standard operating procedure for urine bacteriology, (Quinn *et al.*, 2011, ThermoFisher, 2019) and a commercial automated analyser (Vitek 2, Biomerieux, Hampshire, United Kingdom). Antimicrobial susceptibility testing profiles and minimum inhibitory concentration determination were performed using the same automated analyser. Where multiple organisms were cultured, all organisms were identified and individual AST performed.

3.1.5.2 Voided Samples

Any refrigerated samples were brought to room temperature before being cultured. Samples were gently mixed by inversion prior to inoculation. Using a sterilised calibrated wire loop, 5µL of urine were inoculated onto Columbia agar + 5% sheep blood. Plates were incubated at 37° in 5% CO_2 and examined at 24 and 48 hours post incubation. Quantification of bacterial growth was calculated by multiplying the number of bacterial colonies present on an agar plate by 200. Bacterial growth of $>10^4$ cfu/mL were deemed significant as per current guidelines (Weese *et al.*, 2011). The gross appearance of any colony growth was recorded. Agar plates of positive cultures from voided samples were compared to the corresponding cystocentesis sample agar plates and morphological similarities or differences were recorded.

3.1.5.3 Bacteria Storage

Where a positive urine culture was obtained, up to 6 representative colonies were stored on a slope of nutrient agar (Oxoid Ltd., Hampshire, United Kingdom) at room temperature for proteomic analysis (see section 3.2).

Table 3.1 Identification summary for common UTI pathogens, adapted from Langford Vets Diagnostic Laboratories Standard Operating Procedure for Urine Bacteriology.

Pathogen	**Gram**	**Confirmatory Tests**
Corynebacterium sp.	Positive bacilli	Catalase Positive Confirm ID with Vitek ANC
Enterobacter sp.	Negative bacilli	Oxidase Negative Catalase Positive Indole Negative Confirm ID with Vitek GN
Enterococcus sp.	Positive cocci	Aesculin Positive Lancefield Group D
Escherichia coli	Negative bacilli	Lactose Fermenter Oxidase Negative Indole Positive
Klebsiella sp.	Negative bacilli	Oxidase Negative Catalase Positive Indole Negative Confirm ID with Vitek GN Colonies often very mucoid
Mycoplasma sp.	N/A	Slow growing, no cell wall means not visible with Gram stain
Proteus sp.	Negative bacilli	Non-lactose Fermenter Catalase Positive Indole Negative Usually swarms across plate Confirm ID of non-swarming variants with Vitek GN
Pseudomonas sp.	Negative bacilli	Non-lactose Fermenter Oxidase Positive Metallic sheen to colonies Distinct odour
Staphylococcus sp.	Positive cocci	Catalase Positive DNase Positive Purple Maltose Agar Positive = *S. aureus* Purple Agar Negative = *S. pseudintermedius*
Streptococcus sp.	Positive cocci	Catalase Negative Lancefield Group

3.1.6 Urinalysis

3.1.6.1 Cystocentesis Samples

3.1.6.1.a Visual and Chemical Properties:

Any refrigerated samples were brought to room temperature and gently mixed by inversion prior to analysis. Visual characteristics of colour and turbidity were recorded. One drop of uncentrifuged urine was used to determine USG using a refractometer (Clinical Refractometer T2-NE, Atago Corporation Ltd., Tokyo, Japan). Urine pH was determined by using a benchtop pH meter (Checker®, Hanna Instruments Ltd., Bedfordshire, United Kingdom). Chemical analysis was performed semi-quantitatively using Multistix 10SG dipsticks (Siemens Healthcare Diagnostics Ltd., Surrey, United Kingdom) and measurements of glucose, bilirubin, ketones and blood were recorded by an automatic analyser (Clinitek Status +, Siemens Healthcare Diagnostics Ltd., Surrey, United Kingdom).

Semi-quantitative measures obtained from dipstick readings were converted to a score for statistical analysis as shown in Table 3.2-Table 3.5.

Table 3.2: Scoring chart for glucose measurement obtained by dipstick analysis.
****From Siemens Healthcare Diagnostics, UK.***

Glucose range (mmol/L)	Dipstick Result*	Score
0	Negative	0
≥5.5	Trace	0.5
≥14	+	1
≥28	++	2
≥55	+++	3
≥111	++++	4

Table 3.3: Scoring chart for bilirubin measurement obtained by dipstick analysis.
****From Siemens Healthcare Diagnostics, UK.***

Bilirubin	Dipstick Result*	Score
No range specified	Negative	0
No range specified	+	1
No range specified	++	2
No range specified	+++	3

Table 3.4: Scoring chart for ketone measurement obtained by dipstick analysis.
****From Siemens Healthcare Diagnostics, UK.***

Ketones range (mmol/L)	Dipstick Result*	Score
0	Negative	0
≥0.5	Trace	0.5
≥1.5	+	1
≥4	++	2
≥8	+++	3
≥16	++++	4

Table 3.5: Scoring chart for blood/erythrocyte measurement obtained by dipstick analysis.
****From Siemens Healthcare Diagnostics, UK.***
*****Adapted from Vientos-Plotts et al. (2018).***

Blood range (~Ery/µL)	Dipstick Result*	Score**
0	Negative	0
≥10 – Non-haemolysed	Trace	0.25
≥80 – Non-haemolysed	++	2
≥10 – Haemolysed	Trace	0.5
≥25 – Haemolysed	+	1
≥80 – Haemolysed	++	2
≥200 – Haemolysed	+++	3

3.1.6.1.b Urine Protein-to-Creatinine Measurement:

Five millilitres of urine were centrifuged in a conical tube at 438rcf (1500rpm) for 5 minutes. The supernatant was used to determine the UPC using an automated commercial analyser (KoneLab 60i Prime, Thermo Fisher Scientific Ltd., Leicestershire, United Kingdom). The analyser utilises a pyrogallol red method and an enzymatic method to determine urinary protein and creatinine concentrations respectively (Thermo Scientific, 2014a; Thermo Scientific, 2014b).

Briefly, pyrogallol red molybdate complexes bind to proteins present in a sample. The colour intensity of the pyrogallol-protein complexes is directly proportional to the initial concentration of protein present in the sample and is measured photometrically at 600nm.

Sarcosine is produced from creatinine with creatininase and creatinase acting as catalysts. Sarcosine oxidase then converts sarcosine to glycine, formaldehyde and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminophenazone and 2, 4, 6-triiodo-3-hydroxybenzoic acid to produce a quinone imine chromogen with peroxidase as a catalyst. The colour intensity of the chromogen is directly proportional to the initial concentration of creatinine present in the sample and is measured photometrically at 540nm. Urine creatinine concentrations are converted from mmol/L to mg/dL by the formula shown in Figure 3.1. The UPC is calculated by

dividing the protein concentration in mg/dL by the creatinine concentration in mg/dL as shown in Figure 3.2.

$$\frac{\text{Creatinine (mmol/L)} \times 1000}{88.4} = \text{Creatinine (mg/dL)}$$

Figure 3.1: Formula to convert creatinine concentrations from mmol/L to mg/dL.

$$\text{UPC} = \frac{\text{Protein concentration (mg/dL)}}{\text{Creatinine concentration (mg/dL)}}$$

Figure 3.2: Formula for calculation of the urine protein-to-creatinine ratio (UPC).

3.1.6.1.c Sediment Examination

The urine sediment was re-suspended in 1mL of supernatant by gentle pipetting. One drop of suspended sediment was placed in to 6.6µL containing well (KOVA Slide II with Grid, Hycor Biomedical Incorporation, California, USA) and was examined at low power (x100) for the presence of casts and at high power (x400) for the presence of cells, bacteria, crystals and other components. Ten random representative fields were examined and the average number of components present were recorded as a score as outlined in Table 3.6 and Table 3.7. The presence of pyuria or haematuria was also recorded as shown in Table 3.6. The presence of bacteriuria was recorded where ≥1 bacterium were observed. Where <1ml of urine remained to re-suspend the sediment, components were recorded as either present or absent.

Table 3.6: Scoring chart for visualisation of white and red blood cells on sediment and cytological examination of urine sediment.

WBC/RBC count / HPF	**Qualitative Score**	**Numerical Score**	**Pyuria/Haematuria**
0	Negative	0	0 (No)
<5	Scant	0.5	0
5-20	+	1	1 (Yes)
21-100	++	2	1
>100	+++	3	1
Uncountable	++++	4	1

Table 3.7: Scoring chart for visualisation of epithelial cells or bacteria on sediment and cytological examination of urine sediment.

Epithelial Cell/Bacteria count / HPF	Qualitative Score	Numerical Score
0	Negative	0
<5	Scant	0.5
5-20	+	1
21-100	++	2
>100	+++	3
Uncountable	++++	4

3.1.6.1.d Cytological Examination

Slides for cytological examination were prepared using a Cytospin 4 ™ centrifuge (Thermo Fisher Scientific UK Ltd., Leicestershire, United Kingdom). Four drops of urine preserved in EDTA tubes were centrifuged at 254 rcf, medium acceleration for 5 minutes. Prepared slides were stained semi-automatically with a Modified Wright-Giemsa stain using a Hematek 3000 SlideStainer (Siemens Healthcare GmbH, Erlangen, Germany). Ten random representative fields were viewed at low power (x100) for the presence of casts or large artefacts and at high power (x500) with oil immersion for the presence of cells, bacteria and other components. The average number of individual components present were recorded as a score as outlined in Table 3.6 and Table 3.7.

3.1.6.2 Voided Samples

3.1.6.2.a Visual and Chemical Properties

Visual characteristics and chemical analysis were recorded as described for cystocentesis samples in section 3.1.6.1.a.

3.1.6.2.b Urine Protein-to-Creatinine Measurement

Urine protein-to-creatinine ratios were determined as described for cystocentesis samples in section 3.1.6.1.b.

3.1.6.2.c Sediment Examination

One drop of re-suspended sediment was placed on to a glass slide and covered with a coverslip. Ten random representative fields were examined and components quantified at low power and high-power magnification as described for cystocentesis samples in section 3.1.6.1.c.

3.1.6.2.d Cytological Examination

Slides for cytological examination were prepared as described for cystocentesis samples in section 3.1.6.1.d. Prepared slides were manually stained using a commercial Romanowsky stain as per the manufacturer's guidelines (Rapi-Diff II Stain, Atom Scientific, Cheshire, United Kingdom).

3.1.7 Statistical Analysis

Statistical analyses were performed using a commercially available software package (IBM Corporation. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, New York: United States of America). All non-categorical data were assessed for normality of distribution using the Shapiro-Wilk test. For all analyses, statistical significance was set at $p<0.05$.

3.1.7.1 Sample Size Calculation

The proportion of cats with UTI and subclinical bacteriuria was set at 0.3 and 0.05 respectively. With significance levels of alpha = 0.05 and beta = 0.2, 35 cats per group were needed (Clincalc, 2019).

3.1.7.2 Descriptive Statistics

Descriptive variables described in section 3.1.3 were reported as counts with medians and ranges where appropriate. Individual associations between any historic signs, or current presence of, lower urinary tract signs and positive urine cultures were to be assessed using a Fisher's exact test. Cats classified as having either a urinary tract infection, subclinical bacteriuria or negative culture were reported as counts and differentiated by method of collection.

3.1.7.3 Urine Culture

3.1.7.3.a Cystocentesis Samples

The identity of each organism cultured and AST were reported when requested by the attending clinician.

3.1.7.3.b Voided Samples

Bacterial colony morphological differences between discordant paired samples were described.

3.1.7.4 Urinalysis

Differences in urinalysis parameters between cats with UTI and cats with subclinical bacteriuria were to be assessed by Fisher's exact test.

3.1.8 Follow-up

Cats with positive urine cultures were followed until the end of the study period (September 2019) by reviewing clinical records of hospital visits on a monthly basis between the date of enrolment and study end date. Occurrence of clinical signs, the results of follow up urine cultures and any prescribed treatments were reported where available.

3.2 PROTEOMIC ANALYSIS

3.2.1 Study Design

Proteomic analysis by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS) technology of urine sediments and cultured bacteria were performed to determine whether a biomarker of bacterial infection and/or inflammation could be identified.

3.2.2 Study Population

Cats recruited as described in section 3.1.2 where excess urine was available were used for this study. This study was performed in two runs. Stored bacteria as described in section 3.1.5 were also analysed. Paired urine samples were not required for this study.

3.2.2.1 Urine Sediments

3.2.2.1.a First Run

Samples from cats with a history of lower urinary tract signs or positive urine cultures were analysed first to test the methods being used.

3.2.2.1.b Second Run

Following the acquisition of acceptable data, the remainder of urine samples available irrespective of clinical signs or culture status were analysed. Two samples of deionised water processed in a similar manner to all urine samples were also analysed as control samples on two occasions.

3.2.3 Sample Collection

3.2.3.1 Urine Sediments

Up to 5mL of excess urine were centrifuged at 329 rcf for 5 minutes in a conical tube**.** The supernatant was removed and 30µL of deionised water and 10µL of non-reducing 4X SDS sample buffer (Alfa Aesar, Massachusetts, USA) were added to the centrifuged pellet. These were stored at -20°C until electrophoresis was performed in batches.

3.2.3.2 Bacteria

Stored bacteria were inoculated onto nutrient broth agar plates to give single colonies and cultured overnight at 37°C. Four to six were selected and sub-cultured in 10mL nutrient broth medium and shaken with agitation overnight at 37°C. One-hundred microlitres of the overnight culture samples were diluted 1:10 with nutrient broth medium (Oxoid Ltd., Hampshire, United Kingdom) and further adjusted to give an initial optical density (OD) 600nm of 0.1 in 50mL nutrient broth. These were incubated for approximately 2 hours until an OD 600nm of 0.6-0.8 was reached indicative of the log phase of bacterial growth. Samples were centrifuged at 4500rpm for 10 minutes at 4°C, supernatants were discarded, and pellets kept on ice.

Pellets were resuspended in 30mL of 30mM Tris-hydrochloride pH 8 buffer prior to sonication on ice using a cycle of 1 second on, 1 second off at an amplitude of 63% for 3 minutes by a Sonics Vibracell VC-505TM (Sonics and Materials Incorporated, Connecticut, USA). Sonicated samples were ultra-centrifuged at 8000rpm for 20 minutes at 4°C.

3.2.4 Separation by Electrophoresis

3.2.4.1 Urine Sediments and Deionised Water

Samples were thawed prior to separation by SDS-PAGE as described by Calvopina *et al.* (2017) using an 11% acrylamide and 0.5% bis-acrylamide gel with a 3000X1 Mini-Protean Tetra Cell system (Biorad Laboratories Incorporation, California, USA). Twenty microlitres of combined sample and SDS sample buffer were loaded into each well. Gels were resolved at 180V until each sample had moved approximately 1cm into the separating gel. All gels were stained with Instant Blue dye for a minimum of 30 minutes before being destained with water.

3.2.4.2 Bacteria

One microgram of protein was loaded in to a well for electrophoresis as described for urine sediment samples in section 3.2.4.1.

3.2.5 Liquid Chromatography and Tandem Mass Spectrometry

Prepared gels were analysed by the University of Bristol Proteomics Facility using LC-MS/MS technology as follows:

The 1 cm of gel lane was subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were fractionated separately using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min, 6-15% B over 58 min, 15-32% B over 58 min, 32-40% B over 5 min, 40-90% B over 1 min, held at 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nL/min. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization MS at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.Protein Identification.

3.2.5.1 Urine Sediments and Deionised Water

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt *Felis catus* database using the SEQUEST algorithm or against the SwissProt Eubacteria database using the Mascot algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database

search option was enabled, and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %.

3.2.5.2 Bacteria

Raw data files were quantified as for urine sediments in section 3.2.5.1 but only a search against the SwissProt Eubacteria database was performed.

3.2.6 Data Analysis

3.2.6.1 Urine Sediments

Proteins identified based on fewer than 3 peptide matches were excluded from analysis. The relative abundance for feline proteins identified was calculated by dividing the abundance of an individual protein by the abundance of albumin.

Differential expression of proteins according to method of collection, presence or absence of clinical signs and positive or negative culture status were reported.

3.2.6.2 Bacteria

Proteins identified based on fewer than 3 peptide matches were excluded from analysis. The identity of bacterial proteins identified were recorded. The nature of bacterial proteins were compared to the identity of the cultured organism based on techniques outlined in section 3.1.5.

3.3 The Effect of Non-Absorbent Hydrophobic Sand Litter on Urine Protein-to-Creatinine Measurements

3.3.1 Study Design

A method comparison study evaluating the effect of the non-absorbent hydrophobic sand litter Medicat® on feline UPC measurements was conducted. Paired UPC measures were obtained from urine samples at baseline and after 24 hours contact with non-absorbent litter and results compared any analysed. The study was conducted in 3 stages.

3.3.2 Study Population

Urine samples obtained by cystocentesis as described in section 3.1.4.1 or submitted to the Diagnostic Laboratory from surrounding veterinary practices and marked as urine samples obtained by cystocentesis were collected between February and July 2019.

3.3.3 Data Recording

Descriptive data collected from each cat included age, breed, sex and neuter status. The starting volume and volume recovered for each sample was recorded. Sediment examinations were performed and classified as active or inactive. Active sediments were described as the presence of >5 RBC/HPF or >5 WBC/HPF or the presence of bacteriuria.

3.3.4 Experiment 1 – Pilot Investigation

Thirty-five urine samples obtained by cystocentesis were used. Samples were obtained between February and May 2019 and stored at 4°C until the time of the experiment. Baseline UPCs were measured in duplicate as described in section 3.1.6.1.b and 3mL of urine were placed into a Petri dish containing 20g of non-absorbent litter. Sediment examinations were performed and classified as either active or inactive. Repeat UPC measures were to be obtained in duplicate after 24 hours contact with the non-absorbent litter.

3.3.5 Experiment 2 – 15 Samples

Sufficient volume of 15 samples remained permitting modification of experimental design and investigations to continue. Baseline UPC measurements in the pilot experiment were used for this investigation. Samples had remained stored at 4°C for 2 days prior to restarting the experiment. Up to 3mL (range, 1.5-3.0mL) of urine were placed into a Petri dish containing a reduced amount of 4g of non-absorbent litter, covered and left for 24 hours. Urine was recovered using a pipette and repeat UPCs measured in duplicate. The IRIS category of proteinuria at baseline and 24 hours was also recorded.

3.3.6 Experiment 3 – 25 Samples

Twenty-five urine samples obtained by cystocentesis from 24 cats were analysed. Samples were submitted to the Diagnostic Laboratory either from the Small Animal Teaching Hospital or surrounding veterinary practices between May and July 2019. Samples were stored at -80°C until the time of the experiment where samples were thawed for 1 hour and mixed by inversion. Baseline UPC measurements were obtained in duplicate. Up to 3mL (range, 2.5-3.0mL) of urine were placed into a Petri dish containing 4g of non-absorbent litter, covered and left for 24 hours. Urine was recovered using a pipette and repeat UPCs measured in duplicate. Sediment examinations were performed at 24 hours. The IRIS category of proteinuria at baseline and 24 hours was also recorded.

3.3.7 Statistical Analysis

Statistical analyses were performed using commercially available software packages (IBM Corporation. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, New York, USA and GraphPad Prism version 8.00 for Windows, GraphPad Software, La Jolla, California, USA). All non-categorical data were assessed for normality of distribution using the Shapiro-Wilk test. For all analyses, statistical significance was set at $p<0.05$.

3.3.7.1 Experiment 1 – Pilot Investigation

Descriptive variables described in section 3.3.3 were to be reported as counts. The volume recovered from each Petri dish were to be reported as a mean and range. Median differences in UPC measures and individual components of the ratio were to be assessed using the Wilcoxon signed-rank test. Cohen's kappa method was to be used to measure agreements between IRIS categories at 0 and 24 hours.

3.3.7.2 Experiment 2 and 3

Results from experiments 2 and 3 were combined and analysed together.

Descriptive variables described in section 3.3.3 were reported as counts. The mean and range of volumes recovered from each Petri dish were reported. Median differences in UPC and individual components of the ratio were assessed using the Wilcoxon signed-rank test or sign test where appropriate based on visual assessment of a histogram of difference in measures. Mean changes in urinary protein and creatinine concentrations and UPC were reported as absolute values and absolute percentage changes.

Correlation between UPC measurements at baseline and 24 hours was assessed using Spearman rank-order correlation. Regression analysis was performed using Passing-Bablok regression and acceptability of using 24-hour samples was assessed using a Bland-Altman plot.

