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Absence of bacterial DNA in culture-negative urine from cats with and without lower urinary tract disease

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Abstract

A diagnosis of bacterial cystitis commonly relies on a positive microbiological culture demonstrating the presence of a significant number of colony-forming units/ml urine, as urine within the upper urinary tract, bladder and proximal urethra generally is considered sterile. Recent studies from human and veterinary medicine indicate the presence of non-culturable bacteria in culture-negative urine samples. The aim of the present study was to determine the occurrence of bacterial DNA in culture-negative urine samples from cats with signs of feline lower urinary tract disease (FLUTD) and healthy control cats by 16S ribosomal DNA PCR and subsequent sequencing. The study sample included 38 culture-negative urine samples from cats with FLUTD and 43 culture-negative samples from control cats. Eight culture-positive urine samples from cats with FLUTD were included as external positive controls in addition to negative reaction controls. Of possible methodological limitations, degradation of DNA due to storage, the use of non-sedimented urine for DNA isolation and lack of internal positive reaction controls should be mentioned. The positive controls were recognised, but occurrence of bacterial DNA in culture-negative urine from cats with or without signs of lower urinary tract disease was not demonstrated. However, considering the possible methodological limitations, the presence of bacterial DNA in the urine of culture-negative FLUTD cats cannot be excluded based on the present results alone. Therefore, a prospective study reducing the possibility of degradation of DNA due to storage, in combination with modifications enhancing the chance of detecting even lower levels of bacterial DNA in culture-negative samples, seems warranted.

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Introduction

Urine within the upper urinary tract, bladder and proximal urethra has generally been considered sterile, and most urinary tract infections (UTIs) are caused by retrograde contamination of the urinary tract by bacteria present in faeces or skin.[1–3] A diagnosis of bacterial UTI commonly relies on a positive microbiological culture demonstrating the presence of a significant number of colony-forming units (CFUs)/ml urine.[2] In human medicine, the test efficiency of conventional cultivation methods for detection of bacterial UTI is questioned, as recent research has revealed the presence of intracellular bacteria in uroepithelial cells and bacteria capable of biofilm formation within the urinary tract.[3–6] Further, the presence of viable, but non-culturable bacteria has been demonstrated in urine samples from mice and humans.[7]

The term feline lower urinary tract disease (FLUTD) is a clinical description of cats with signs of lower urinary tract disease and does not in itself represent a specific diagnosis. In a large proportion of cats with signs of FLUTD, thorough diagnostic investigation does not reveal a specific cause, and these patients are classified as having feline idiopathic cystitis (FIC).[8–10] A recent pilot study reported the detection of bacterial DNA in culture-negative urine from cats diagnosed with FIC,

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indicating similarities to the results published from other species.[11]

The aim of the present study was to determine whether bacterial DNA could be detected in culture-negative urine samples from Norwegian cats with signs of lower urinary tract disease and healthy control cats by PCR and subsequent sequencing.

Materials and methods

Study sample

The urine samples included in the present study stem from a larger study of FLUTD in Norwegian cats. The study sample consisted of client-owned cats presented at the clinics at the Small Animal Section, Department of Companion Animal Clinical Sciences, Norwegian School of Veterinary Science (NVH) from 2003–2009. In addition to the owners' informed consent, the inclusion criteria were clinical signs of FLUTD, for example dysuria, stranguria, haematuria, pollakiuria and/or periuria, a diagnosis consistent with FLUTD and urine samples negative by microbiological culturing. Exclusion criteria were treatment that could interfere with the diagnostics (antimicrobial medication, hormones or medication altering blood pressure, urine production and/or composition) and concurrent diseases likely to influence the urinary findings, such as chronic kidney disease, diabetes mellitus or hyperthyroidism. Only cats aged 7 months or older were included; no sex or breed restrictions were made.

The healthy control cats were recruited among patients brought to the clinic in the same period of time for healthcare reasons that required sedation or anaesthesia, and would not interfere with the urinalyses (castration, spaying, dental problems or minor surgery). With the exception of clinical signs and a diagnosis consistent with FLUTD, the cats in the control group met the same inclusion and exclusion criteria as the FLUTD cats. Permission to obtain the data was granted from the committee of research and ethics at NVH.

In order to verify the methodology, FLUTD cats with positive urine cultures that otherwise met the inclusion and exclusion criteria were found eligible as positive controls.

Methods

A standardised questionnaire was used to register age, breed, sex, reproductive status, body weight, prior health history and information concerning housing conditions and feeding regime for each cat. A veterinarian performed a clinical examination, and blood samples for routine haematology and biochemistry were collected. Abdominal radiography or ultrasound was included as part of the diagnostic investigation. All urine samples were obtained by cystocentesis. After sedation, the cats were placed in dorsal recumbency and the skin clipped and prepared aseptically. A ventral midline approach was used, and cystocentesis performed blindly or with ultrasound guidance with a 23 G needle attached to a 10 ml syringe. Standard urinalysis was performed, including commercial urine dipstick analysis (Krulab; Kruuse), urine specific gravity measured with refractometer (URC-Ne; Atago) and microscopic examination of the urine sediment (native wet samples and wet samples stained with Sternheimer-Malbins).

The urine samples were obtained in the acute phase when the cat was admitted to the clinic, and all samples were cultured on the day of collection. Quantitative bacteriology was performed by streaking 1 µl of urine onto blood agar (Blood Agar Base II; Difco), and qualitative bacteriology by cultivation on blood agar and selective bromo-thymol agar (Difco) from sediments after centrifugation, incubated aerobically at 37°C in a 5% CO_2-enriched atmosphere. The phenotypic identity of each bacterial isolate was determined by standard biochemical procedures.[12] For each urine sample, a non-sedimented subsample aliquoted prior to centrifugation was frozen immediately for later isolation of viral and bacterial DNA. The samples were stored at −20°C for a maximum of 7 years.

Not all the information from the questionnaire and clinical investigation was used in the present study; other aspects of the obtained data were previously published for subsets of the cats included in the present study.[13–16]

DNA preparation

DNA was isolated from 200 µl well-mixed non-sedimented urine using the QIAamp DNA mini kit (Qiagen) following the manufacturer's instructions, with the exception of the amount of elution buffer used in the final steps. A reduced volume of 50 µl elution buffer was used in order to increase the final DNA concentration in the eluate. Isolated DNA was not stored prior to the initial round of PCR.

PCR and gel electrophoresis

A 16S ribosomal DNA (rDNA) PCR was performed using the forward primer fD1 and the reverse primer rP1 (Eurogentec) as described by Weisburg et al.[17]

A total of 4 µl of DNA template was used for all samples, DNA concentration was not measured. A reaction volume of 50 µl was used, containing 5 µl 10 × PCR buffer, 2 µl $MgCl_2$ (50mM), 1 µl dNTP, 0.4 µl Taq, 2 µl of each primer, 4 µl DNA template and dH_2O.

Negative controls (as described above without template) and external positive controls in the form of urine samples positive by conventional culturing and biochemical identification were run for each gel.

Cycling conditions were 94°C for 5 mins, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 mins, and a final extension of 72°C for 5 mins.

Amplified products were resolved by electrophoresis for 60 mins (90 V) on a 1% agarose gel (Ultrapure agarose; Invitrogen) with 6 × Orange Loading Dye and a 1 kb ladder (Thermo Fisher Scientific).

A second round of PCR was run for samples returning weak but visible bands after the initial PCR. These amplicons were purified using the Qiaquick Gel Extraction kit (Qiagen). Of the extracted material (approximately 50 µl/sample), 4 µl was used for PCR with primers, reaction volume and reaction conditions as described for the initial PCR. Isolated DNA extracted after gel electrophoresis was stored at −4 °C for a maximum of 5 days prior to the second round of PCR.

Sequencing

The amplicons from the positive controls and other amplicons of potential interest were purified using the Qiaquick Gel Extraction kit (Qiagen) and verified by Sanger sequencing (GATC Biotech),[18] and, subsequently, alignment in Basic Local Alignment Search Tool-Nucleotide database (BLASTn).[19]

Evaluation of sensitivity of the assay

A 10-fold dilution series was prepared by adding *Escherichia coli* (strain ATCC 11303) to culture-negative feline urine previously found negative by PCR for bacterial DNA. Microbiological culturing and DNA isolation, PCR and gel electrophoresis as described above were performed in duplicate for each level of dilution. Weak, but visible, bands were formed at gel electrophoresis for bacteria levels as low as 10^2–10^3 CFU/ml urine, and the amplicons were verified by sequencing as *E coli*.

Statistical analyses

The results are presented as frequencies of occurrence, expressed in percentages. Statistical analyses were performed in JMP 8 (SAS Institute).

Results

Study sample

A total of 46 cats with signs of FLUTD were included, 38 with culture-negative urine samples and eight with positive cultures, functioning as positive controls. The number of culture-negative, healthy control cats was 43. Information concerning sex, reproductive status, age, breed, weight and, for the FLUTD cats, final diagnosis, is listed in Table 1.