Cohen's kappa method was used to measure agreements between IRIS categories for proteinuria at 0 and 24 hours.

3.4 The Effect of Urine Sample Collection Technique on Common Urinalysis Parameters

3.4.1 Study Design

A study comparing measurements of USG, urine pH, UPC, dipstick parameters (pH, glucose, bilirubin, ketones, blood), urine sediment examination findings, cytological examination findings and growth on urine culture between samples obtained by cystocentesis and voided methods was conducted.

3.4.2 Study Population

The population of cats recruited in section 3.1.2 were used for this study. An additional inclusion criterion of paired sampled being obtained within a 24-hour time period was applied to this investigation.

3.4.3 Data Recording

Results of a full urinalysis including sediment examination, UPC measurement, cytological examination and urine culture were recorded for each method of collection.

3.4.4 Procedures

All procedures were performed with the same methods described in section 3.1.5 and 3.1.6.

3.4.5 Statistical Analysis

Statistical analyses were performed using commercially available software packages (IBM Corporation. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, New York, USA and GraphPad Prism version 8.00 for Windows, GraphPad Software, La Jolla, California, USA). All non-categorical data were assessed for normality of distribution using the Shapiro-Wilk test. For all analyses, statistical significance was set at $p<0.05$.

3.4.5.1 Urine Specific Gravity

Median differences between USG depending on method of collection were assessed using the Wilcoxon signed-rank test or sign test where appropriate. Descriptions of the change in USG depending on method of collection were reported.

3.4.5.2 pH

Median differences between pH depending on method of collection were assessed using the Wilcoxon signed-rank test or sign test where appropriate. Descriptions of the change in pH depending on method of collection were reported.

3.4.5.3 Urine Protein-to-Creatinine Ratio

Median differences between UPC depending on method of collection were assessed using the Wilcoxon signed-rank test or sign test where appropriate. The mean change in urinary protein and creatinine concentrations and UPC measurements were reported as integers and percentage changes. Correlation between UPC measurements obtained by cystocentesis and voided methods was assessed using Spearman rank-order correlation. Regression analysis was performed using Passing-Bablok regression and acceptability of using voided samples was assessed using a Bland-Altman plot. Cohen's kappa method was used to measure agreements between IRIS categories for proteinuria between samples obtained by cystocentesis and voided methods.

Passing-Bablok analysis, Bland-Altman plots and Cohen's kappa measures of agreement were performed for all samples and then separately for urine samples with active and inactive sediments.

3.4.5.4 Dipstick Parameters

Semi-quantitative measures obtained from dipstick readings were converted to a score for statistical analysis as shown in Table 3.2-Table 3.5. The number and nature of discordant paired samples were reported.

3.4.5.5 Sediment and Cytological Examination

The average number of components identified in 10 random representative fields were recorded as a score as outlined in Table 3.6-Table 3.7. The presence or absence of pyuria, haematuria (as outlined in Table 3.6) or bacteriuria were also recorded. Median scores and ranges for each variable were reported as well as descriptions of changes observed in each variable depending on method of collection.

3.4.5.6 Urine Culture

McNemars test was used to determine if there was a significant difference between method of collection for any bacterial growth and any significant bacterial growth (i.e. $>10^3$ cfu/mL for cystocentesis samples and $>10^4$ cfu/mL for voided samples). The number of cases in which any, and significant, bacterial growth for each method of collection were reported. Where positive cultures were obtained, bacterial colony morphology and suspected organism identity were reported.

Chapter 4 RESULTS

4.1 THE DIAGNOSTIC APPROACH TO FELINE SUBCLINICAL BACTERIURIA AND URINARY TRACT INFECTIONS

4.1.1 Study Population

Eighty-seven cats had cystocentesis performed as part of their medical investigations during the study period. Thirty-one cats were excluded from analysis for either recently receiving antimicrobials (n=3), already being enrolled in the study (n=2) or not being able to collect a voided sample (n=26). Fifty-six cats met all inclusion criteria hence the results of 112 urinalyses were available for analysis as outlined in Figure 4.1.

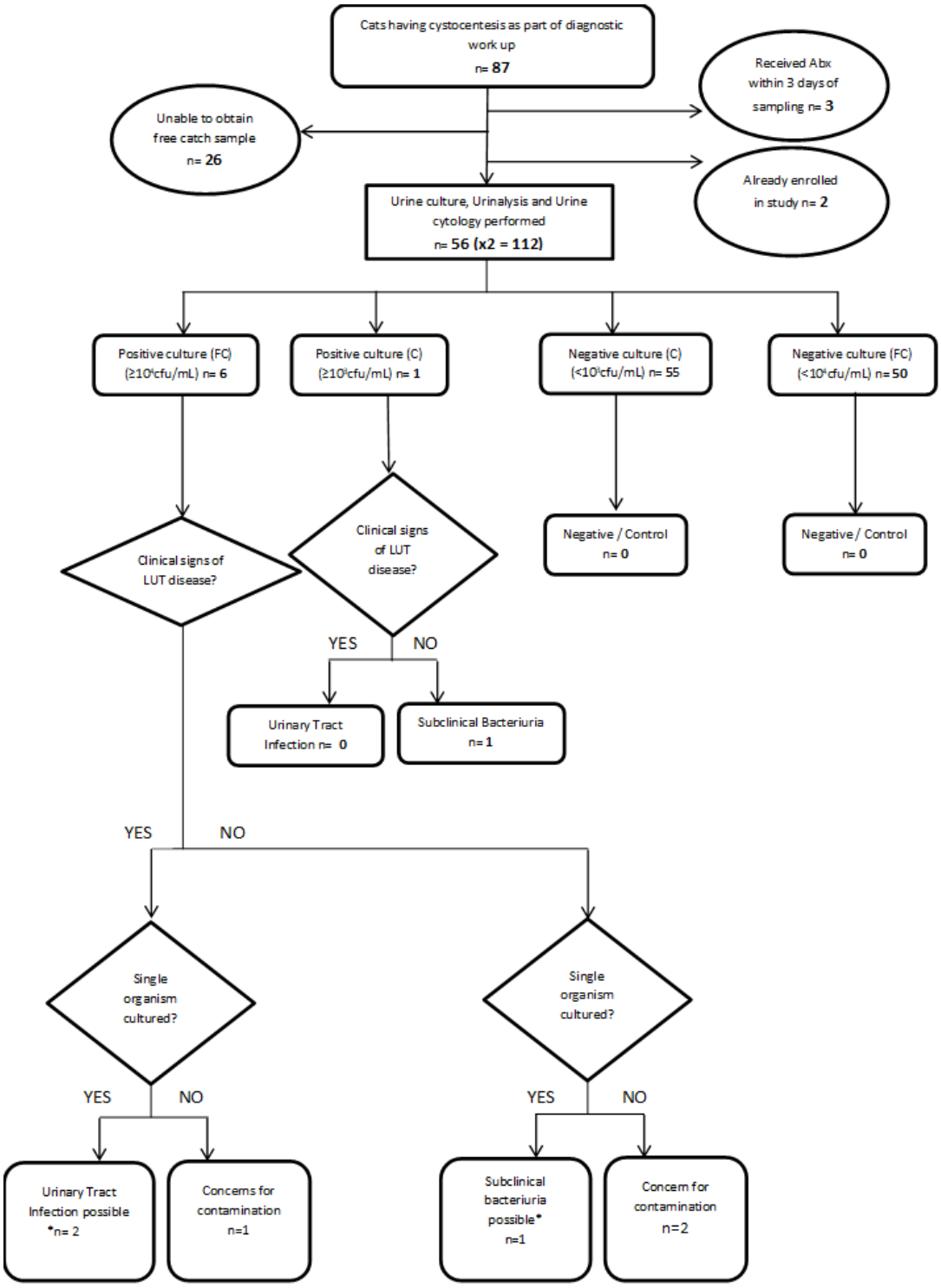

Figure 4.1: Flow chart showing how cats were included in the study and how their urine samples were categorised based on method of collection and urine culture result. In the upper half of the figure, n= number of cats. In the lower half of the figure n= number of urine samples.
(FC) – Free catch sample, (C) – Cystocentesis or Subcutaneous bypass system sample.
LUT – Lower urinary tract.
****Urinary tract infection and subclinical bacteriuria are only considered possible in these cases as samples obtained by cystocentesis are required by definition for these categories (Weese et al., 2019)***

Median age at the time of enrolment was 11.5 years (range, 1-18). Of the 56 cats, 28 were male and 28 were female. All cats were neutered. Forty-one (73%) cats were domestic shorthair cats, 6 (11%) were domestic longhair cats and 1 of each of the following breeds were recruited: domestic medium hair, Bengal, Bengal-cross, British shorthair, Burmese, Maine Coon, Persian, Persian-cross and Tiffany. Table 4.1 shows the numbers of cats enrolled stratified by gender and age category.

Table 4.1: Gender and life stage of the study population.
****From International Cat Care, 2017.***

Sex / Life Stage*	**Junior (6m-2y)**	**Prime (3-6y)**	**Mature (7-10y)**	**Senior (11-14y)**	**Super Senior (≥15y)**	**Total**
Male	3	1	5	16	3	28
Female	0	4	5	16	3	28
Total	3	5	10	32	6	56

The most common reason for presentation was to assess the suitability of radioactive iodine (RAI) treatment for feline hyperthyroidism. The reason for presentation or final diagnosis reached in all recruited cats are presented in Table 4.2.

Table 4.2: : The reason for presentation or final diagnosis of the 56 cats enrolled in the study.
RAI – Radioactive iodine, SUB – Subcutaneous ureteral bypass system, CNS – Central nervous system.

Reason for presentation	**Number of cats (%)**
Assessment for RAI treatment	36 (64.3)
SUB device flush	4 (7.1)
Anaemia	2 (3.6)
Abdominal distension	1 (1.8)
Aspiration pneumonia	1 (1.8)
Chronic kidney disease	1 (1.8)
CNS Lymphoma	1 (1.8)
Constipation	1 (1.8)
Diabetes mellitus	1 (1.8)
Diabetic ketoacidosis	1 (1.8)
Haematuria	1 (1.8)
Hypercalcaemia	1 (1.8)
Lethargy and inappetence	1 (1.8)
Pancreatitis	1 (1.8)
Thyroid carcinoma	1 (1.8)
Ureteric obstruction	1 (1.8)
Vomiting	1 (1.8)

Voided urine samples were obtained first in 37 cases. Cystocentesis or SUB device samples were obtained first in 19 cases (16 cystocentesis and 3 SUB device samples). The median time delay between collecting paired samples was 11 hours (range, 1 to 2160 hours). One voided sample was collected by an owner at home and posted to the Diagnostic Laboratory, the remainder were collected within the hospital.

Fifteen cats (27%) had lower urinary tract clinical signs described in their medical histories. Up to 8 cats (14.3%) displayed lower urinary tract clinical signs at the time of urine sampling. One cat had no lower urinary tract clinical signs at presentation for a SUB device flush but subsequently developed pollakiuria within 24 hours of being discharged; the voided urine sample was obtained whilst pollakiuria was present.

Periuria and haematuria were each reported as the only clinical sign in 2 cats. Haematuria was also reported in conjunction with stranguria and pollakiuria in 1 cat. Pollakiuria and dysuria were each reported as the only clinical sign in 1 cat.

As outlined in Table 4.3, of the 7 cats that displayed lower urinary tract clinical signs at the time of obtaining cystocentesis or SUB device urine samples, 0 had a positive urine culture. Of the 49 cats without lower urinary tract clinical signs, 1 was found to have a positive urine culture.

As outlined in Table 4.4, of the 8 cats that displayed lower urinary tract clinical signs at the time of obtaining voided urine samples, 3 (37.5%) were found to have a positive urine culture. Of the 48 cats without lower urinary tract clinical signs, 3 (6.2%) were found to have a positive urine culture. There was a statistically significant association between the presence of lower urinary tract clinical signs and a positive urine culture, p=0.032.

Table 4.3: Frequencies of cats presenting with or without LUT signs at the time of cystocentesis or SUB device sampling and the respective urine culture status.
LUT – Lower urinary tract, SUB – Subcutaneous ureteral bypass system.

Current LUT clinical signs / Culture status	**Culture positive**	**Culture negative**
LUT clinical signs present	0	7
LUT clinical signs absent	1	48

Table 4.4: Frequencies of cats presenting with or without LUT signs at the time of free catch urine sampling and the respective urine culture status.
LUT – Lower urinary tract, SUB – Subcutaneous ureteral bypass system.

Current LUT clinical signs / Culture status	Culture positive	Culture negative
LUT clinical signs present	3	5
LUT clinical signs absent	3	45

The study group assigned to each cat (i.e. negative, subclinical bacteriuria or urinary tract infection) based on urine culture result varied depending on method of sample collection. Study group assignment differentiated by method of collection is outlined in Table 4.5.

Table 4.5: Study group assignment differentiated by urine sample method of collection.
SUB – Subcutaneous ureteral bypass system.

Method of collection / Study group	Negative	Subclinical bacteriuria considered	Urinary tract infection considered	Contamination considered
Cystocentesis / SUB device	55	1	0	0
Free catch	50	1	2	3

4.1.2 Subclinical Bacteriuria

4.1.2.1 Cystocentesis Samples

One cat was diagnosed with subclinical bacteriuria based upon a positive urine culture obtained from a SUB device urine sample and is hereafter referred to as 'Cat A'. Cat A was an 8-year female neutered domestic longhair weighing 3.2kg and was presented for a routine flush of a SUB device that had been placed 30 months prior due to nephrolithiasis. An isolate of *Enterococcus faecalis* susceptible to clavulanate-potentiated amoxicillin and ampicillin, intermediate susceptibility to enrofloxacin and marbofloxacin and resistant to penicillin G had been cultured at the time of the SUB device placement.

The SUB device was flushed with taurolidine three months post-placement due to persistence of *E. faecalis* infection. The subsequent urine culture documented a *Staphylococcus aureus* isolate susceptible to erythromycin and trimethoprim/sulphonamide, intermediately susceptible to enrofloxacin and resistant to clindamycin, cefoxitin, gentamicin and penicillin G.

Enterococcus spp. were documented at the subsequent SUB device flush 3-months later and persisted until the end of the study period. This isolate was susceptible to clavulanate-

potentiated amoxicillin, ampicillin and nitrofurantoin and intermediately susceptible to enrofloxacin and trimethoprim/sulphonamide.

Co-morbidities at the time of presentation included CKD (IRIS stage II, non-hypertensive [treating] and proteinuric [treating]) and hypercalcaemia (total calcium 3.58mmol/L [ref: 2.3-2.5] and ionised calcium 1.55mmol/L [ref: 1.1-1.4]). Current medications included amlodipine as well as being fed a prescription renal diet.

Notable urinalysis results obtained from the SUB device sample and voided sample are presented in Table 4.6. The voided sample was collected 12 hours after the SUB device sample. The urine culture from the SUB device sample documented a pure growth of *Enterococcus* spp*.* The predominant organism cultured from the voided sample looked morphologically similar to the *Enterococcus* spp*.* with smaller growths of yellow colonies also documented. A comparison of the blood agar plates can be seen in Figure 4.2.

Table 4.6: Comparison of SUB device and free catch urinalyses results for Cat A.

Parameter / Method of collection	**SUB device sample**	**Voided sample**
USG	1.016	1.018
pH (pH meter)	6.4	6.2
UPC	1.04	0.84
Dipstick analysis		
Blood	+++	+++
Sediment examination		
White blood cells	++	+++
Red blood cells	+++	+++
Bacteria	++	+++
Calcium phosphate crystals	Scant	-
Cytology examination		
White blood cells	+++	++++
Red blood cells	+++	+++
Bacteria	++++	++++
Urine culture		
Quantity	Profuse	Profuse
Purity	Pure	Mixed
Species / Morphology	*Enterococcus* spp*.*	Profuse growth of small grey colonies and scant large yellow colonies.

Figure 4.2: A comparison of the blood agar plates cultured from Cat A.
Left – SUB device sample. A pure growth of Enterococcus spp. was identified.
Right – Free catch sample. Note the similarity in morphology of the grey colonies with the additional scant growth of larger yellow colonies.

4.1.2.2 Voided Samples

Three cats were diagnosed with subclinical bacteriuria based upon positive urine cultures obtained from voided samples. One sample was from Cat A described in section 4.1.2.1. Two urine culture results from voided samples were discordant with corresponding cystocentesis samples.

The two additional cats were domestic shorthair cats aged between 13 and 15 years. Both cats were female and neutered and were presented for assessment of suitability for RAI treatment. Urine samples were collected in the hospital with a time delay between obtaining paired samples of 6 and 1512 hours. Both cats had no prior history of lower urinary tract clinical signs reported.

'Cat B' was female neutered and weighed 3.6kg. Chronic kidney disease (IRIS Stage I borderline proteinuric, pre-hypertensive) was documented at the time of presentation. Urine samples were collected 6 hours apart with the voided sample obtained first. Voided urine remained in the litter tray for approximately 5 hours prior to collection and analysis. Glucosuria was documented on the voided urine sample (result: '++') but not on the cystocentesis sample. Bacteriuria on sediment exam was documented to be 'scant' and '+' on voided and cystocentesis samples respectively. Cytological examination documented bacteria on the voided sample only and all bacteria were associated with squamous epithelial cells. Scant red blood cells were also documented on cytological examination of the voided sample. A comparison of urinalysis results for Cat B is shown in Table 4.7 and the morphology of colonies

cultured can be seen in Figure 4.3. A growth of approximately 13000 cfu/mL was documented and the predominate colonies cultured were suspected to be coliform in nature. A repeat urine culture of a voided sample 1-month post-inclusion was culture negative.

Table 4.7: Comparison of discordant urinalyses results from Cat B.

Parameter / Method of collection	**Cystocentesis sample**	**Voided sample**
USG	> 1.050	> 1.050
pH (pH meter)	6.2	6.9
UPC	0.42	0.38
Dipstick analysis		
Glucose	-	++
Sediment examination		
White blood cells	Scant	-
Red blood cells	-	-
Bacteria	+	Scant
Fat droplets	++++	+++
Cytology examination		
White blood cells	-	-
Red blood cells	-	Scant
Bacteria	-	++
Urine culture		
Quantity	Negative	Moderate
Purity	N/A	Mixed
Species / Morphology	N/A	Predominately larger grey colonies with 2 smaller lighter grey colonies.

Figure 4.3: Blood agar plate from the voided urine sample of Cat B.

'Cat C' was female neutered and weighed 3.6kg. An enteropathy and clinical signs of lower respiratory tract disease were documented at presentation. Urine samples were collected 1512 hours apart. The cystocentesis sample was collected first at the assessment visit and was culture negative. The voided sample was not obtained until Cat C re-presented to the Small Animal Teaching Hospital for RAI treatment. Clinical signs at the time of re-presentation included: occasional wheezing, nausea and inappetence. Voided urine remained in the litter tray for approximately 1-hour prior to collection and analysis. Pyuria and bacteriuria were documented on sediment examination of the voided sample only. Cytological evidence of inflammation and infection were also evident on the voided sample only. A monomorphic population of bacilli were reported with documentation of intra-cellular bacteria suggestive of genuine infection. Culture on blood agar plate documented a profuse, pure growth of a bacteria suspect to be coliform in nature as shown in Figure 4.4. A two-week course of clavulanate-potentiated amoxicillin was prescribed by the referring veterinary surgeon. Two subsequent urine cultures, one voided sample and one cystocentesis sample, each documented significant growth of *E. coli*. The isolates AST profile documented susceptibility to amoxicillin, clavulanate-potentiated amoxicillin, cephalexin, pradofloxacin, marbofloxacin, cefovecin and trimethoprim/sulphonamide and resistance to clindamycin. No clinical signs of lower urinary tract disease have been reported in Cat C. Radioactive iodine therapy has currently been withheld from Cat C and monitoring of urine culture status and renal parameters has been advised.

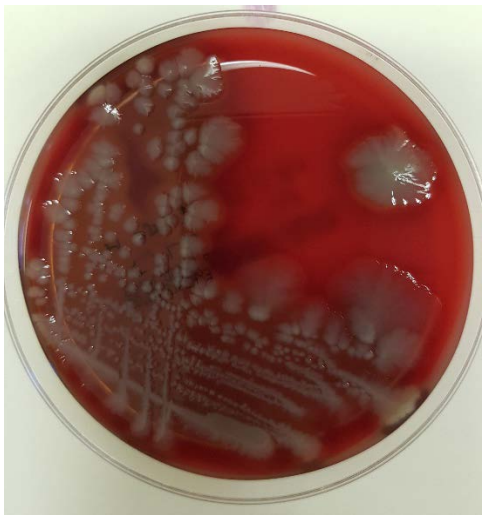

Figure 4.4: Blood agar plate from the voided urine sample of Cat C.

4.1.3 Urinary Tract Infections

4.1.3.1 Cystocentesis Samples

As outlined in Table 4.5, no cats were diagnosed with a clinical UTI based on culture results of cystocentesis samples at the time of inclusion into the study.

4.1.3.2 Voided Samples

Three cats were diagnosed with a clinical UTI based upon positive cultures obtained from voided samples. Corresponding cystocentesis or SUB device samples were culture negative in all cases at the time of inclusion.

All cats were domestic shorthair cats aged between 6 and 17 years. Two were female and one was male, all were neutered. Two voided samples were collected in the hospital 12 and 72 hours post SUB device and cystocentesis samples respectively and one was collected by an owner at home and posted to Diagnostic Laboratory 96 hours post discharge. Two cats were presented for routine flushing of SUB devices whilst the third was presented for treatment of aspiration pneumonia.

'Cat D' presented for routine flushing of bilateral SUB devices that had been placed 2 months prior. Urine culture from a cystocentesis sample at the time of placement was negative. No clinical signs of lower urinary tract disease had been documented prior to presentation. Urine cultures from samples obtained from both SUB devices were negative at presentation. No sediment examination of the SUB device urine was performed but cytological evidence of haematuria and inflammation were documented as shown in Figure 4.5. Two days post discharge, Cat D represented to the Small Animal Teaching Hospital with clinical signs of pollakiuria, lethargy and inappetence. A voided urine sample was obtained at the representation. Bacteriuria was documented on both sediment examination and cytological examination of the voided sample. A monomorphic population of bacilli were documented with intra-cellular bacteria observed as shown in Figure 4.6. Urine culture documented a profuse growth of *E. coli* demonstrating resistance to ampicillin and cefalexin but susceptible to the all other antimicrobials tested against. Cat D was prescribed clavulanate-potentiated amoxicillin at a dose rate of 15mg/kg TID for 4 weeks.

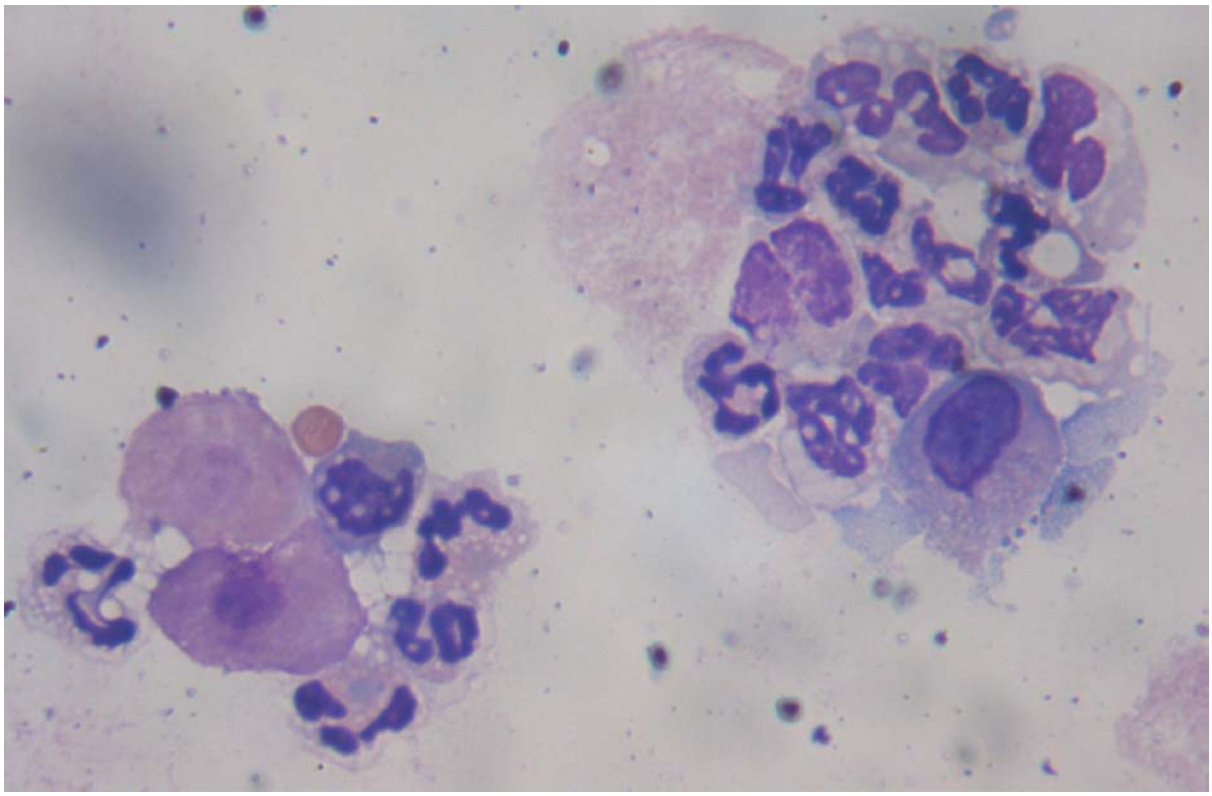

Figure 4.5: Neutrophilic inflammation and haematuria documented on cytological examination of urine obtained from the SUB device of Cat D.
Image magnification x1000.
SUB – Subcutaneous ureteral bypass system.

Repeat culture of urine samples obtained from both SUB devices 1-month later were negative. No clinical signs of lower urinary tract disease were reported after beginning antimicrobial therapy. Cytological evaluation of urine obtained from both SUB devices at this time documented marked neutrophilic inflammation with rare extracellular bacilli noted. A further 4 weeks of clavulanate-potentiated amoxicillin was prescribed at the same dose rate.

A further repeat urine culture 1-month later was negative from urine obtained from the right SUB device. Culture of urine obtained from the left SUB device documented growth of *E. coli* with the same AST profile as the isolate cultured 8 weeks prior. Ultrasound examination of the left SUB device documented kinking of the cystostomy tube within subcutaneous tissues. Patency of the left ureter was confirmed by contrast radiography and the left SUB device was subsequently removed. The SUB device, nephrostomy tube and cystostomy tube were cultured and each documented a profuse *E. coli* growth with the same AST profile. Cat D was prescribed clavulanate-potentiated amoxicillin at an increased dose rate of 25mg/kg TID for 7 days with a view to re-culture urine after this course.

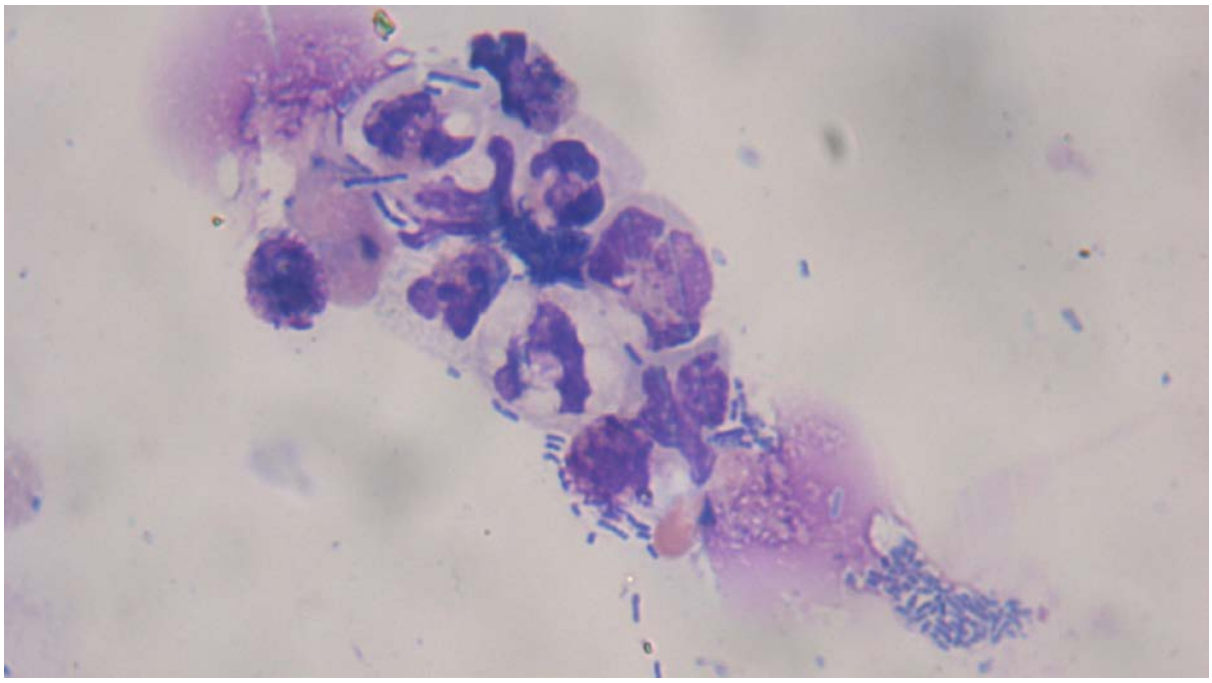

Figure 4.6: Septic neutrophilic inflammation and haematuria documented on cytological examination of voided urine obtained from Cat D.
Image magnification x1000.

'Cat E', had demonstrated signs of periuria and haematuria 3 weeks prior to presentation and had recently been treated for pyelonephritis caused by *E. coli* with marbofloxacin. At presentation the SUB device sample was culture negative. A voided sample was collected at home and posted to the Diagnostic Laboratory 96 hours post discharge. It was unknown how long the sample remained in the litter tray prior to collection. A comparison of urinalysis results obtained by each method of collection for Cat E are shown in Table 4.8. The blood agar plate is shown in Figure 4.7, colonies were non-haemolytic and catalase negative. Gram staining, aesculin testing and Lancefield Group testing were not performed and so either an *Enterococcus* spp. or *Streptococcus* spp. were suspected.

Table 4.8: Comparison of discordant urinalyses results from Cat E.

Parameter / Method of collection	**SUB device sample**	**Voided sample**
Colour	Red	Pink
USG	1.005	1.019
pH (pH meter)	6.6	5.8
UPC	10.40	1.03
Dipstick analysis		
Blood	+++	+++
Sediment examination		
White blood cells	Scant	Scant
Red blood cells	++++	+
Bacteria	-	++
Struvite crystals	-	Scant
Cytology examination		
White blood cells	Scant	-
Red blood cells	+	-
Bacteria	-	++++
Urine culture		
Quantity	No growth	Profuse
Purity	N/A	Mixed
Species / Morphology	N/A	Predominate growth of non-haemolytic small grey colonies. Scant growth of larger cream/yellow colonies.

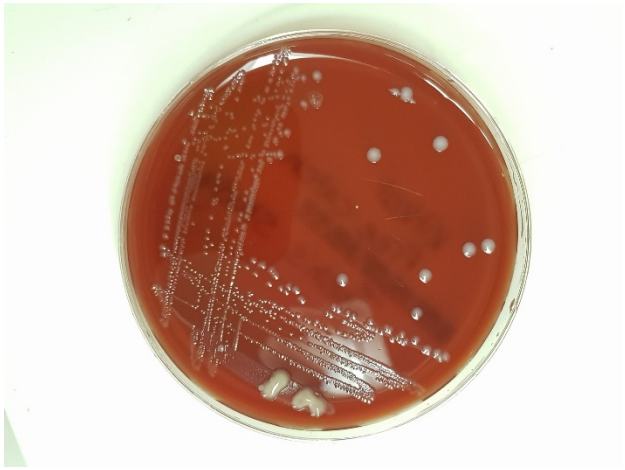

Figure 4.7: Blood agar plate from the voided urine sample of Cat E.

A follow-up urine culture obtained from the SUB device of Cat E 8 months post-inclusion documented a polymicrobial infection with *Streptococcus* spp. (Lancefield group G) and two morphologically different *E. coli*. The *E. coli* isolates had the same AST profiles and were susceptible to all antimicrobials tested against. The *Streptococcus* isolate demonstrated resistance to marbofloxacin confirmed by Kirby-Bauer disc diffusion testing. Cat E is currently being treated with marbofloxacin (2mg/kg PO, SID) and clavulanate-potentiated amoxicillin (20mg/kg PO, TID) with a 3-week course of both antimicrobials prescribed.

The cat referred for treatment of aspiration pneumonia, hereafter referred to as 'Cat F', had recently displayed dysuria and had a prior history of haematuria. Co-morbidities identified at presentation included: CKD (IRIS Stage II proteinuric [treating], non-hypertensive), chronic pancreatitis, degenerative joint disease and unilateral laryngeal paralysis. Paired urine samples in this case were collected 12 hours apart with the cystocentesis sample being collected first. Voided urine remained in the litter tray for approximately 2.5 hours prior to collection and analysis. Intravenous fluid therapy was being administered at the time of sample collection. Free catch urinalysis results documented a urine specific gravity of 1.017, urine pH of 6.0 and UPC of 0.23. No notable findings on dipstick analysis, sediment examination or cytological examination were reported. Cystocentesis urinalysis results were similar. The cultured organism from the voided sample was suspect to be a *Proteus* spp. due to the swarming documented on the blood agar plate. The urine culture from the cystocentesis sample was negative. The positive culture from the voided sample was suspected to represent contamination.

4.2 PROTEOMIC ANALYSIS

4.2.1 Study Population

Urine samples from 22 cats were analysed in two runs for this study. This included 20 paired samples (voided and cystocentesis) and 2 unpaired samples. Four cultured bacteria were also analysed.

4.2.1.1 Urine Sediments

4.2.1.1.a First Run

Samples from 5 cats presenting with lower urinary tract signs or positive urine culture were analysed. Table 4.9 shows the breakdown of samples analysed for the first run based upon presence of lower urinary tract clinical signs and urine culture status. One paired sample analysed produced discordant culture results. Four cats had clinical signs consistent with lower urinary tract disease at the time of inclusion. Three cats had a positive urine culture although one case was suspected to represent contamination.

Median age at the time of enrolment was 11 years (range, 2-18 years). Of the 5 cats, 3 were female and 2 were male. All cats were neutered. Three cats were domestic shorthair cats and 2 were domestic longhair cats.

Table 4.9: Nature of clinical signs and culture status for cats analysed in the first run of proteomic analysis. LUT – Lower urinary tract, (C) – Cystocentesis sample, (FC) – Free catch sample.
**** A Proteus spp. was cultured from this samples and was suspected to represent contamination.***
 # Denotes cultured bacteria were available for proteomic analysis.

Presence of LUT signs / Culture status	**Culture positive**	**Culture negative**
Current LUT signs	1 unpaired sample# (C) Half of a paired sample (FC)*	2 paired samples Half of a paired sample (C)
No LUT signs	1 paired sample#	

4.2.1.1.b Second Run

Samples from 17 cats were analysed in the second run. This included 14 cats that had no prior history of lower urinary tract clinical signs and 3 cats presenting with lower urinary tract clinical signs. A voided sample from a cat with no lower urinary tract clinical signs was positive on urine culture, all other urine cultures from cats without lower urinary tract clinical signs were negative. Of the three cats presenting with lower urinary tract clinical signs, bacterial cystitis was documented in one case. Table 4.10 shows the breakdown of samples analysed in the second run.

Median age at the time of enrolment was 12 years (range, 5-18 years). Of the 17 cats, 11 were female and 6 were male. All cats were neutered. Sixteen cats were domestic shorthair cats and 1 was a domestic medium hair cat.

Table 4.10: Nature of clinical signs and culture status for cats analysed in the second run of proteomic analysis. LUT – Lower urinary tract, (C) – Cystocentesis sample, (FC) Free catch sample, (SUB) Subcutaneous ureteral bypass system sample.
[#] Denotes cultured bacteria were available for proteomic analysis.

Presence of LUT signs / Culture status	**Culture positive**	**Culture negative**
Current LUT signs	1 unpaired sample[#] (FC)	2 paired samples
No LUT signs	Half of a paired sample[#] (FC)	13 paired samples Half of a paired sample (C)

4.2.1.2 Bacteria

4.2.1.2.a First Run

Two bacteria were available for analysis (marked by # in Table 4.9). One *E. coli* isolate obtained from the unpaired cystocentesis sample from a cat with UTI and one suspected *Enterococcus* sp. obtained from the voided sample of the cat with subclinical bacteriuria (Cat A).

4.2.1.2.b Second Run

Two bacteria were available for analysis (marked by # in Table 4.10). Both were *E. coli* isolates obtained from voided samples. One cat had confirmed UTI (Cat D), the other is suspected to have subclinical bacteriuria (Cat C).

4.2.2 Urine Sediment - Uniprot *Felis catus* Searches

4.2.2.1 First Run

Described below (Sections 4.2.2.1.a - 4.2.2.1.c) are the number and nature of feline proteins identified for each study group in the first run of analyses. Any differences in protein expression according to method of collection are also highlighted. A summary table is provided in section 4.2.2.1.d.

4.2.2.1.a Culture Negative Cats with Lower Urinary Tract Clinical Signs

Three paired samples were compared from cats that presented with lower urinary tract clinical signs.

One hundred and nineteen and one hundred and twenty-five feline proteins with unique accession numbers were identified in the cystocentesis and voided samples respectively. Twenty-seven and twenty-eight proteins identified in the cystocentesis and voided samples

respectively were uncharacterised by the software and removed from further analysis. Seventy-seven proteins were identified in both the cystocentesis and voided samples of all three pairs.

Uromodulin was the most abundant protein in all voided samples and was on average 9.5x more abundant than albumin. Carboxylicesterases and haemoglobin-derived proteins were frequently identified in greater abundance than albumin. Haematuria was reported as a historical clinical sign in 2 cats and as a current clinical sign in the third.

Of note, fibronectin was identified in all cystocentesis and voided samples in cats presenting with lower urinary tract disease with a mean abundance of 0.007 and 0.032 respectively relative to albumin. A full description of proteins identified in this group can be found in Supplementary Table S1.

4.2.2.1.b Culture Positive Cats with Lower Urinary Tract Clinical Signs

One cystocentesis sample from a cat with confirmed bacterial cystitis was analysed. Five hundred and fifty-six feline proteins with unique accession numbers were identified, eighty-two of which were uncharacterised and excluded from further analysis.

Actin was the most abundant protein identified in this sample followed by myeloperoxidase and uromodulin at abundances of 4.05, 2.93 and 2.5 respectively relative to albumin.

Up to 9 histone proteins were differentially identified in the cat with a positive urine culture compared to culture negative cats. An elastase expressed by neutrophils was also identified in this sample at an abundance of 2.2 relative to albumin. This protein was not identified in samples obtained from culture negative cats. A full description of proteins identified in this group can be found in Supplementary Table S2.

4.2.2.1.c Culture Positive Cats with No Lower Urinary Tract Clinical Signs

One paired sample from Cat A in section 4.1.2 with subclinical bacteriuria was analysed. The cystocentesis sample was collected 5-weeks after the voided sample. Positive urine cultures with growth of *Enterococcus* spp. were identified in both samples.

Two hundred and twenty-five unique accession numbers were identified in the cystocentesis sample and three hundred and fifty-five were identified in the voided sample. One hundred and ninety-three feline proteins with unique accession numbers were common to both the cystocentesis and voided samples.

Haemoglobin derived proteins were the most abundant protein type in the voided sample with an abundance of 2.0 relative to albumin. These proteins were also present in the cystocentesis sample but at an abundance of 0.5 relative to albumin. Haematuria was documented in the urinalysis of both samples.

The neutrophil-expressed elastase identified in the clinical UTI sample was also identified in both the voided and cystocentesis sample.

One hundred and seventeen unique accession numbers were identified in this case of subclinical bacteriuria that were not identified in the clinical UTI sample. Thirty-five of these were uncharacterised by the identification software. The most abundant unique proteins identified were haemoglobin-derived proteins, actin and fragments of IgG lambda chain regions. A full description of proteins identified in this group can be found in Supplementary Table S3.

4.2.2.1.d Summary Table

	Cystocentesis samples	Voided samples	Common to both
Culture negative with LUT signs (n=3)	119	125	77
Culture positive with LUT signs (n=1)	556	N/A	N/A
Culture positive without LUT signs (n=1)	225	355	193

Table 4.11: The number of feline proteins identified in each group of cats from the first run for cystocentesis samples, voided samples and common to both methods of collection.
LUT – Lower urinary tract.