Microbiological culture-positive urine samples: positive controls

Amplicons of the expected size, approximately 1500 base pairs (bp), were found by gel electrophoresis and the specificities of the amplicons were verified by nucleotide sequencing. There was agreement between these results and the previous traditional biochemical identification: four samples with *Escherichia* species and one each with *Enterococcus* species, *Proteus* species, *Pasteurella* species and *Pseudomonas* species. The number of CFU/ml urine was between 10^4 and 10^6 in the samples used as positive controls.

Microbiological culture-negative urine samples

After the initial first round of DNA isolation and PCR for detection of 16S rDNA in the urine, 26/38 (68%) of the culture-negative samples from FLUTD cats and 39/43 (91%) of the samples from healthy control cats were found on gel electrophoresis to be negative for amplicons (Table 2).

Two of the samples from culture-negative FLUTD cats returned clearly visible bands on gel electrophoresis, indicating amplicons with an approximate size of 750 bp. These were sequenced and alignment in BLASTn showed obvious sequence similarities to feline DNA (*Felis catus*).

Fourteen culture-negative samples returned bands of various strengths in the area between 250 and 1500 bp (denoted as 'intermediate' in Table 2). These samples were included in a second round of PCR in order to amplify the DNA. Nine of the culture-negative samples from FLUTD cats and three of the samples from healthy control cats returned bands on gel electrophoresis in the area of approximately 750 bp. After the second round of PCR, sequencing showed that the DNA was of feline origin (*F catus*), as above. One culture-negative FLUTD cat and one control cat returned, after the second round of PCR, amplicons of 250 bp and 1500 bp, respectively. Alignment in BLASTn showed that this most likely was contamination with human DNA (250 bp) and contamination with a marine fish bacterium (*Aliivibrio wodanis*) (1500 bp) most likely originating from fish disease-related research co-located in the laboratory.

Discussion

In this study, there was no indication of non-culturable bacteria in urine samples from cats with negative microbiological cultures. These results are not in agreement with recent publications in human and veterinary medicine. A diverse microbiota has been demonstrated in culture-negative urine from healthy humans, as well as women with interstitial cystitis, and non-culturable bacteria have been identified in urine from mice and humans.[3,4,7] Studies from human, as well as veterinary, medicine have detected bacterial DNA in culture-negative urine samples by using similar technology as applied in the present study – 16S rDNA PCR. Imirzalioglu et al[4] found fastidious and anaerobic bacteria in 22% of their culture-negative human urine samples when using PCR.

Table 1 Feline lower urinary tract disease (FLUTD) cats (n = 46) and controls (n = 43): sex, reproductive status, age, breed, diagnosis and weight

Variable	FLUTD cats		Controls	
	n (%)	Mean (SE)	n (%)	Mean (SE)
Sex/reproductive status				
Intact male	3 (7)		5 (12)	
Castrated male	31 (67)		11 (26)	
Intact female	3 (7)		17 (40)	
Castrated female	9 (19)		10 (23)	
Age				
⩽2 years	8 (17)		22 (51)	
3–6 years	22 (48)		15 (35)	
7–10 years	11 (24)		5 (12)	
⩾11 years	5 (11)		1 (2)	
Breed				
Pure breed	8 (17)		5 (12)	
Mixed breed	38 (83)		38 (88)	
Diagnosis				
Bacterial cystitis	8 (17)		NA	
Urolith	1 (2)		NA	
Urethral plug	3 (7)		NA	
Feline idiopathic cystitis	34 (74)		NA	
Weight		5.2 (0.20)		3.4 (0.20)

NA = not applicable

Table 2 Feline lower urinary tract disease (FLUTD) cats and controls: results from the initial round of DNA isolation, polymerase chain reaction (PCR) and gel electrophoresis

	Result of PCR and gel electrophoresis	n (%)
FLUTD, positive bacterial culture (n = 8)	Positive	8/8 (100)
FLUTD, negative bacterial culture (n = 38)	Positive	2/38 (5.3)
	Intermediate*	10/38 (26.3)
	Negative	26/38 (68.4)
Control, negative bacterial culture (n = 43)	Intermediate*	4/43 (9.3)
	Negative	39/43 (90.7)

*Weaker bands on gel electrophoresis of various molecular sizes

In a study performed by Dorsch et al,[11] a higher proportion of cats with bacterial DNA from organisms closely related to *Rhodococcus* species in their urine was found among the cats with FIC than the healthy controls.[11] In contrast to the present study, DNA was isolated from urine sediments in the study performed by Dorsch et al,[11] and a different set of primers was used, which may explain the different outcome. There are, however, also studies reporting negative results that have examined the presence of specific bacteria by similar methodology. A study of *Mycoplasma* species and *Ureaplasma* species in urine from cats with FLUTD concluded that these organisms play no causative role in FLUTD.[20]