4.2.2.2 Second Run

Described below (Sections 4.2.2.2.a - 4.2.2.2.e) are the number and nature of feline proteins identified for each study group in the second run of analyses. Any differences in protein expression according to method of collection are also highlighted. A summary table is provided in section 4.2.2.2.f.

4.2.2.2.a Culture Negative Cats without Lower Urinary Tract Clinical Signs

Thirteen paired samples from culture negative cats without lower urinary tract clinical signs were analysed. One hundred and five and one hundred and fifteen unique accession numbers were identified in all thirteen cystocentesis and voided samples respectively. Eighty-two and

eighty-four proteins identified in the cystocentesis and voided samples respectively were uncharacterised by the software used and removed from further analysis. Forty-nine proteins were identified in both the cystocentesis and the voided sample.

Uromodulin was the most abundant protein in voided samples and carboxylicesterases were more abundant in cystocentesis samples. Haemoglobin-derived proteins were identified in all samples despite no cat having any history of haematuria reported.

Fibronectin was identified in all samples with a mean abundance of 0.012 relative to albumin for both cystocentesis and voided samples. A full description of proteins identified in this group can be found in Supplementary Table S4.

4.2.2.2.b Culture Negative Cats with Lower Urinary Tract Clinical Signs

Two paired samples from cats with lower urinary tract clinical signs but negative urine cultures were analysed. One hundred and twenty-one unique accession numbers were identified both cystocentesis samples and one hundred and forty-three were identified in both voided samples. Ninety-six proteins were common to all samples in this group. The most abundant proteins identified were a haemoglobin-derived protein and uromodulin in cystocentesis and voided samples respectively.

Sixty-six protein accession numbers identified in cats with lower urinary tract clinical signs were also identified the culture negative cats without lower urinary tract clinical signs. After visual inspection of remaining accession numbers and protein descriptions, 2 unique proteins were identified in culture negative cats with lower urinary tract clinical signs that were not identified in culture negative cats without lower urinary tract clinical signs. These were actin and an ATP synthase beta subunit.

Fibronectin was identified in all samples with a mean abundance of 0.005 and 0.02 relative to albumin for cystocentesis and voided samples respectively. A full description of proteins identified in this group can be found in Supplementary Table S5.

4.2.2.2.c Culture Positive Cats with Lower Urinary Tract Clinical Signs

One voided urine sample from Cat D in section 4.1.3 with an *E. coli* UTI was analysed. Four hundred and thirty-nine unique accession numbers were identified in this sample. The most abundant protein identified was albumin followed by an uncharacterised protein, uromodulin and haemoglobin-derived proteins.

Two hundred and sixty-two of the accession numbers identified in this sample were also identified in the UTI sample described in 4.2.2.1.b. Of note, these included: actin, IgG chain

fragments, haptoglobin, myeloperoxidase, fibrinogen, transferrin, RBP and neutrophil-expressed elastase. A full description of proteins identified in this group can be found in Supplementary Table S6.

4.2.2.2.d Culture Positive Cats without Lower Urinary Tract Clinical Signs

One voided urine sample from Cat C in section 4.1.2.2 with suspected subclinical bacteriuria due to *E. coli* was analysed. Four hundred and twenty-six proteins were identified in this sample, one hundred and sixty-nine of which were also identified in both samples from the culture positive cats without lower urinary tract clinical signs described in section 4.2.2.1.c.

One hundred and eleven of the accession numbers identified in all suspected cases of subclinical bacteriuria were also identified in at least of one the culture negative cats without lower urinary tract clinical described in section 4.2.2.2.a.

Fifty-seven accession numbers were identified in suspected cases of subclinical bacteriuria that were not identified in culture negative cats with no lower urinary tract clinical signs. Two of the fifty-seven accession numbers were identified in at least one of the , culture negative cats with lower urinary tract signs described in sections 4.2.2.1.a and 4.2.2.2.b respectively

Fifty-five accession numbers identified in cases of subclinical bacteriuria were also identified in cases of UTI. Two unique accession numbers were solely identified in all cases of subclinical bacteriuria, both proteins were uncharacterised by the software used but had molecular weights of 43.4 kDa and 59.6 kDa. Accession numbers, descriptions, number of amino acids and the molecular weight of both proteins are reported in Table 4.12. The Basic Local Alignment Search Tool (BLAST) of the Uniprot database was used to search for regions of similarity between sequences of the uncharacterised proteins identified in this study and other proteins within the database. For the accession number A0A2I2V2Y7, 61% of the sequence was identical to human complement factor H (Uniprot, 2019). A full description of proteins identified in this group can be found in Supplementary Table S7.

Table 4.12: Description of the two unique proteins identified in only cases of suspected subclinical bacteriuria. # AAs – Number of amino acids, MW – Molecular weight, kDa – Kilodalton.

Accession Number	Description	# AAs	MW (kDa)
A0A2I2V2Y7	Uncharacterized protein OS=Felis catus OX=9685 PE=4 SV=2 - [A0A2I2V2Y7_FELCA]	380	43.4
M3VUG0	Uncharacterized protein OS=Felis catus OX=9685 GN=LOC105259896 PE=3 SV=3 - [M3VUG0_FELCA]	560	59.6

4.2.2.2.e Deionised Water Controls

Forty-two accession numbers were identified in both control samples. Uromodulin and two haemoglobin subunit proteins were identified at a greater abundance than albumin. Ten of the forty-two accession numbers corresponded to keratin proteins. Eight of the accession numbers were uncharacterised by the identification software.

Analysis of control samples was repeated. Prior to the repeat analysis samples were frozen at -20°C for 5 weeks. Ten accession numbers were identified in both control samples in the repeat analysis. Two of these accession numbers were not identified in the first analysis and these accession numbers both corresponded to uncharacterised proteins. Seven of the ten accession numbers identified in both control samples corresponded to keratin proteins, two corresponded to uncharacterised proteins and one corresponded to albumin.

A further 8 accession numbers were identified in the cystocentesis control sample that were not identified in the voided control sample. These included: uromodulin, haemoglobin subunit proteins, proteins involved in desmosome formations and uncharacterised proteins.

4.2.2.2.f Summary Table

Table 4.13: The number of feline proteins identified in each group of cats from the second run for cystocentesis samples, voided samples and common to both methods of collection.
LUT – Lower urinary tract.

	Cystocentesis samples	**Voided samples**	**Common to both**
Culture negative without LUT signs (n=13)	105	115	49
Culture negative with LUT signs(n=2)	121	143	96
Culture positive with LUT signs (n=1)	N/A	439	N/A
Culture positive without LUT signs (n=1)	N/A	426	N/A

4.2.3 Urine sediment - SwissProt Eubacteria Searches

4.2.3.1 First Run

Described below are the number and nature of bacterial proteins identified for each study group in the first run of analyses. Any differences in protein expression according to method of collection are also highlighted. A summary table is provided in section 4.2.3.1.d.

4.2.3.1.a Culture Negative Cats with Lower Urinary Tract Clinical Signs

Three paired samples were analysed. A mean of 310 bacterial proteins were identified in each individual sample. On average, 71 more bacterial proteins were identified in cystocentesis samples than in voided samples.

One hundred and seventeen unique accession numbers were identified in all samples. One hundred (85%) of these proteins were identified as *Pseudomonas* spp. proteins. These included: membrane proteins, elongation factors, ATP synthase subunits, ATP binding proteins, ribosomal proteins, polymyxin-resistance proteins, transcription factors, DNA binding proteins and an RNA polymerase.

Other genera identified in all samples included: *Enterobacter, Escherichia, Francisella, Hahella, Marinobacter, Pseudoalteromonas, Psychrobacter, Serratia, Shewanella* and *Teredinibacter*. All genera identified in all samples belonged to the Proteobacteria phylum. A full description of proteins identified in this group can be found in Supplementary Table S8.

4.2.3.1.b Culture Positive Cats with Lower Urinary Tract Clinical Signs

One cystocentesis sample from a cat with confirmed *E. coli* bacterial cystitis was analysed. Sixty-six bacterial proteins were identified in this sample, thirteen of which were identified as *E. coli* proteins. These included: membrane proteins, elongation factors, ATP synthase subunits and ribosomal proteins.

Thirty proteins identified in this sample belonged to the *Pseudomonas* genus. These included: membrane proteins, elongation factors, ATP synthase subunits, ribosomal proteins, DNA binding proteins and an RNA polymerase.

Proteins belonging to *Yersinia, Serratia, Shigella, Xanthobacter, Streptococcus, Clostridium and Azotobacter* genera were also identified in this sample. A full description of proteins identified in this group can be found in Supplementary Table S9.

4.2.3.1.c Culture Positive Cats without Lower Urinary Tract Clinical Signs

One paired sample from Cat A in section 4.1.2 with *Enterococcus* spp. subclinical bacteriuria was analysed. Three hundred and thirty bacterial proteins were identified in the cystocentesis sample and one hundred and forty-two in the voided sample. One hundred and thirty-seven proteins were identified in both samples.

Of the proteins identified in both samples, 83 belonged to the *Pseudomonas* genus.

Sixty-two proteins identified in both samples from this cat were also identified in all cases of culture negative with lower urinary tract clinical signs cats described in section 4.2.3.1.a, fifty-seven of which belonged to the *Pseudomonas* genus.

Three *Enterococcus* spp. bacterial proteins were identified in the voided sample of this cat. No *Enterococcus* spp. proteins were identified in the cystocentesis sample. A full description of proteins identified in this group can be found in Supplementary Table S10.

4.2.3.1.d Summary Table

Table 4.14: The number of bacterial proteins identified in each group of cats from the first run for cystocentesis samples, voided samples and common to both methods of collection.
LUT – Lower urinary tract.

	Cystocentesis samples	Voided samples	Common to both
Culture negative with LUT signs (n=3)	188	126	117
Culture positive with LUT signs (n=1)	66	N/A	N/A
Culture positive without LUT signs (n=1)	330	142	137

4.2.3.2 Second Run

Described below are the number and nature of bacterial proteins identified for each study group in the second run of analyses. Any differences in protein expression according to method of collection are also highlighted. A summary table is provided in section 4.2.3.2.f.

4.2.3.2.a Culture Negative Cats without Lower Urinary Tract Clinical Signs

Of 13 paired urine samples analysed up to 51 bacterial proteins were identified in 5 cystocentesis and up to 78 proteins were identified in 7 voided samples. In 4 cases, bacterial proteins were identified in both the voided and cystocentesis samples. In 3 cases, bacterial proteins were identified in the voided sample only and in 1 case bacterial proteins were identified in the cystocentesis sample only.

Fifteen bacterial proteins were identified in all 5 of the bacterial protein-positive cystocentesis samples. Bacterial proteins identified largely belonged to the Enterobacteriaceae family with *Serratia, Salmonella and Klebsiella* being the most commonly identified genera.

No protein identified was common to the 7 bacterial protein-positive free catch samples. A protein identified as an elongation factor of *Serratia proteamaculans* was identified in 6 of 7 samples. Nine proteins identified were common to five voided samples. Each protein identified common to 5 or more samples was of a unique genus. Proteins identified most commonly belonged to the Enterobacteriaceae family. A full description of proteins identified in this group can be found in Supplementary Table S11.

4.2.3.2.b Culture Negative Cats with Lower Urinary Tract Clinical Signs

Two paired samples were analysed from cats currently displaying clinical signs of lower urinary tract disease but with negative urine cultures.

In one paired sample, 0 and 9 bacterial proteins were identified in the cystocentesis and voided samples respectively. Two proteins were identified as *E. coli* proteins. Other genera identified included: *Erwinia, Magnetospirillum, Rhodobacter, Rhodopseudomonas, Serratia, Xanthobacter* and *Yersinia*, all of which belong to the Proteobacteria phylum.

In the second paired sample analysed, 33 and 6 bacterial proteins were identified in the cystocentesis and voided samples respectively. Six *Serratia spp.* and five *E. coli* proteins were identified in the cystocentesis sample. Of note, a protein identified as a *Mycobacterium bovis* protein was identified in the cystocentesis sample.

Of the 6 proteins identified in the voided samples, 5 were also identified in the cystocentesis sample. A *Rhodobacter* spp. protein was identified in the voided sample only. A full description of proteins identified in this group can be found in Supplementary Table S12.

4.2.3.2.c Culture Positive Cats with Lower Urinary Tract Clinical Signs

One voided urine sample from Cat D in section 4.1.3 with an *E. coli* UTI was analysed. Four hundred and seventy-three bacterial proteins were identified in this sample. Of which 380 (80%) were identified as *E. coli* proteins.

Citrobacter, Enterobacter, Klebsiella and *Salmonella* proteins were frequently identified. Only two proteins were identified as *Pseudomonas* spp. proteins. A full description of proteins identified in this group can be found in Supplementary Table S13.

4.2.3.2.d Culture Positive Cats without Lower Urinary Tract Clinical Signs

One voided urine sample from Cat C in section 4.1.2.2 with suspected subclinical bacteriuria due to *E. coli* was analysed. Four hundred and eighty-seven bacterial proteins were identified in this sample. Of which 395 (81%) were identified as *E. coli* proteins. Two proteins were identified as *Pseudomonas* spp. proteins. A full description of proteins identified in this group can be found in Supplementary Table S14.

4.2.3.2.e Deionised Water Controls

One hundred and twelve bacterial proteins were identified in both deionised water control samples. A further 88 bacterial proteins were identified in the sample prepared as a cystocentesis sample and a further 9 bacterial proteins were identified in the sample prepared as a voided sample. Bacterial proteins identified largely belonged to the Enterobacteriaceae family with *Enterobacter, Escherichia, Serratia and Yersinia* being commonly identified genera. Sixteen proteins belonging to the genus *Pseudomonas* were also identified.

Analysis of control samples was repeated. Prior to the repeat analysis samples were frozen at -20°C for 5-weeks. Three and one bacterial proteins were identified the cystocentesis control and voided control samples respectively. A carbapenem-hydrolysing beta-lactamase from a *Klebsiella* spp. was identified in both samples. A membrane protein from an *Escherichia* spp. and a nitrile hydratase from a *Rhodococcus* spp. were identified in only the cystocentesis control sample.

4.2.3.2.f Summary Table

Table 4.15: The number of bacterial proteins identified in each group of cats from the second run for cystocentesis samples, voided samples and common to both methods of collection.
LUT – Lower urinary tract.

	Cystocentesis samples	**Voided samples**	**Common to both**
Culture negative without LUT signs (n=13)	Up to 51 in 7 samples	Up to 78 in 5 samples	0
Culture negative with LUT signs (n=2)	0 33	9 6	0 5
Culture positive with LUT signs (n=1)	N/A	473	N/A
Culture positive without LUT signs (n=1)	N/A	487	N/A

4.2.4 Bacterial Analysis

4.2.4.1 First Run

Eight hundred and sixty-four bacterial proteins were identified from the *E. coli* isolated from the cystocentesis sample of the cat with bacterial cystitis. Of these, 721 (83%) were identified as *E. coli* proteins. Eleven of the proteins identified from the *E. coli* isolate were also identified in the corresponding urine sediment. These included: membrane proteins, an elongation factor, 30S and 50S ribosomal proteins and pyruvate dehydrogenase complex proteins.

Two hundred and fifty-three bacterial proteins were identified from the *Enterococcus spp.* isolated from Cat A. Of these, 139 (55%) were identified as *Enterococcus faecalis* proteins, 85 (34%) were identified as *E. coli* proteins. Twelve other genera of bacteria were also identified.

The 3 *Enterococcus faecalis* proteins identified in the corresponding urine sediment were identified in the bacterial analysis. These were an elongation factor, an enolase and a triosephosphate isomerase.

4.2.4.2 Second Run

Four hundred and eighty-one bacterial proteins were identified from the *E. coli* isolated from the voided sample of Cat D. Of these, 342 (71%) were identified as *E. coli* proteins. One hundred and ninety-eight proteins identified from the *E. coli* isolate were also identified in the corresponding urine sediment.

Eight proteins were identified from the *E. coli* isolated from the voided sample of the Cat C with subclinical bacteriuria. Of these, 2 (25%) were identified as *E. coli* proteins. Other genera identified included: *Enterobacter, Erwinia, Klebsiella, Photorhabdus, Serratia* and *Sodalis.* One of the *E. coli* proteins identified was also identified in the corresponding urine sediment.

4.3 The Effect of Non-Absorbent Hydrophobic Sand Litter on Urine Protein-To-Creatinine Measurements

4.3.1 Experiment 1 – Pilot Investigation

Thirty-five urine samples obtained by cystocentesis were used for the pilot investigation. Three millilitres of urine were placed into a Petri dish containing 20g of non-absorbent litter. After 24 hours, no urine was available for recovery from any sample. All urine appeared to have absorbed into the litter or evaporated.

4.3.2 Experiment 2 and 3 – 40 Samples

4.3.2.1 Study Population

Forty urine samples were obtained from thirty-nine cats over a six-month period. All urine samples were obtained by cystocentesis. Of the 40 urine samples obtained, 34 were obtained from the Small Animal Teaching Hospital and 6 were submitted to the Diagnostic Laboratory from external veterinary practices.

Median age at the time of enrolment was 11 years (range, 1-18). Of the 39 cats, 21 (54%) were male and 18 (46%) were female. One female cat was entire and the sex status of one male was not recorded. All other cats were neutered. Of the 39 cats, 29 (74%) were domestic shorthair cats, 3 (8%) were domestic longhair cats, 2 (5%) were Persian and Bengal and 1 (3%) of each of the following breeds were included: British Blue, Maine Coon cross and Siamese.

4.3.2.2 Volume of Samples

From the 3mL placed into each Petri dish, a mean volume of 2.5mL (range, 1.0-2.9) were recovered after 24 hours.

4.3.2.3 Sediment Examination

Of the 40 samples analysed, 27 (67.5%) were classified as having an inactive sediment and 13 (32.5%) were classified as having an active sediment.

4.3.2.4 Urine Protein-to-Creatinine Ratios

The median baseline UPC was 0.17 (range, 0.05-5.59). The median baseline urine protein and urine creatinine concentrations were 38.13mg/dL (range, 7.35-277.90) and 215.58mg/dL (range, 17.53-640.21) respectively. The median UPC at 24 hours was 0.16 (range, 0.05-5.22). The median urine protein and urine creatinine concentrations at 24 hours were 37.85mg/dL (range, 5.9-279.00) and 215.67mg/dL (range, 18.10-657.01) respectively.

Out of 40 samples, a decrease in UPC after 24 hours was observed in 29 (72.5%) cases. An increase in UPC was observed in 3 (7.5%) cases and no change in UPC was observed in 8 (20%) of cases. There was a statistically significant median decrease in UPC of 0.01 after urine was exposed to non-absorbent hydrophobic sand litter for 24 hours, $p<0.0005$.

A decrease in urinary protein concentration after 24 hours was observed in 26 (65%) cases and an increase in concentration was observed in 14 (35%) cases. There was no statistically significant change in urinary protein concentration observed after urine was exposed to non-absorbent hydrophobic sand litter for 24 hours, $p=0.082$.

An increase in urinary creatinine concentration was observed in 34 (85%) cases and a decrease observed in 6 (15%) cases. A statistically significant median increase in urinary creatine concentration measurement of 5.76mg/dL was observed after urine was exposed to non-absorbent hydrophobic sand litter for 24 hours, $p<0.0005$.

The absolute mean difference in UPC observed over 24 hours was 0.04, equivalent to 8.89% absolute mean change. Absolute percentage changes observed in paired UPC samples ranged from 0% to 31.38%. Absolute mean percentage changes observed at 24 hours in urinary protein and creatinine concentrations were 6.86% and 7.25% respectively.

There was a statistically significant, strong positive correlation between UPC measured at baseline and UPC measured after urine was exposed to non-absorbent hydrophobic sand litter for 24 hours, $r_s=0.988$, $p<0.0005$.

Passing-Bablok regression analysis revealed minimal constant and proportional error. The regression equation of y= -0.0038 + 0.9606x with 95% confidence intervals (CI) for the intercept and slope of -0.0126 to 0.0040 and 0.9198 to 1.0046 respectively, suggests that UPC measurements at baseline or after 24-hour exposure to non-absorbent hydrophobic litter can be used interchangeably. A scatter plot with the regression line and 95% CI are shown in Figure 4.8.