The lack of positive results in the present study may be owing to technical aspects. Urine samples were stored pending further analyses, including detection of viral and bacterial DNA, and possibly also additional tests of urine components. When the first samples were frozen, a temperature of −20°C was chosen in order to preserve cells without adding, for instance, glycerol, which is recommended for deep freezing at lower temperatures such as −80°C. In retrospect, as the analyses carried out were detection of DNA only, preservation in the form of deep freezing or vacuum drying should have been chosen.[21] The effect of storage in terms of degradation of DNA would probably have been reduced. However, during the period of data collection, in order to ensure equal

conditions for all samples included, the procedure was not changed. Although the positive controls were recognised, which indicates a certain level of preservation of DNA, a negative impact of lengthy storage, especially on samples with low levels of bacteria, cannot be ruled out.

In the study of bacterial phylogeny and taxonomy, 16S rRNA gene sequences are considered the most commonly used housekeeping genetic marker owing to their well-preserved function and presence in almost all bacteria.[22] The primers used in the present study have previously been shown to recognise most known bacteria and, again, recognised the positive controls.[17] Thus, if unculturable bacteria were present in the urine samples, the primers would be expected to pick up at least some of them, unless the DNA isolation kit should prove ineffective for bacterial groups not previously tested.

Bacterial levels below the threshold of the test are a possible reason for false-negative results. There is also a possibility of fluctuating loads of bacteria in the urine, for instance in the case of the presence of intracellular bacteria, and even intracellular bacteria protected by biofilm, as demonstrated in humans.[6] The sensitivity evaluation of the methodology applied in the present study indicated a cut-off level of detection by PCR at approximately 10^2–10^3 CFU/ml urine. In addition to the limited number of cases in the present study, some methodological limitations may have resulted in failure to detect lower levels of uncultivable bacteria, although a second round of PCR was run in order to increase the DNA isolated from some of the urine samples. However, the second round of PCR was only based on DNA extracted from the visible agarose separated amplicons resulting from the first round of PCR, and may have been more efficient if the entire PCR product from the first round of PCR had been included. While non-sedimented urine was used in the present study, the inclusion of urine sediments for DNA extraction instead would potentially have increased the sensitivity of the methodology. Applying real-time PCR instead of conventional PCR would also enhance the possibility of detecting and also quantifying very low levels of bacterial DNA in culture-negative samples.[23]

Further, the possibility of factors in the urine inhibiting the PCR reaction to some degree must be considered. While the external positive controls indicated minimal inhibition in the culture-positive samples, no internal positive controls were applied and thus occurrence of inhibiting substances in the culture-negative samples cannot be excluded.[24]

In the present study, the detection levels of microbiological cultures and PCR techniques were similar. While dissimilar factors may affect the sensitivity of the two methods, the latter is commonly accepted as the most sensitive diagnostic method. However, previous studies have shown that traditional culturing on agar plates remains one of the most relevant methods for quantification of bacteria from tissue or liquid material from animals, for instance in the case of blood samples, as haemoglobin is a known inhibitor of PCR reactions.[25,26]

Although human and feline urine may not necessarily be directly comparable, the similarities in clinical presentation between interstitial cystitis in women and FIC merit comparison of the aetiology. Siddiqui et al compared the microbiota of urine from healthy women and women with interstitial cystitis by 16S rDNA PCR followed by 454 pyrosequencing and analyses using a suite of bioinformatics tools previously described by this group of researchers.[3,27] They found reduced microbial diversity and richness in urine from women with interstitial cystitis, along with a higher abundance of *Lactobacillus* species compared with healthy women. These findings indicate that further studies of feline urine applying even more advanced methodology may be warranted in the future. The human urine samples were, however, collected as midstream urine by the clean catch method, and therefore may represent the microbiota of the urethra and proximal vagina, as well as the urinary bladder. In order to study the environment of the urinary bladder alone, previous human studies have applied PCR or microbiological cultivation to bladder biopsies. These studies have so far given conflicting results, as pathogenic agents were detected only in some of the studies.[28–30]

Conclusions

The results from the present study did not demonstrate occurrence of bacterial DNA in culture-negative urine from cats with or without signs of lower urinary tract disease. However, owing to several methodological considerations, the occurrence of bacterial DNA in culture-negative urine from cats with FLUTD cannot be excluded based on the results from the present study alone. Therefore, a prospective study reducing the possibility of degradation of DNA due to storage, in combination with modifications enhancing the chance of detecting even lower levels of bacterial DNA in culture-negative samples, seems warranted.

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Conflict of interest The authors do not have any potential conflicts of interest to declare.

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