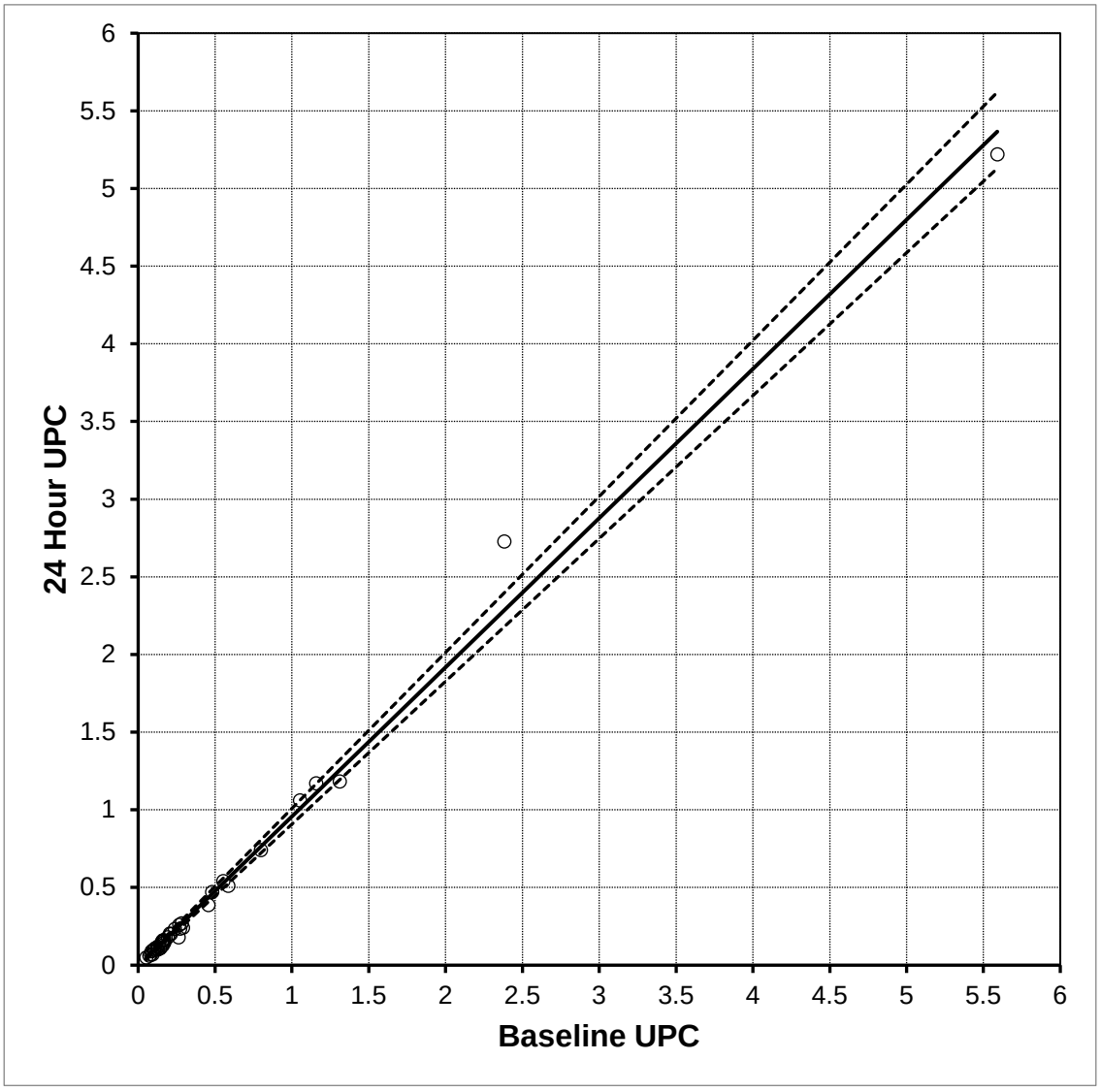

Figure 4.8: Passing-Bablok regression analysis of UPC measured at baseline and after 24-hour exposure to non-absorbent hydrophobic sand litter. n=40; UPC range 0.05-5.59; Spearman correlation coefficient 0.988, p<0.0005. Scatter chart with regression line and confidence bands for regression line shown. Regression line is solid. Regression line equation: y= -0.0038 + 0.9606x: 95% CI for intercept -0.0126 to 0.0040 and for slope 0.9198 to 1.0046.

A difference versus average Bland-Altman plot (Figure 4.9) demonstrated agreement between the UPCs obtained at baseline and after 24 hours exposure to non-absorbent hydrophobic sand litter for samples with UPC <2. The mean bias of the differences of paired UPC measures was -0.02075 and the 95% limits of agreement ranged from -0.1896 to 0.1481. The 95% CI for the calculated mean bias and 95% limits of agreement are outlined in Table 4.16. No real bias was evident as the 95% CI for the mean bias included 0 (Table 4.16), thereby demonstrating excellent agreement between the two measurements. Visual assessment of the plot suggested a possible negative proportional bias trend; however this is limited by the low numbers of samples with UPC >2.

Two paired UPC measures fell outside of the 95% limits of agreement. These values had a larger difference in UPC observed at 24 hours of -0.37 and 0.35. Actual values of UPC decreased from 5.59 to 5.22 and increased from 2.38 to 2.73 equivalent to absolute % changes of 6.62% and 14.71% respectively. Both sediments were classified as active with haematuria and pyuria documented in one case and bacteriuria in the other. The IRIS category for proteinuria did not change in either case.

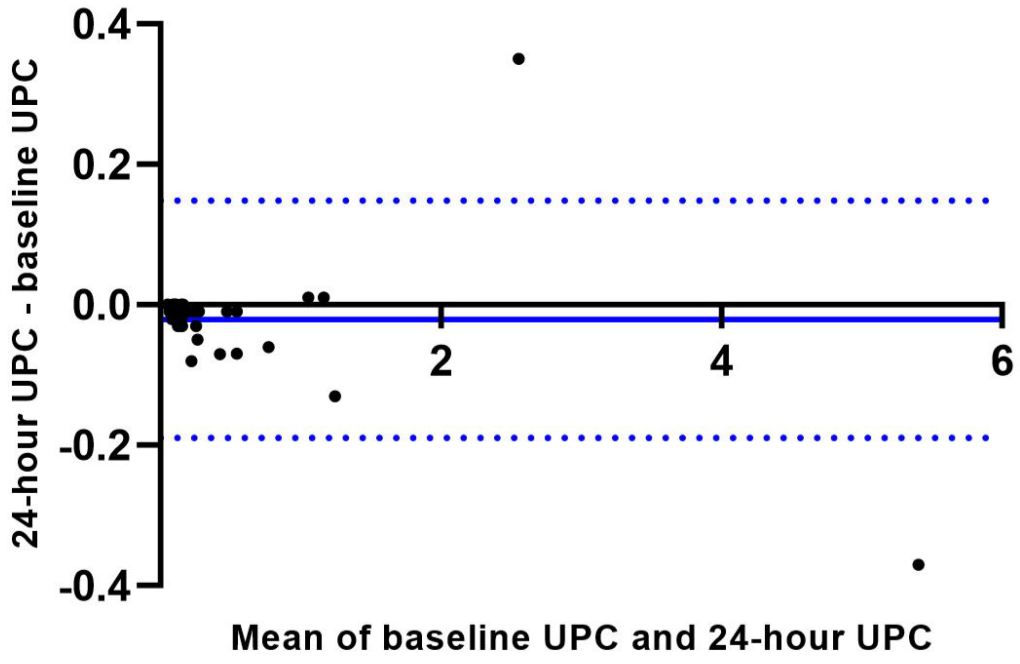

Figure 4.9: Difference versus average Bland-Altman plot of UPC measures obtained at baseline and after 24 hours exposure to non-absorbent hydrophobic sand litter. N=40.
Solid blue line represents the mean of the difference (bias) = -0.02075
Blue dashed lines represent the 95% limits of agreement from -0.1896 to 0.1481

Table 4.16: Mean bias and 95% limits of agreement (LOA) for Bland-Altman analysis with 95% confidence intervals (CI) reported to two decimal places.

Mean Bias (95% CI)	Lower LOA (95% CI)	Upper LOA (95% CI)
-0.02 (-0.05 to 0.01)	-0.19 (-0.24 to -0.14)	0.15 (0.10 to 0.19)

Of the 40 samples, at baseline, 22 (55%) were classified as non-proteinuric, 8 (20%) were classified as borderline proteinuric and 10 (25%) were classified as proteinuric as defined by IRIS (outlined in Table 4.17). Two samples changed IRIS category at 24 hours. One from borderline proteinuric to non-proteinuric and one from proteinuric to borderline proteinuric. The number of samples recorded within each IRIS category at baseline and 24 hours is shown in Table 4.18.

Table 4.17: Classification of feline proteinuria as defined by the International Renal Interest Society.

UPC Measurement	IRIS category of proteinuria
<0.2	Non-proteinuric
0.2 – 0.4	Borderline proteinuric
>0.4	Proteinuric

Table 4.18: Number of samples within each IRIS proteinuria category at baseline and at 24 hours. Discordant results are highlighted.

		IRIS proteinuria category at 24 hours		
		Non-proteinuric	**Borderline proteinuric**	**Proteinuric**
IRIS proteinuria category at baseline	**Non-proteinuric**	22	0	0
	Borderline proteinuric	1	7	0
	Proteinuric	0	1	9

IRIS categories for proteinuria were concordant in 38 (95%) cases. IRIS category changed by no more than one level for any discordant case. The kappa coefficient was $\kappa=0.915$, $p<0.0005$, corresponding to almost perfect agreement according to Landis and Koch (1977) between IRIS categories obtained at baseline and 24 hours.

4.4 The Effect of Urine Sample Collection Technique on Common Urinalysis Parameters

4.4.1 Study Population

Paired urinalyses results from 43 cats were used for this investigation. The mean time delay between obtaining paired urine samples was 9.7 hours (range, 1-23 hours). The voided sample was obtained first in 35 cases and the cystocentesis sample in 8 cases. No samples obtained from SUB devices were used for this investigation.

Median age at the time of enrolment was 12 years (range, 2-18). Of the 43 cats, 21 were male and 22 were female. All cats were neutered. Of the 43 cats, 32 (74%) were domestic shorthair cats, 4 (9%) were domestic longhair cats and 1 of each of the following breeds were included: domestic medium hair, Bengal, British shorthair, Burmese, Maine Coon, Persian and Persian-cross.

4.4.2 Urine Specific Gravity, pH and Urine Protein-to-Creatinine Ratios

The difference between paired measures of all continuous variables were not normally distributed as assed by the Shapiro-Wilk test. The differences in parameters observed according to method of collection were not symmetrically distributed when assessed visually on a histogram for USG and pH. The differences observed were symmetrically distributed when assessed visually on a histogram for UPC.

4.4.2.1 Urine Specific Gravity

The median USG recorded for voided samples and cystocentesis samples was 1.035 (range, 1.007 to >1.050) and 1.036 (range, 1.008 to >1.050) respectively. Out of 43 paired samples, a higher USG was observed in the voided sample in 18 (42%) cases. A higher USG was observed in the cystocentesis sample in 16 (37%) cases and no change in USG was observed in 9 (21%) cases. There was no statistically significant median change in USG when free catch techniques were used to obtain urine, p=0.864.

In 4 cases, a USG of ≥1.035 was observed in cystocentesis samples and a USG of <1.035 observed in corresponding voided sample. In two cases, a USG of ≥1.035 was observed in the voided samples and a USG of <1.035 observed in corresponding cystocentesis samples.

4.4.2.2 pH

The median pH recorded for voided samples and cystocentesis samples was 6.7 (range, 5.8-8.5) and 6.4 (range, 5.1-8.1) respectively. Out of 43 paired samples, a higher pH was observed in the voided sample in 22 (51%) cases. A higher pH was observed in the cystocentesis sample in 12 (28%) cases and no change in pH was observed in 9 (21%) cases. There was no statistically significant difference in urine pH when free catch techniques were used to obtain urine, p=0.123.

4.4.2.3 Urine Protein-to-Creatinine Ratios

The median UPC recorded for voided samples and cystocentesis samples was 0.24 (range, 0.06 to 0.99) and 0.27 (range, 0.09 to 0.79) respectively. Out of 43 paired samples, a higher UPC was observed in the voided sample in 8 (18.6%) cases. A higher UPC was observed in the cystocentesis sample in 30 (70%) cases and no change in UPC was observed in 5 (12%) cases. There was a statistically significant median decrease in UPC of 0.04 when free catch techniques were used to obtain urine, $p<0.0005$.

There was a statistically significant, strong positive correlation between UPCs determined from voided urine samples and from cystocentesis samples, $r_s=0.937$, $p<0.0005$.

For all samples, Passing-Bablok regression analysis revealed minimal constant and proportional error. The regression equation of y= -0.0019 + 0.8596x with 95% CI for the intercept and slope of -0.04 to 0.0257 and 0.7619 to 1.00 respectively. A scatter plot with the regression line and 95% CI are shown in Figure 4.10.

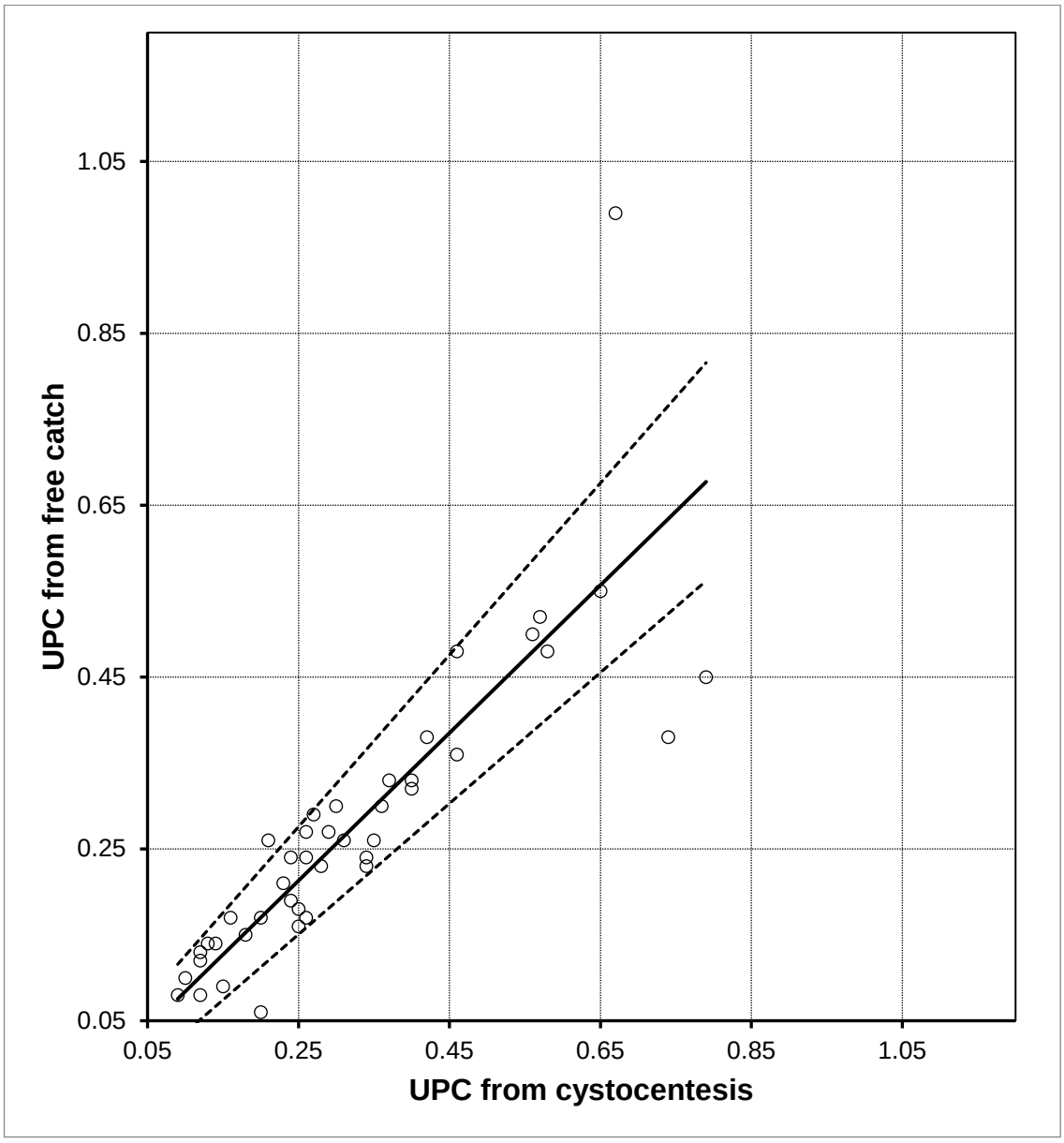

Figure 4.10: Passing-Bablok regression analysis of UPCs determined from voided urine samples and from cystocentesis samples. n=43; UPC range 0.06-0.99; Spearman correlation coefficient 0.937, p<0.0005. Scatter chart with regression line and confidence bands for regression line shown. Regression line is solid. Regression line equation: y= -0.0019 + 0.8596x: 95% CI for intercept -0.04 to 0.0257 and for slope 0.7619 to 1.0000.

A difference versus average Bland-Altman plot (Figure 4.11) was used to judge acceptability between the UPCs determined from voided samples and cystocentesis samples. For all samples, the mean bias of paired UPC measures was -0.05 and the 95% limits of agreement ranged from -0.24 to 0.14. The 95% confidence intervals for the calculated mean bias and 95% limits of agreement are outlined in Table 4.19. Three paired UPC measures fell outside of the 95% limits of agreement. These values had a larger difference in UPC observed between methods of collection of -0.36, -0.34 and 0.32. These differences were equivalent to absolute % changes of 47.76%, 48.65% and 43.04% respectively. All urine samples that fell outside the 95% limits of agreement had active sediments and a mean UPC of >0.55.

Table 4.19: Mean bias and 95% limits of agreement (LOA) for Bland-Altman analysis with 95% confidence

Sediment status (n=)	**Mean Bias (95% CI)**	**Lower LOA (95% CI)**	**Upper LOA (95% CI)**
All Samples (43)	-0.05 (-0.08 to -0.02)	-0.24 (-0.29 to -0.19)	0.14 (0.09 to 0.19)
Active (20)	-0.06 (-0.12 to 0.01)	-0.32 (-0.43 to -0.21)	0.21 (0.10 to 0.32)
Inactive (23)	-0.04 (-0.06 to -0.02)	-0.13 (-0.16 to -0.09)	0.05 (0.02 to 0.09)

intervals (CI) reported to two decimal places

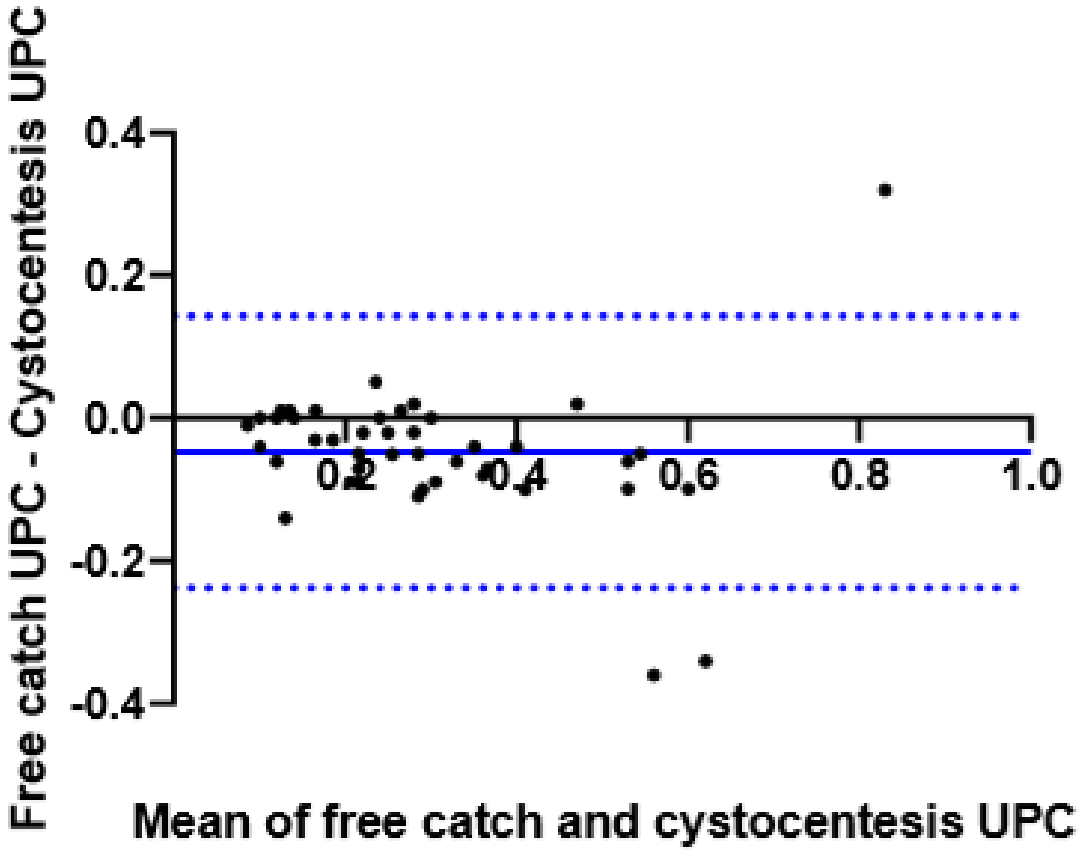

Figure 4.11: Difference versus average Bland-Altman plot of UPC measures obtained from free catch urine and cystocentesis samples. n=43
Solid blue line represents the mean of the difference (bias) = -0.05
Blue dashed lines represent the 95% limits of agreement from -0.24 to 0.14.

For samples with active sediments, the Passing-Bablok regression equation was y= -0.0136 + 0.8800x with 95% CI for the intercept and slope of -0.0136 to 0.0944 and 0.6429 to 1.1176 respectively. Despite still including 0, the 95% CI for the slope in the subset of sample with active sediments were wider than that for the subset of samples with inactive sediments. A scatter plot with the regression line and 95% CI are shown in Figure 4.12.

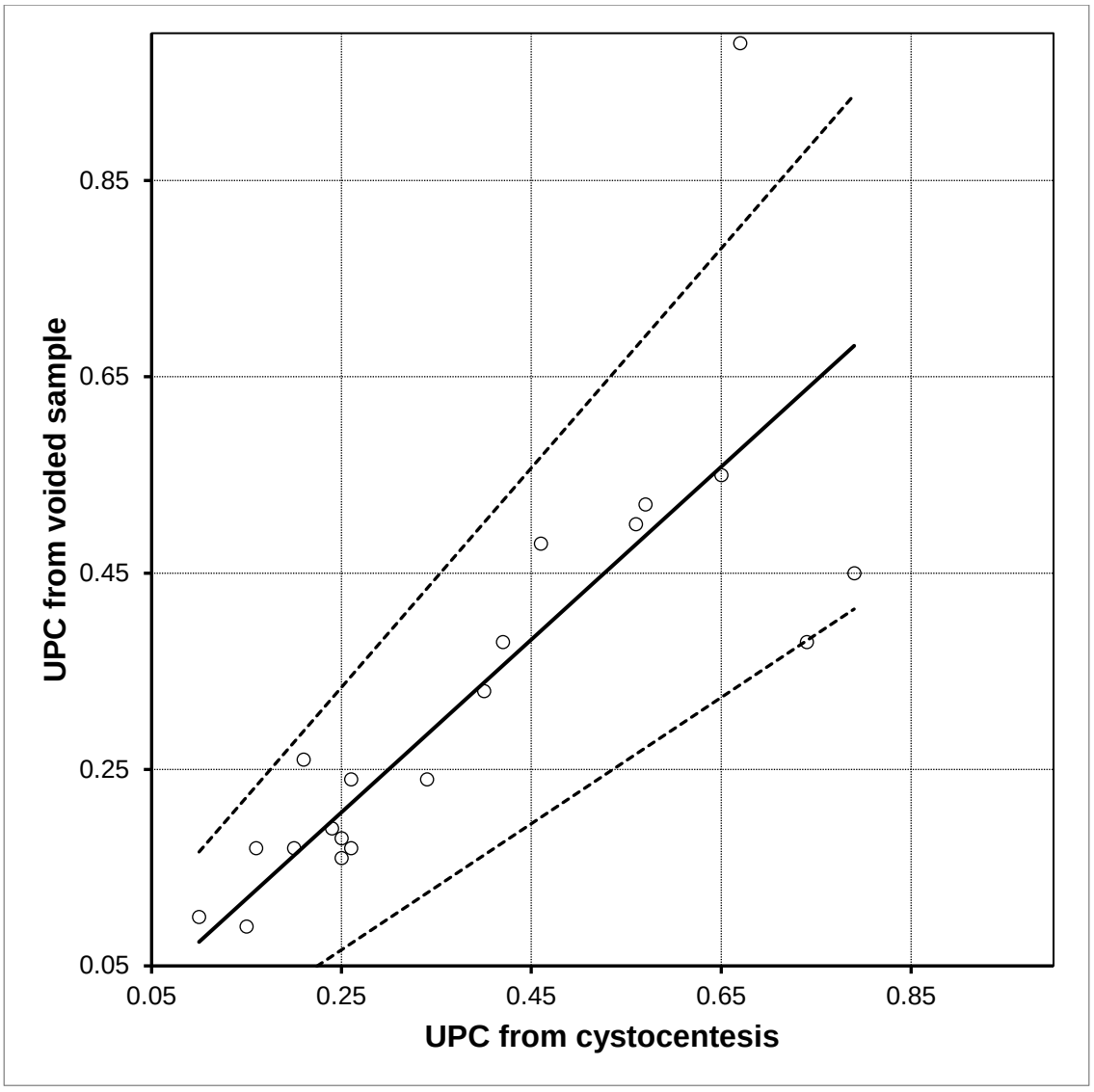

Figure 4.12: Passing-Bablok regression analysis of UPCs determined from voided urine samples and from cystocentesis samples with active sediments. n=20; UPC range 0.09-0.99; Spearman correlation coefficient 0.875, p<0.0005. Scatter chart with regression line and confidence bands for regression line shown. Regression line is solid.
Regression line equation: y= -0.0136 + 0.8800x: 95% CI for intercept -0.0944 to 0.0543 and for slope 0.6429 to 1.1176.

Visual inspection of the Bland-Altman plot for urine samples with active sediments revealed poor agreement between methods of collection for UPCs. For samples with active sediments, the 95% limits of agreement calculated from the Bland-Altman plot span 0.53, which would be unacceptable for UPC measurements. A minimum of 40 samples with active sediments would however be needed to draw meaningful conclusions from this subset.

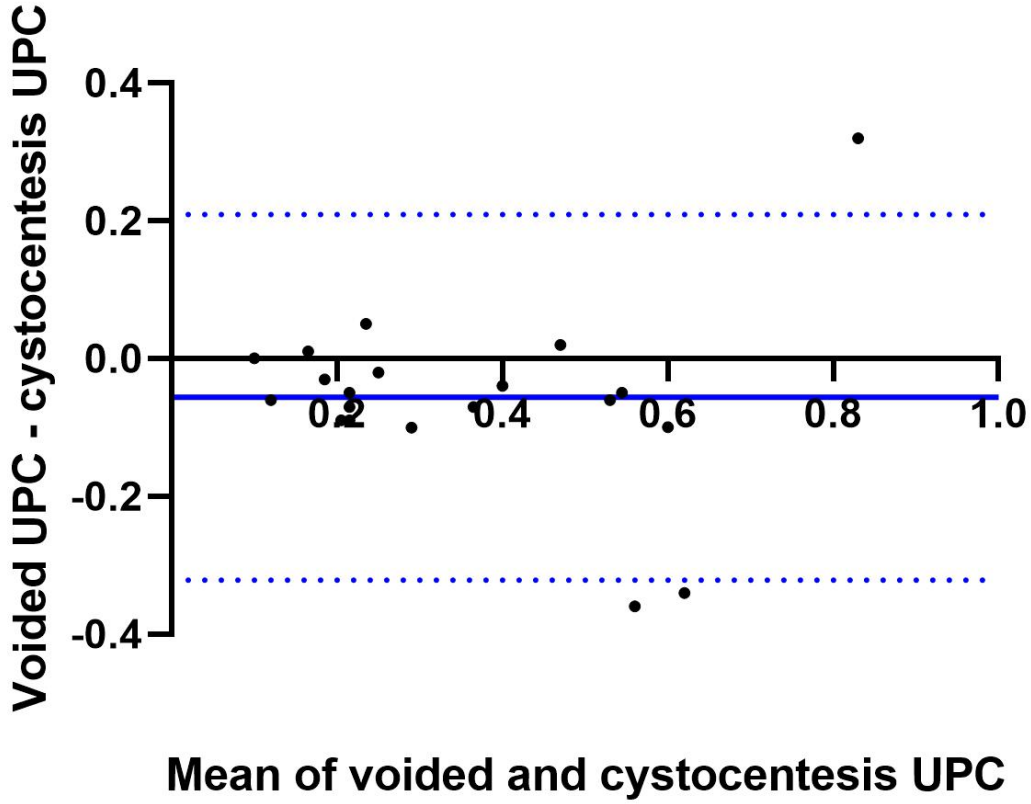

Figure 4.13: Difference versus average Bland-Altman plot of UPC measures obtained from voided urine samples and cystocentesis with an active sediment. n=20
Solid blue line represents the mean of the difference (bias) = -0.06
Blue dashed lines represent the 95% limits of agreement from -0.32 to 0.21.

For samples with inactive sediments, the Passing-Bablok regression equation was y= -0.0160 + 0.8000x with 95% CI for the intercept and slope of -0.0300 to 0.0469 and 0.6875 to 1.000 respectively. A scatter plot with the regression line and 95% CI are shown in Figure 4.12.

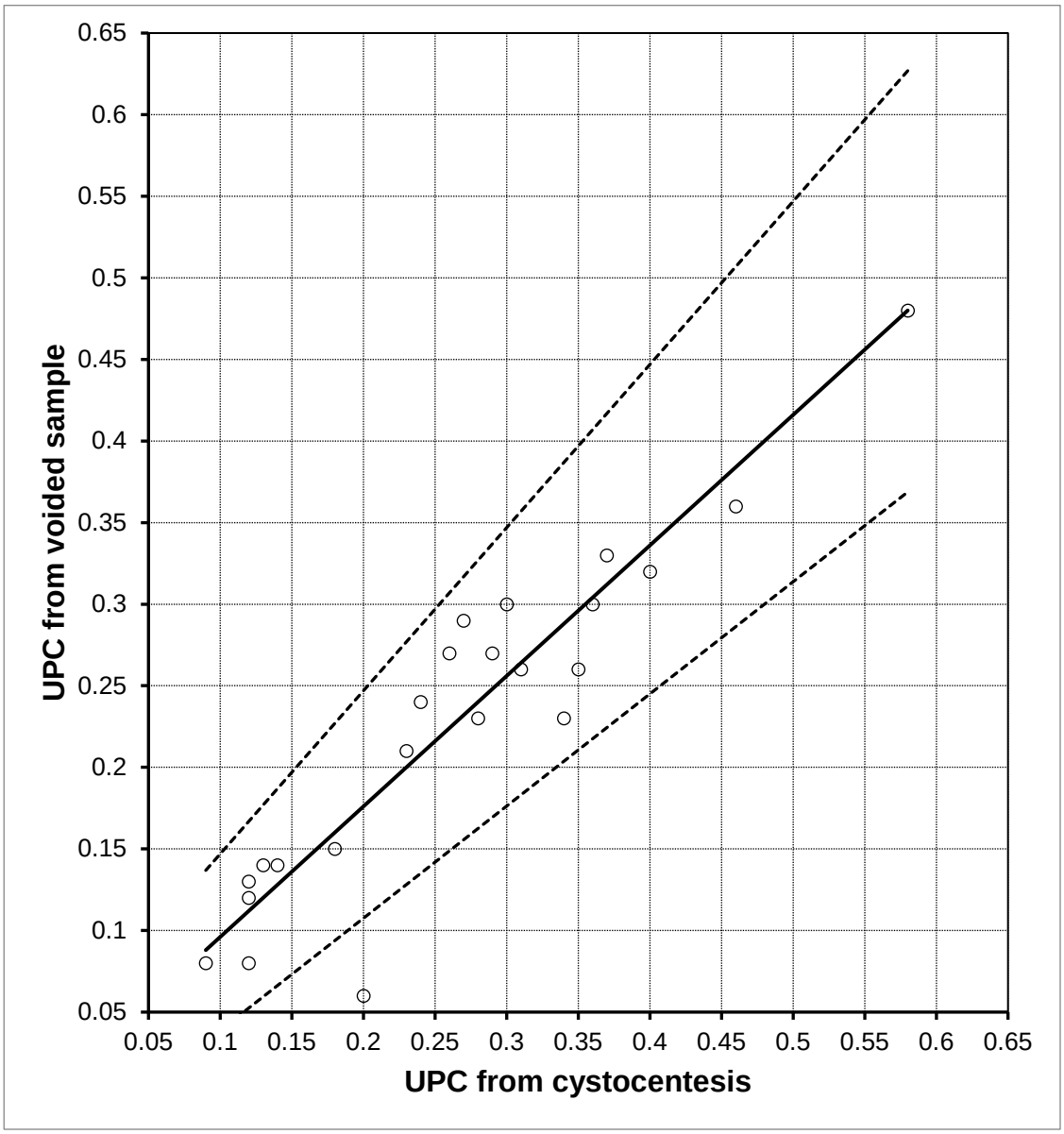

Figure 4.14: Passing-Bablok regression analysis of UPCs determined from voided urine samples and from cystocentesis samples with inactive sediments. n=23; UPC range 0.06-0.58; Spearman correlation coefficient 0.900, p<0.0005. Scatter chart with regression line and confidence bands for regression line shown. Regression line is solid.
Regression line equation: y= 0.0160 + 0.8000x: 95% CI for intercept -0.0300 to 0.0469 and for slope 0.6875 to 1.0000.

Visual inspection of the Bland-Altman plot for urine samples with inactive sediments revealed good agreement between methods of collection for samples with a UPC <0.5. For samples with inactive sediments, the 95% limits of agreement calculated from the Bland-Altman plot span 0.18. One data point fell outside the 95% limits of agreement. The cystocentesis UPC was 0.20 and the voided sample was 0.06 in this case. A minimum of 40 samples with inactive sediments would however be needed to draw meaningful conclusions from this subset.

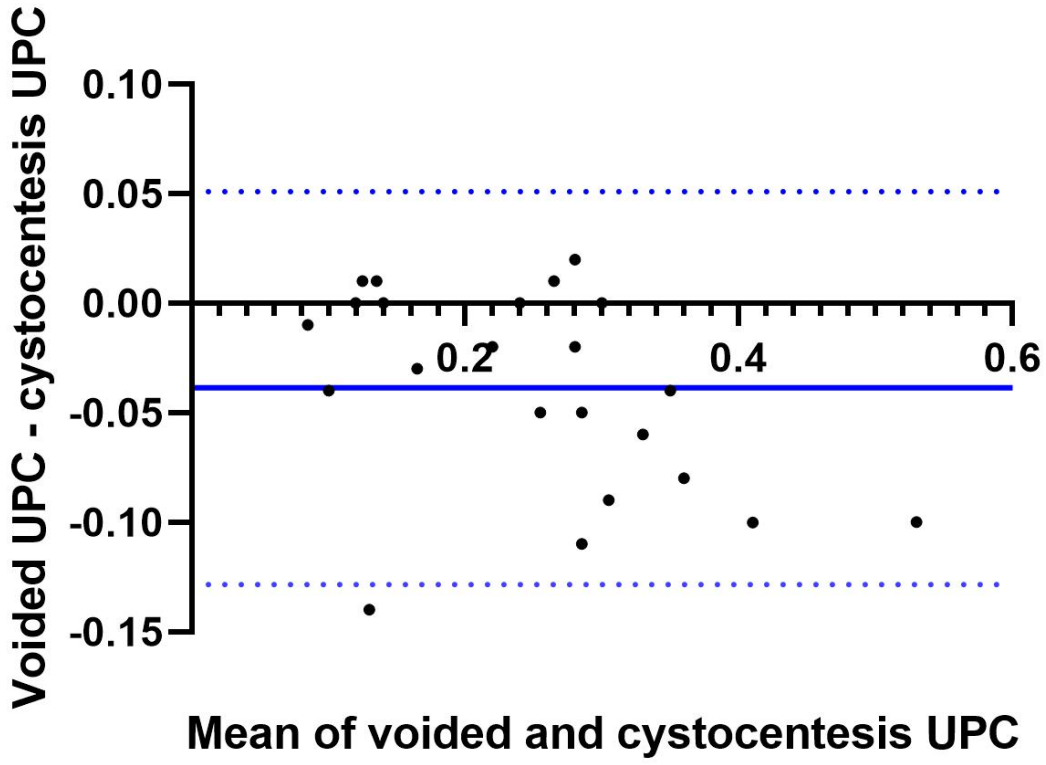

Figure 4.15: Difference versus average Bland-Altman plot of UPC measures obtained from voided urine samples and cystocentesis samples with inactive sediments. n=23
Solid blue line represents the mean of the difference (bias) = -0.04
Blue dashed lines represent the 95% limits of agreement from -0.13 to 0.05.

For all samples, Cohen's kappa coefficient was κ=0.666, p<0.0005, corresponding to substantial agreement according to Landis and Koch (1977) between voided urine samples and cystocentesis samples for IRIS classification of proteinuria. This reduced to κ=0.489 for urine samples with active sediments and increased to κ=0.833 for urine samples with inactive sediments, corresponding to moderate and almost perfect agreement respectively (Landis and Koch, 1977).

4.4.3 Dipstick Parameters

4.4.3.1 pH

Out of 43 samples, a higher pH was observed in the voided sample in 18 (42%) cases. A higher pH was observed in the cystocentesis sample in 2 (5%) cases and no change in pH was observed in 23 (53%) cases. In seven cases, a pH difference of ≥ 1 unit was observed and in two cases a pH difference of 2 units was observed. In both cases where a difference of 2 units was observed, a higher pH was recorded in the voided sample.

4.4.3.2 Glucose

Forty and thirty-nine cystocentesis samples and voided samples respectively were negative for glucose. Tied observations for glucose measurement were recorded for 40 of 43 paired samples. In two of the discordant cases, glucosuria was documented in the voided sample only with 'trace' and '++' results.

4.4.3.3 Ketones

Ketonuria was not detected in any sample.

4.4.3.4 Bilirubin

All 43 paired samples matched for bilirubin measurements. Forty-two paired samples were negative, and a '+' result was recorded in one paired sample.

4.4.3.5 Blood

The median recorded score for blood was 0 for both voided samples and cystocentesis samples. Twenty-nine scores of 0 for both voided and cystocentesis samples were recorded. Fourteen samples scores ranged from 0.25-3 ('Trace' – '+++') for each method of collection. Out of 43 paired samples, an increased amount of blood was observed in the voided sample in 5 (12%) cases. An increased amount of blood was observed in the cystocentesis sample in 6 (14%) cases and no difference in the amount of blood present between the voided sample and cystocentesis sample was observed in 32 (74%) cases.

4.4.4 Sediment Examination

4.4.4.1 White Blood Cells

The median score recorded for the presence of white blood cells in both voided samples and cystocentesis samples was 0 (range, 0-1). Out of 43 paired samples, an increased amount of white blood cells was observed in the voided sample in 3 (7%) cases. An increased amount of

white blood cells was observed in the cystocentesis sample in 13 (30%) cases and no difference in the amount of white blood cells present was observed in 27 (63%) cases.

When evaluating the difference in the observation of pyuria recorded between voided and cystocentesis samples, matched observations were recorded in 42 of 43 paired cases. In 41 cases, no pyuria was observed and in 1 case, pyuria was documented.

In the discordant pair, pyuria was observed in the cystocentesis sample whilst only scant (Table 3.6) white blood cells were observed in the voided sample. In this case, the voided sample remained in the litter tray for approximately 2 hours prior to collection and analysis. It is therefore possible some degeneration of any leukocytes present may have occurred.

4.4.4.2 Red Blood Cells

The median score recorded for the presence of red blood cells in voided samples and cystocentesis samples was 0 (range, 0-3) and 0 (range, 0-4) respectively. Out of 43 paired samples, an increased amount of red blood cells was observed in the voided sample in 7 (16%) cases. An increased amount of blood was observed in the cystocentesis sample in 10 (23%) cases and no difference in the amount of red blood cells present was observed in 26 (60%) cases.

When evaluating the difference in the observation of haematuria recorded between voided and cystocentesis samples, matched observations were recorded in 39 of 43 paired cases. In 30 cases, no haematuria was observed and in 9 cases, haematuria was documented.

Of the 4 discordant pairs, haematuria was documented twice in only cystocentesis samples and twice in only voided samples. Paired scores for discordant results were 0.5 and 1 in two cases and 0 and 2 in two cases. In 3 cases the voided urine sample was obtained first.

4.4.4.3 Squamous Epithelial Cells

The median score recorded for the presence of squamous epithelial cells in voided samples and cystocentesis samples was 0.5 (range, 0-0.5) and 0 (range, 0-0.5) respectively. Out of 43 samples, an increased number of squamous epithelial cells was observed in the voided sample in 20 (47%) cases. An increased number of squamous epithelial cells was observed in the cystocentesis sample in 3 (7%) cases and no difference in the number of squamous epithelial cells present was observed in 20 (47%) cases. All observations were deemed normal findings with no more than 5 squamous epithelial cells per HPF seen in any sample.

4.4.4.4 Transitional Epithelial Cells

The median score recorded for the presence of transitional epithelial cells in voided samples and cystocentesis samples was 0 (range, 0-0.5) and 0 (range, 0-0.5) respectively. Out of 43 samples, an increased number of transitional epithelial cells was observed in the voided sample in 3 (7%) cases. An increased number of transitional epithelial cells was observed in the cystocentesis sample in 6 (14%) cases and no difference in the amount of transitional epithelial cells present was observed in 34 (79%) cases. All observations were deemed normal findings with no more than 5 transitional epithelial cells per HPF seen in any sample.

4.4.4.5 Bacteria

The median score recorded for the presence of bacteria in voided samples and cystocentesis samples was 0 (range, 0-3) and 0 (range, 0-1) respectively. Out of 43 samples, an increased number of bacteria was observed in the voided sample in 5 (12%) cases. An increased number of bacteria was observed in the cystocentesis sample in 4 (9%) cases and no difference in the number of bacteria present was observed in 34 (79%) cases.

When evaluating the difference in the observation of bacteriuria recorded between voided and cystocentesis samples, matched observations were recorded in 37 of 43 paired cases (90%). In 31 cases no bacteriuria was observed and in 6 cases bacteriuria was documented. In the discordant pairs, bacteriuria was observed in the voided sample only or cystocentesis sample only in 4 and 2 cases respectively.

4.4.4.6 Casts

Significant cylindruria was not observed in any sample. The scant presence of casts was documented in voided or cystocentesis samples in 10 and 14 cases respectively. For voided samples this was due to the presence of hyaline casts, coarse granular casts and fine granular casts in 3, 1 and 5 cases respectively. In 1 voided sample the scant presence of hyaline casts and fine granular casts were documented. For cystocentesis samples, hyaline casts, coarse granular casts and fine granular cast were documented in 6, 3 and 5 cases respectively.

4.4.4.7 Crystals

Scant crystalluria was documented in voided or cystocentesis samples in 12 and 20 cases respectively. Discordant results were obtained in 6, 1 and 18 cases for struvite, bilirubin and calcium phosphate crystalluria respectively.

Of the 6 discordant struvite crystalluria cases, crystals were documented in the voided sample only in 4 cases.

In the case of bilirubin crystalluria, scant crystals were noted in the voided sample but not the cystocentesis sample. Both the voided and cystocentesis sample were positive for bilirubin on dipstick analysis.

Of the 18 discordant cases of calcium phosphate crystalluria, crystals were documented in the cystocentesis samples only in 14 cases.

4.4.5 Cytological Examination

4.4.5.1 White Blood Cells

The median score recorded for the presence of white blood cells in voided samples and cystocentesis samples was 0 (range, 0-0.5) and 0 (range, 0-0.5) respectively. For voided samples, no white blood cells were documented in 42 of 43 cases. A score of 0.5 was recorded in 1 case. For cystocentesis samples scores of 0 and 0.5 were recorded in 37 and 6 cases respectively. Pyuria based upon cytological examination as defined in Table 3.6 was not documented in any case.

4.4.5.2 Red Blood Cells

The median score recorded for the presence of white blood cells in voided samples and cystocentesis samples was 0 (range, 0-4) and 0 (range, 0-4). For voided samples, a score of >0.5 was documented in 7 (16%) cases. For cystocentesis samples score of >0.5 was recorded in 9 (21%) cases.

Discordant results for the observation of haematuria based upon cytological examination was documented in 6 cases. In 4 of the 6 cases, haematuria was observed in the cystocentesis sample only. In 5 of the 6 cases, the voided sample was obtained first.

For cystocentesis samples, of the 9 cases with cytological evidence of haematuria, 7 had evidence of haematuria on sediment examination. All 7 voided samples with cytological evidence of haematuria had evidence of haematuria on sediment examination.

4.4.5.3 Squamous Epithelial Cells

The median score recorded for the presence of squamous epithelia cells in voided samples and cystocentesis samples was 0.5 (range, 0-1) and 0 (range, 0-0.5) respectively. For voided samples, a score of 1 was documented in 5 (12%) cases. In 3 of the 5 cases, a score of 0.5 was recorded for the corresponding cystocentesis sample.

4.4.5.4 Transitional Epithelial Cells

The median score recorded for the presence of transitional epithelia cells in voided samples and cystocentesis samples was 0 (range, 0-0.5) and 0 (range, 0-1) respectively. For voided samples a score of 0 was documented in all but one case. For cystocentesis samples scores of 0.5 and 1 were documented in 3 and 1 cases respectively.

4.4.5.5 Bacteria

Bacteria were observed in 11 and 2 voided samples and cystocentesis samples respectively. In the 2 cases bacteria were observed in the cystocentesis sample, bacteria were also observed in the voided sample.

In the 11 voided samples, bacteria were specifically reported as being associated with squamous epithelial cells in 5 cases. Significant bacterial growth on urine culture was reported in only one case in which cytological evidence of bacteriuria was present.

Of the 2 cystocentesis samples, scores of 0.5 and 3 were reported, no intra-cellular bacteria were reported in either case. No bacterial growth was reported on urine culture for either case.

4.4.6 Urine Culture

Bacterial growth was significantly more likely in voided samples compared to cystocentesis samples, $p<0.0005$, and was reported in 37 (86%) of 43 cases.

When significant quantitative bacterial growth cut-off levels were applied, there was no significant difference in positive growth results between method of collection, $p=0.5$. Significant growth was only reported in 2 voided urine samples. In one case, a mixed growth of approximately 13000 cfu/mL was documented. The predominate organism was suspected to be coliform in nature. A profuse growth of a *Proteus* spp. was suspected in the other case due to the swarming nature of growth noted on the blood agar plate.

No bacterial growth was observed in any cystocentesis sample.

Chapter 5 DISCUSSION

5.1 THE DIAGNOSTIC APPROACH TO FELINE SUBCLINICAL BACTERIURIA AND URINARY TRACT INFECTIONS

The main objectives of identifying urinalysis results predictive of subclinical bacteriuria and comparing methods of urine collection within this were not achieved due to the lack of subclinical bacteriuria cases recorded in this study. Instead, cases that may have been interpreted as subclinical bacteriuria or clinical UTI were described as a case series with differences between methods of urine collection highlighted.

Genuine subclinical bacteriuria, as defined by Weese *et al.* (2019), was suspected in 2 cats (Cat A and Cat C in Section 4.1.2) in this study giving a prevalence of 4%. This falls within the wide prevalence range previously reported of 0.9%-28.8% and compares to the recently reported prevalence from a prospective study of 6.1% (Eggertsdottir *et al.*, 2007, Litster *et al.*, 2009, Moberg *et al.*, 2019).

A post-hoc sample size calculation with significance levels of alpha = 0.05 and beta = 0.2 showed that 43 cats per study group would have been required to make statistically powerful conclusions regarding differences in urinalysis results between cats with subclinical bacteriuria and cats with UTI (Clincalc, 2019). The small number of urine culture-positive cats is a major limitation of this study but recruitment of such a sample size would not have been possible within the study period.

All 4 cats with genuine subclinical bacteriuria or UTI in this study were female, which is consistently reported as a risk factor for their development (Lekcharoensuk *et al.*, 2001, Litster *et al.*, 2009).

Out of 36 hyperthyroid cats screened, 1 case (2.7%) of subclinical bacteriuria was suspected. This is much lower than the reported prevalence from retrospective studies of positive urine cultures in hyperthyroid cats of 12%-17% (Mayer-Roenne *et al.*, 2007, Bailiff *et al.*, 2008). At the authors institution, cystocentesis and urine culture were part of the RAI suitability assessment. This was stopped approximately 5 months into the study and the number of hyperthyroid cats recruited reduced after this point. This likely contributed to the reduced prevalence of subclinical bacteriuria observed in this study.

In this study, 53% of cats had CKD (of any IRIS stage) documented as a primary reason for presentation or a co-morbidity. Both cats with suspected subclinical bacteriuria had CKD of IRIS

stage 2 and 3 respectively. Only one previous retrospective study has identified CKD as a significant risk factor for subclinical bacteriuria (Puchot *et al.*, 2017). In two larger scale retrospective studies, severity of azotaemia was not identified as a significant risk factor for positive urine culture, nor was CKD identified as a risk factor for subclinical bacteriuria in a recent prospective study of 179 cats (Bailiff *et al.*, 2008, Mayer-Roenne *et al.*, 2007, Moberg *et al.*, 2019).

Moberg *et al.* (2019) recently identified hepatic disease as a risk factor for subclinical bacteriuria, although this was not a finding in this study with both subclinical bacteriuria cats having alanine aminotransferase (ALT) and alkaline phosphatase (ALP) enzyme activities within laboratory reference ranges. The study by Moberg *et al.* (2019) was underpowered but with an increased prevalence of asymptomatic bacteriuria reported in human patients with hepatic cirrhosis, further studies evaluating hepatic disease as a risk factor for subclinical bacteriuria in cats seem warranted.

A profuse growth of an *Enterococcus* sp. was documented in the confirmed case of subclinical bacteriuria in this study (Cat A in Section 4.1.2.1). *Enterococcus* spp. are reported to account for up to 27% of positive feline urine cultures (Mayer-Roenne *et al.*, 2007) and are significantly more likely to be isolated from cats with subclinical bacteriuria compared to other pathogens (Teichmann-Knorrn *et al.*, 2018). In a review of *Enterococcus* spp. isolated from canine and feline urine samples, KuKanich and Lubbers (2015) reported that no clinical signs of lower urinary tract disease were recorded in over 54% of cats with *E. faecalis*-positive urine cultures. Interestingly Kopecny *et al.* (2019) suggested enterococcal infections in cats with SUB devices may be more associated with lower urinary tract clinical signs, although clinical signs of lower urinary tract disease were not reported in Cat A in this study. The AST of the *Enterococcus* isolate in this study documented resistance to marbofloxacin and intermediate susceptibility to enrofloxacin and although the use of fluoroquinolones is not recommended for enterococcal UTI (Weese *et al.*, 2019), the risk of transmission of antimicrobial resistant bacteria from animal to human is of concern (Dotto *et al.*, 2018).

Although the cystocentesis urine sample of Cat C (Section 4.1.2.2) was culture negative, due to repeated positive follow up urine cultures, it is suspected the voided sample genuinely represented subclinical bacteriuria. It is unknown whether subclinical bacteriuria developed during the time between obtaining cystocentesis and voided samples (2160 hours) or whether this represents transient infection. Transient asymptomatic bacteriuria with *E. coli* has been described in human medicine (Biggel *et al.*, 2019) and transient subclinical bacteriuria has been

described infrequently by White *et al.* (2016) although the causative pathogen was not reported in this study.

It is likely the *Enterococcus* sp. or *Streptococcus* sp. suspected in the case of Cat E (Section 4.1.3.2) represents contamination due to this sample being a voided sample. With the sample being posted to the laboratory it is possible the sample was not processed for up to 48 hours post-voiding and with lack of refrigeration throughout posting, caution should be exercised when interpreting this culture result. It is possible that a false negative result was obtained from SUB device sample in this case as false negative results from cystocentesis samples in dogs have also infrequently been reported (Sorensen *et al.*, 2016). Financial considerations precluded the identification of bacterial species cultured from voided samples in this study.

The AST of one *E. coli* isolate was performed at an external laboratory as requested by the referring veterinary surgeon for the case. The isolate was reported as susceptible to amoxicillin, clavulanate-potentiated amoxicillin, cephalexin, pradofloxacin, marbofloxacin, cefovecin and trimethoprim/sulphonamide and resistant to clindamycin. The methodology used to perform the AST was not provided by the external laboratory making comparisons between this isolate and the susceptibilities of two *E. coli* tested in-house difficult. Both isolates tested in-house were from cats with clinical UTIs. One isolate demonstrated resistance to ampicillin and cephalexin and susceptibility to the remainder of antimicrobials tested against. The second isolate demonstrated full susceptibility, which has been reported in up to 67.8% of *E. coli* isolated from canine and feline UTIs in the UK (Marques *et al.*, 2016).

Both cats classified as having UTIs (Cat D and Cat E, Section 4.1.3.2) had SUB devices fitted at the time a positive culture was documented. At the author's institute, antimicrobials are not typically prescribed post-operatively to patients undergoing SUB device placement. In a retrospective study of 43 cats that had either ureteral stents or SUB devices placed, Kopecny *et al.* (2019) identified the post-operative use of antimicrobials significantly reduced the risk of subclinical bacteriuria or UTI post-discharge. Due to the retrospective nature of this study, antimicrobial prescribing was not standardised and so prospective studies aiming to identify appropriate protocols whilst avoiding overuse of antimicrobials are needed.

Measurement of UPC appeared to be a good screening tool for positive urine cultures for cystocentesis samples. Using a cut-off of >0.4, sensitivity and specificity were calculated as 100% and 69% respectively. For voided samples sensitivity and specificity were calculated as 66% and 80% but these values are likely influenced by the number of false positive urine cultures obtained using voided samples. These values should be interpreted with caution however

given Meindl *et al.* (2019) calculated sensitivity and specificity of proteinuria predicting a positive urine culture to be 43.5% and 37.2% respectively in a population of 394 dogs. The discrepancy in such values is likely due to the small sample size of this study.

Bacteriuria defined by sediment examination was present in all but one case of positive urine cultures. In this study the sensitivity and specificity of sediment examination for bacteriuria were superior for cystocentesis samples (100% and 85% respectively) compared to voided samples (83% and 78% respectively). The sensitivity of cytological evaluation for bacteriuria was equivalent to sediment examination for both methods of collection. Specificity of cytological evaluation increased to 96% for cystocentesis samples but reduced slightly to 76% for voided samples. One voided sample documented a growth of *Proteus* spp., in which bacteriuria was not documented on sediment or cytological examination. Quantification of culture for *Proteus* spp. is difficult when only blood agar plates are used due to the swarming nature of their growth (Schaffer and Pearson, 2015). It is possible that low numbers of bacteria present precluded their identification on sediment or cytological examination in this case (Reppas and Foster, 2016b).

From six positive urine cultures obtained from voided samples, three false positive results were suspected. Sensitivity and specificity were calculated to be 100% and 94% respectively and the positive predictive value was calculated to be 50%, suggesting positive cultures from voided samples should be interpreted with caution given the low prevalence of feline UTI and subclinical bacteriuria. This is in contrast to dogs where Sorensen *et al.* (2016) reported a sensitivity and specificity of 94% and 94% respectively and positive and negative predictive values of 88% and 97% respectively when using voided samples to determine significant bacteriuria. The study by Sorensen *et al.* (2016) included 94 dogs in which UTI was a differential diagnosis and so inclusion of the same number of cats in this study would be preferable in order to better compare the sensitivity and specificty of voided urine samples being used to diagnose UTI.

5.2 PROTEOMIC ANALYSIS

This pilot investigation into the use of proteomics within feline subclinical bacteriuria and UTI aimed to identify potential biomarkers that could be more thoroughly investigated in future research. Protein searches against feline and bacterial databases were conducted to explore possible host or bacterial markers. Two unique proteins were identified in the 3 samples of subclinical bacteriuria analysed. Bacterial proteins were identified in most urine samples although the predominate genera present varied between the two runs of samples.

When comparing voided samples to cystocentesis samples, up to 60% of proteins identified in each cat were present in both samples. Whilst moderate agreement between methods of collection was present, to ensure identification of true urinary biomarkers, future work should focus on samples obtained by cystocentesis mitigating the possible risk of sample contamination by commensal bacteria of the skin or distal urinary tract (Litster *et al.*, 2011).

Uromodulin was frequently identified as the most abundant protein in all study groups. Uromodulin is produced exclusively in the kidney, specifically by the tubular cells of the thick ascending limb of the loop of Henle, and is the most abundant protein identified in the urine of many mammalian species (Devuyst *et al.*, 2017, Jepson *et al.*, 2016). Although its full function has yet to characterised, uromodulin is thought to play a protective role in the prevention of UTI by binding pathogenic bacteria within the distal tubules and collecting ducts of the kidney (Vyletal *et al.*, 2010). Garimella *et al.* (2017) demonstrated that increased concentrations of urinary uromodulin appeared to decrease the risk of UTI development in an elderly human population. Whether this finding is also true in cats is yet to be studied.

Urinary fibronectin was identified in samples obtained from cats with and without lower urinary tract clinical signs. Fibronectin has previously been suggested as a marker for idiopathic cystitis in cats, with urinary concentrations found to be significantly higher in cats with idiopathic cystitis compared to cats with urolithiasis and bacterial cystitis (Lemberger *et al.*, 2011). Treutlein *et al.* (2013) later showed fibronectin concentrations did not decrease significantly over a 6 month follow up period in cats with idiopathic cystitis and were comparable to control cats beyond 14 days post-presentation. In this study, there were cases where the abundance of fibronectin relative to albumin were higher in cats without lower urinary tract clinical signs than cats with lower urinary tract clinical signs, although whether this difference was significant or not was not tested. Although urinary fibronectin concentrations may reflect underlying bladder pathology (Lemberger *et al.*, 2011), its use as a biomarker to differentiate causes of lower urinary tract disease requires further study.

The identification of a neutrophil-expressed elastase in the urine of cats with suspected subclinical bacteriuria (Section 4.2.2.1.c) supports previous work showing that subclinical infections can incite an inflammatory response (Litster *et al.*, 2009). Although in this study, this protein was identified in both cats with UTI and subclinical bacteriuria, the relative abundance was greater in cats with UTI and it is possible differential expression of inflammatory proteins may prove useful to differentiate cats with subclinical bacteriuria and UTI. In human medicine, urinary concentrations of interleukin-6 (IL-6) have been shown to discriminate between UTIs, asymptomatic bacteriuria and pyelonephritis in elderly patients (Kjolvmark *et al.*, 2016). Interleukin-6 was not identified in any cat with a positive urine culture through proteomic analysis in this study although it's lack of identification does not exclude its presence. Enzyme-linked immunosorbent assays (ELISA) for IL-6 are available with detection limits of 1pg/mL for human urine (Kjolvmark *et al.*, 2016), and it would prove interesting evaluate IL-6 concentrations in urine samples from this study. Although an ELISA for the detection of feline serum IL-6 is commercially available, its ability to detect urinary IL-6 concentrations has yet to be validated (Habenicht *et al.*, 2013). Given the day-to-day variation that exists in urinary protein excretion (Russo *et al.*, 1986), repeated analysis of successive urine samples from the same cat would help to quantify variation in excretion of specific proteins, which would be required to validate any potential biomarker.

The enzyme myeloperoxidase was identified in all cases of positive urine cultures. Myeloperoxidase was not identified in the deionised water control samples. Myeloperoxidase is a marker of inflammation and increased enzyme activity in urine has been identified in humans with UTI. Recent studies have suggested myeloperoxidase may serve as a useful non-invasive test to diagnose UTI in human patients with a test sensitivity and specificity of 87% and 100% reported in one trial (Masajtis-Zagajewska and Nowicki, 2017). Myeloperoxidase was however also identified in many culture negative samples from cats with lower urinary tract clinical signs in this study (data not shown).

There were two unique feline proteins that were identified in only cases of subclinical bacteriuria. These were of molecular weight 43.4kDa and 59.6kDa (Table 4.12). At present, the function of these proteins has yet to be elucidated. Further characterisation of the feline urinary proteome in larger cohorts of both healthy and diseased cats could potentially aid in identification of these proteins. Proteins of this molecular weight are freely filtered by healthy glomeruli (Brandt *et al.*, 2014) although a post-renal origin cannot be excluded. Proteins of this molecular weight may also represent cleavage products of larger proteins due to the presence of endogenous proteases found in urine (Jepson *et al.*, 2013). The BLAST search function of the

Uniprot database identified the uncharacterised protein with accession number A0A2I2V2Y7 as having 61% of its sequence identical to human complement factor H, a regulator of the complement cascade (Makou *et al.*, 2013, UniProt, 2019).

For the second run of samples, two control samples of deionised water prepared in a similar manner to urine samples were analysed. The results of these analyses proved disappointing as the finding of both feline and bacterial proteins suggest that contamination had occurred at some point during preparation. Deionised water was aspirated from sterilised beakers with sterile needles and syringes. When control samples were first analysed, they were run on a gel with 3 urine samples. Following the unexpected acquisition of bacterial proteins in these samples, analysis was repeated. Control samples were run on a single gel with no other samples for the second analysis. The reduced number of proteins identified on the second analysis suggests contamination occurred during gel loading or during separation by electrophoresis. The Proteomics Facility mitigates the risk of sample contamination by running blank/control samples in between each test sample and this was confirmed during both runs of analysis. Given the same batch of reagents and buffers were used for the entire second run of analyses, the risk of contamination is a major limitation to this study and precludes valid conclusions being drawn from results of this run. Repeat analysis of all samples in the second run with fresh reagents would have helped confirm if this was the source of potential contamination. The keratin proteins identified in the second analysis of control samples are likely of human origin.

In human medicine, the concept and understanding of a urinary microbiome has evolved rapidly over the last 10 years (Wolfe and Brubaker, 2019). Studies concerning the characterisation of the microbiome largely utilise genomic techniques, as is the case for the canine urinary microbiome (Burton *et al.*, 2017). The canine urinary microbiome has been shown to contain bacteria largely belonging to the Proteobacteria phylum. This was consistent with our proteomic study where *Pseudomonas* spp. proteins were frequently identified (Section 4.2.3.1). These data suggest a urinary microbiome of a similar nature may exist in cats, although the bacterial genera identified in this study did not compare to those identified by Vester Boler *et al.* (2018). In that study, although bacteria belonging to the Proteobacteria phylum were the most abundant*, Staphylococcus* spp. were identified in each individual cat. That finding was not mirrored in this study where *Staphylococcus* spp. were rarely identified. Differences could be explained by the populations studied as Vester Boler *et al.* (2018) only evaluated healthy cats with no history of lower urinary tract disease, which was not the case for this study. Changes in the urinary microbiome have been reported in associations with

lower urinary tract clinical signs in humans (Komesu *et al.*, 2018), and the same may be true in cats. Further proteomic and genomic studies of cats with and without lower urinary tract clinical signs seem warranted to gain an understanding of what constitutes a normal urinary microbiome and whether detectable changes occur with various disease states. Given the identification of bacterial proteins identified on analysis of deionised water, contamination must also be considered as an explanation for the differences observed, particularly for the second batch of analyses.

5.3 The Effect of Non-Absorbent Hydrophobic Sand Litter on Urine Protein-to-Creatinine Measurements

As this study was purely analytical in nature, no exclusions were applied regarding age, sex, neuter status, health status or urine sediment status.

No data was available for analysis from the pilot investigation as all urine appeared to either be absorbed by the litter or evaporate over 24 hours. The initial amount of 20g of litter was based upon estimating the ratio of a typical voided urine volume of 42mL (Pelligand *et al.*, 2011) per 500g bag of litter used per tray. The reduced amount of 4g used in the later experiments was based upon the approximate surface area one 500g bag covered of a litter tray. As Petri dishes for this experiment were not covered, it is difficult determine whether absorption or evaporation contributed more to the loss of sample. As a 500g bag of litter does not often fully line a litter tray, it was theorised the non-absorbent litter may aid urine recovery by repelling urine from the litter surface to an unlined area of the tray.

For the 40 samples analysed as part of experiment 2 and 3 (Section 4.3.2), it is difficult to explain the increase in creatinine concentrations observed in the 24-hour samples. This finding disagrees with a study of 50 canine urine samples, in which urine creatinine concentrations were shown to remain stable at room temperature for up to 72 hours (Rossi *et al.*, 2012). Protein concentrations would have been expected to increase in a similar fashion if sample dehydration was the sole explanation and covering of all Petri dishes with lids should also have mitigated this possibility. Sample dehydration alongside protein adsorption to litter cannot be fully excluded as in two control urine samples (covered in a Petri dish without any litter), although not significant, increases in both urinary protein and creatinine concentrations were observed at 24 hours. Absolute mean % changes for urinary protein and creatinine concentrations were with the allowable total error for analytical variability of serum analytes of <10% and <20% respectively as defined by the American Society of Veterinary Clinical Pathology Quality Assurance and Laboratory Standards Committee (Harr *et al.*, 2013).

Confidence intervals for the Passing-Bablok regression equation intercept and slope were subjectively narrow and included 0 and 1 respectively. This suggests minimal constant and proportional error when using urine exposed to non-absorbent hydrophobic sand for 24 hours for UPC measurements compared to cystocentesis samples.

Inspection of the Bland-Altman plot (Figure 4.9) revealed agreement between UPC at baseline and 24 hours. The two measurements that fell outside the 95% limits of agreement according

to the Bland-Altman plot had mean UPCs of a much larger magnitude than the rest of the data set and are considered outliers. Absolute % changes for these values of 14.7% and 6.6% were below the observed total error for UPC values in their range of 18.87% and so differences observed between these paired samples may purely reflect analytical variability.

Agreement for IRIS classification of proteinuria was almost perfect as measured by Cohen's kappa according to Landis and Koch (1977). The two samples that changed classification had baseline UPCs of 0.26 and 0.46 and 24-hour UPCs of 0.18 and 0.39 respectively. Changes in IRIS proteinuria category would therefore not have occurred if values were reported to one decimal place. These changes corresponded to absolute % changes of 31.38% and 15.22% respectively. Guidelines for total allowable error for UPC measurement do not exist in veterinary medicine or human medicine (CLIA, 1992, Harr *et al.*, 2013). Because of this, the calculated observed total error of the Diagnostic Laboratory analyser for UPC measurements was used to compare against % changes observed in paired samples. Observed total error was calculated by combining low and high range quality control materials for urinary protein and urinary creatinine supplied by the analyser manufacturer. Low control targets for protein and creatinine were 8mg/dL and 78.05mg/dL respectively, giving a low-range target UPC of 0.10. High control targets for protein and creatinine were 70mg/dL and 177.60mg/dL respectively, giving a high-range target UPC of 0.39. The inter-assay observed total error for low- and high-range UPCs were calculated to be 29.39% and 18.87% respectively. Analytical variability alone may therefore be responsible for the misclassification of the latter sample and 31.38% is considered acceptably close to 29.39%. Although the precision of UPC measurements at the authors institute appears superior to comparable studies, the magnitude of the inter-assay CV (11.48%) supports the recommendations of Giraldi *et al.* (2018) that repeated UPC measures should be obtained when ratios are close to the clinical threshold values of 0.2 and 0.4.

The agreement measures of Bland-Altman analysis and Cohen's kappa for IRIS proteinuria classification both suggest that the use of non-absorbent sand litter has minimal effect on UPC measurement in feline urine and that samples exposed to this litter substrate are acceptable for analysis.

5.4 The Effect of Urine Sample Collection Technique on Common Urinalysis Parameters

In this study, identical urinalyses were performed on voided urine samples and cystocentesis samples obtained from the same cat within a 24-hour period. Few studies exist evaluating the effect of method of collection on various urinalysis parameters.

Regarding USG, although overall no significant difference in USG was observed between methods of collection, as 1.035 is generally accepted as adequate urine concentrating ability in the cat, changes crossing this threshold were also evaluated (Sparkes *et al.*, 2016). However, in this study, observations regarding water intake and the use of IVFT were not standardised and these will have impacted some of the changes seen more so than the method of urine collection making conclusions regarding the effect of sample collection technique on USG difficult.

No significant difference in urinary pH was observed between voided samples and cystocentesis samples when using the benchtop pH meter. Differences in pH of larger magnitudes were observed when using the urine dipstick. Correlations between the benchtop meter and dipstick were not calculated for this study but previous studies have demonstrated greater inaccuracy and imprecision for urine dipsticks (Raskin *et al.*, 2002). Timing of urine sampling in relation to eating was not monitored in this study. Increases in urinary pH can be seen following eating, the so-called 'post-prandial alkaline tide' (Reppas and Foster, 2016a). Food was withheld from all cats prior to cystocentesis as cats were sedated or anaesthetised for this procedure. Increased urinary pH may have been observed in voided samples if food was not restricted around the time of sample collection.

To the best of the authors' knowledge only two studies exist comparing UPCs of urine obtained by cystocentesis and free catch methods in cats. In experimental conditions, Monroe *et al.* (1989) showed no significant difference between UPCs for urine samples obtained by cystocentesis and for voided samples collected from a modified plastic oil pan in 12 cats. Although not significant, a trend of increased UPCs in voided samples was observed. In a second study, Vilhena *et al.* (2015) demonstrated linear correlation between UPCs from cystocentesis samples and urine obtained by manual bladder expression in 43 cats.

In the present study, although agreement between methods of collection for all samples appeared acceptable, agreement varied considerably according to sediment status. Three data points fell outside the 95% limits of agreement in the Bland-Altman plot for all samples. In two

cases, the IRIS category for proteinuria did not change. In the third, the voided sample was classified as borderline proteinuric and the cystocentesis classified as proteinuric. However, if UPCs were reported on only one decimal place, then no change in IRIS category for proteinuria would have occurred.

Superior agreement between methods of collection was found for samples with inactive sediments compared to active sediments. For urine samples with an active sediment, the width of the 95% limits of agreement calculated by Bland-Altman analysis (-0.3359 to 0.2087) was large and greater than that of inactive sediments (-0.1284 to 0.05099). Agreement between methods of collection for IRIS classification of proteinuria was also superior for urine samples with an inactive sediment compared to samples with an active sediment. Although the Cohen's kappa coefficient for all samples represented substantial agreement, a coefficient of 0.666 indicates that up to 65% of the data may be erroneously categorised (McHugh, 2012). Cohen's kappa coefficient for inactive sediments was 0.833 corresponding to almost perfect agreement, whilst for active sediments the coefficient was 0.489 corresponding to only moderate agreement (Landis and Koch, 1977). The author believes this to be the first study where voided samples collected using a non-absorbent litter have been compared to cystocentesis samples and this further supports earlier work by demonstrating both correlation and agreement between UPCs determined from voided samples and cystocentesis samples with inactive sediments and UPC <0.5 (Monroe *et al.*, 1989, Vilhena *et al.*, 2015).

For the dipstick parameters of glucose, ketones, bilirubin and blood. Almost all results obtained were negative irrespective of method of collection. Two cases of glucosuria were documented in voided samples only. Boag *et al.* (2019) demonstrated that improper use of the dipstick by dripping sample onto reagent pads instead of submerging the dipstick is more likely to produce a positive reaction for glucose. In this study however, all urinalyses were performed according to dipstick manufacturer guidelines by submerging reagent pads in the sample. Transient glucosuria of low magnitude can occur in cats due to stress (Piech and Wycislo, 2018) and this may explain the discordant results observed regarding glucosuria. This study demonstrated good agreement between methods of urine collection for negative results of the aforementioned dipstick parameters but more data spanning a wider range of results would be needed to comment on agreement for positive results.

Very few cases of pyuria were documented in this study with only one paired sample producing discordant results for sediment examination. No cases of pyuria were documented using cytology suggesting good agreement between sediment examination and cytological

findings for the absence of pyuria. Similarly, haematuria was documented in ~20% samples for each method of collection on sediment examination. For sediment examination, agreement in findings for haematuria was documented in 90% cases. Three-quarters of these cases were non-haematuric and so more data would be needed to confirm agreement between methods of collection for haematuric urine samples. Cytological evidence of haematuria was also present in ~20% of cases. In only two cases was cytological evidence of haematuria present that was not evidence on sediment examination. These data suggest good agreement between both methods of collection and sediment and cytological examination for the absence of pyuria and haematuria.

Bacteria were observed more frequently in voided samples compared to cystocentesis samples. In human medicine, low numbers of bacteria are frequently observed on microscopic examination of voided urine samples (Ringsrud, 2001). In 8 cases, bacteriuria was documented on sediment examination in which corresponding urine cultures were negative. This highlights the imperfect nature of sediment exam being able to predict culture status previously reported (O'Neil *et al.*, 2013). The use of cytological examination has been shown to improve the sensitivity and specificity of being able to predict culture status (Swenson *et al.*, 2011), although due to the low number of positive urine cultures, this was not evident in this study.

Only low numbers of bacteria were observed in voided specimens with a maximum score for any sample of 0.5 for cytological examination. In 45% of cases these bacteria were associated with squamous epithelial cells suggestive of contamination rather than true infection. No intra-cellular bacteria were noted in the two cystocentesis samples in which bacteria were observed (Section 4.4.4.5). More samples positive for bacteria, particularly with intra-cellular bacteria documented, are required to comment on agreement between voided urine samples and cystocentesis samples for identification of bacteria on cytological examination.

Regarding urine culture, in this study 86% of voided samples resulted in bacterial growth. However only 2 of these resulted in significant bacterial growth of $>10^4$ cfu/mL as per Weese *et al.* (2011). No bacterial growth was documented in any sample obtained by cystocentesis suggesting the two positive cultures from voided samples represent contamination. Magnitude of bacterial growth is often low with contamination, but Lees *et al.* (1984) demonstrated that contaminant growths of $>10^4$ cfu/mL do occur infrequently. In a study comparing methods of urine collection in 79 cats, van Duijkeren *et al.* (2004) showed that voided samples often produced mixed cultures making interpretation difficult. This was evident in this study with one growth obviously mixed in nature as stated in Section 4.4.6. It was unclear whether the

culture containing the *Proteus* sp. was pure as its swarming nature on blood agar can outcompete other bacterial species that may be present (Schaffer and Pearson, 2015). Contaminant bacterial species cultured from voided specimens can often be uropathogens in their own right (Lees *et al.*, 1984, Sorensen *et al.*, 2016), as was the case in this study. These results suggest that cystocentesis should continue to be the preferred sampling technique when bacterial culture is required in cats.

Chapter 6 FUTURE WORKS

Limitations to each study reported in this thesis have been addressed in Chapter 5 . Yet despite these limitations, several areas for future research have also been highlighted as a result of this work.

The number of cases of suspected subclinical bacteriuria and UTI in this study precluded meaningful statistical analyses of urinalysis results. Due to the apparent low prevalence of subclinical bacteriuria, the recruitment of a powerful enough sample size within a reasonable study period does not seem feasible in many situations. This difficulty was also encountered by Moberg *et al.* (2019) in a prospective study of 179 cats in which only 11 cats with subclinical bacteriuria were identified. To overcome this, collaborative studies between multiple centres have the potential to achieve larger recruitment targets. Depending on study aims, institutes contributing to multi-centre studies concerning bacterial infections such as subclinical bacteriuria should ideally be in the same geographical area given the temporal variation in uropathogen prevalence and antimicrobial resistance patterns (Marques *et al.*, 2018). Alternatively, risk factor analysis for subclinical bacteriuria, particularly in the United Kingdom, could occur through the use of veterinary practice records surveillance programmes such as the Veterinary Companion Animal Surveillance System (VetCompass™) or the Small Animal Veterinary Surveillance Network (SAVSNET).

Subclinical bacteriuria and UTI occur frequently in cats with urological implants (Kopecny *et al.*, 2019). At the authors institute, post-operative antimicrobial therapy is not standard procedure following the placement of any urological implant, yet anecdotally the rate of post-operative subclinical bacteriuria or UTI does not appear higher than institutes where post-operative antimicrobials are prescribed. There is therefore an urgent need for a prospective study evaluating the effect of peri- and post-operative antimicrobial therapy for urological implant surgeries. A comparison of prescribing protocols could help to refine antimicrobial prescribing patterns and reduce any unnecessary antimicrobial use.

The risk of sample contamination during proteomic analysis in Section 4.2 is a major limitation of the study and as stated, prevents valid conclusions being drawn from the results. The results of the feline and bacterial protein database searches do however suggest that a complex urinary proteome and urinary microbiome are present in cats, as has been shown in other species (Brandt *et al.*, 2014, Brubaker and Wolfe, 2016, Burton *et al.*, 2017). Better characterisation of the feline urinary proteome in both health and diseased states has the

potential to identify a whole host of biomarkers that could be exploited to develop diagnostic tests for subclinical bacteriuria, UTI and other diseases.

The enzyme myeloperoxidase was identified frequently in cats with subclinical bacteriuria and UTI in this study. The enzyme was also identified in cats presenting with lower urinary tract clinical signs that had negative urine cultures. Through proteomic techniques alone, urinary myeloperoxidase activity in samples analysed could not be established. In human medicine, increased urinary myeloperoxidase activity has been shown to be able to discriminate between sterile and infected urine samples (Ciragil *et al.*, 2014). Although myeloperoxidase activity has been shown to be a non-specific inflammatory maker in humans (Masajtis-Zagajewska and Nowicki, 2017) establishment of activity levels in feline urine may prove useful in differentiating the various causes of lower urinary tract disease.

An increased understanding of the human urinary microbiome has furthered understanding of a number of urinary tract disease pathogeneses (Wolfe *et al.*, 2012). A definitive aetiology for the leading cause of feline lower urinary tract disease, idiopathic cystitis, has yet to be identified (Buffington, 2011). With feline lower urinary tract disease accounting for up to 5% of primary care veterinary practices feline caseload (Sparkes, 2018), there is a clear need for further research in this area. Combining proteomic and genomic techniques could give an increased understanding of what constitutes a healthy feline urinary microbiome; understanding how this microbiome changes in disease states would almost certainly increase the understanding of feline lower urinary tract disease.

Finally, this study was able to show that the use of non-absorbent hydrophobic sand litter had minimal effect on UPC measurements (Section 4.3). When this was taken further to compare the UPC between voided urine samples and cystocentesis samples (Section 4.4.2.3), improved agreement between methods of collection was seen for samples with inactive sediments compared to active. When analyses of active and inactive sediments were performed separately, the sample sizes were 20 and 23 respectively. A minimum of 40 samples are recommended for method comparison studies (Jensen and Kjelgaard-Hansen, 2006), and so a further 20 samples with active sediments and 17 samples with inactive sediments should be analysed to confirm the agreement reported in this study.

Chapter 7 APPENDICES

7.1 APPENDIX A

Does your cat need antibiotics to treat their cystitis?

About the project:

Although there can be many reasons your cat may develop cystitis; bacterial infection is often not a major cause. We are seeing an alarming increase in antimicrobial resistance in both veterinary and human medicine and the unnecessary use of antimicrobials contributes to this problem. Our study aims to better identify patients that require antimicrobial therapy to treat their cystitis, and those that do not. In human medicine there are well defined guidelines for determining these patients and we hope to develop a similar set of guidelines for our feline patients.

Your pet can be included in this study if:

- Your cat is showing signs of cystitis and will have a urine sample taken directly from their bladder as part of their investigations.
- Your cat is being brought to the hospital for a reason other than cystitis but will also have a urine sample taken directly from their bladder in order to exclude an infection.
- We are sorry but your cat **cannot** be included if we are unable to obtain a sample of urine directly from your cat's bladder or if your cat has previously participated in this study.

What we will do:

- Urine samples will be obtained directly from your cat's bladder using a small needle and syringe. This will already be performed as part of routine investigations in to your cat's illness.
- These urine samples will be compared to urine passed freely by your cat in to their litter tray.
- You will only need to attend hospital appointments as scheduled by the Veterinary Surgeon treating your cat. No extra visits to the hospital will be required by participating in this study.
- Only excess routine diagnostic samples will be used for testing. Your cat will not have any additional tests or procedures carried out by enrolling in this study.

Benefits to you and your pet:

- If test results from urine obtained from your cat's litter tray prove as useful as results from urine obtained directly from your cats' bladder, there will be less need for cats to undergo such procedures in the future.
- Current tests to identify a bacterial infection in urine can take over 48 hours to obtain results. Excess urine samples will be analysed to try and develop a quicker method of confirming the presence of a bacterial infection.
- By quickly and correctly identifying patients with bacterial cystitis, we should be able to prescribe antimicrobials only to those that cats that require them, thereby reducing the risks of antimicrobial resistance developing.

Study has been reviewed and approved by University of Bristol Ethics Committee VIN/17/037 valid until 29/9/2020.

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Langford Vets | University of BRISTOL

Small Animal Referral Hospital, Langford Vets in conjunction with the University of Bristol Vet School, Langford, BS40 5DU

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Figure 7.1: Client information leaflet designed to aid recruitment for the study.

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