The Veterinary Journal 206 (2015) 149–153

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Detection of feline upper respiratory tract disease pathogens using a commercially available real-time PCR test

A. Litster [a,*], C.C. Wu [b,1], C.M. Leutenegger [c]

[a] Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Purdue University, 625 Harrison St., West Lafayette, IN 47907, USA
[b] Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA
[c] IDEXX Laboratories, Inc., 2825 KOVR Drive, West Sacramento, CA 95605, USA

ARTICLE INFO

Article history:
Accepted 2 August 2015

Keywords:
Cats
Feline upper respiratory tract disease
FURTD
Real-time PCR
Shelter

ABSTRACT

Feline herpesvirus (FHV-1), feline calicivirus (FCV), *Bordetella bronchiseptica* (*Bb*), *Chlamydia felis* (*Cf*) and *Mycoplasma felis* (*Mf*) are common infectious agents identified in cats with upper respiratory tract disease (URTD). Each of these agents can either act as primary pathogens or cause subclinical infections, and pathogen identification can be used to prevent disease transmission in shelters, or to manage individual cats with recurrent URTD. The aim of this study was to compare pathogen detection rates using real-time PCR testing and virus isolation (VI) or bacterial culture in conjunctival, nasal and oropharyngeal swabs from 18 shelter-housed cats with clinical URTD.

Co-infections were common; FHV-1 was most prevalent and *Cf* and FCV were least prevalent. Agents detected by PCR were FCV 2/18 (11%), FHV-1 17/18 (94%), *Bb* 8/18 (44%) and *Mf* 15/18 (83%). Agents detected by VI and bacterial culture were FCV 1/18 (6%), FHV-1 12/18 (67%), *Bb* 8/18 (44%) and *Mf* 12/18 (67%). Agreement between PCR results and the other two methods was: FHV-1, 57.4%; FCV, 98.1%; *Bb*, 75.0%; *Mf*, 60.0%. Discordancies included PCR-positive, VI-negative (FCV, $n = 1/54$, 1.9%; FHV-1, $n = 23/54$, 42.6%), PCR-positive, culture-negative (*Bb*, $n = 6/36$, 16.7%; *Mf*, $n = 13/36$, 36.1%) or PCR-negative, culture-positive (*Bb*, $n = 3/36$, 8.3%; *Mf*, $n = 2/36$, 5.6%) results. A combination of an oropharyngeal swab and either a conjunctival or a nasal swab submitted for PCR testing was able to detect all infectious agents tested for in each cat. PCR testing was a sensitive and convenient method of detection of infectious agents in cats with clinical signs of URTD.

Introduction

Feline upper respiratory tract disease (URTD) is a commonly diagnosed condition, especially in high stress, crowded environments. Although often clinically manageable, URTD is a leading cause of euthanasia in shelters due to high morbidity, cost of veterinary care and potentially reduced adoptability (Bannasch and Foley, 2005; Edwards et al., 2008; Dinnage et al., 2009). The five most prevalent feline URTD pathogens are feline herpesvirus (FHV-1), feline calicivirus (FCV), *Bordetella bronchiseptica* (*Bb*), *Chlamydia felis* (*Cf*) and *Mycoplasma* spp. (Sykes et al., 1999; Pedersen et al., 2004; Bannasch and Foley, 2005; Helps et al., 2005; Johnson et al., 2005). While clinical signs of URTD such as conjunctivitis, sneezing, ocular and/or nasal discharge and coughing are readily recognized by veterinarians and shelter staff, diagnostic testing to identify infectious agents is usually only performed in the face of an outbreak (Dinnage et al., 2009). This is partly because although each of these pathogens can cause primary disease, they are often present in subclinical carrier states, so that pathogen identification does not necessarily establish an etiological diagnosis (Veir and Lappin, 2010). Local prevalence data in individual shelter populations have the potential to optimize management and treatment regimens and reduce disease spread. This is particularly the case where feline URTD prevalence is high, thereby increasing the cost benefit of diagnostic testing for URTD either during an outbreak or on a periodic basis.

Since there is considerable overlap between the clinical signs of URTD regardless of the infectious agent(s) involved, laboratory testing is required for accurate pathogen identification (Sykes et al., 1999). Real-time polymerase chain reaction (PCR)-based assays are commercially available and can rapidly screen for all five major URTD pathogens in a single specimen. Virus isolation (VI) and bacterial culture are considered reference standard methods with very high positive predictive values, but their relative lack of sensitivity and delays between specimen collection and the receipt of final results can pose challenges for clinical case management.

Although PCR testing indicates the presence of target sequences of microbial DNA or RNA, it does not confirm pathogen viability or provide antimicrobial susceptibility information for

* Corresponding author. Tel.: +1 765 418 3186.
E-mail address: catvet@purdue.edu (A. Litster).
[1] Present address: School of Veterinary Medicine, National Taiwan University, Roosevelt Rd., Taipei 10617, Taiwan.

http://dx.doi.org/10.1016/j.tvjl.2015.08.001
1090-0233/

bacteria. The increased sensitivity and fast turn-around time of PCR (Veir and Lappin, 2010; Cai et al., 2014) makes it the preferred method for virus detection in biological samples (Thiry et al., 2009). Traditional gel-based PCR, which sometimes produced false positive results because of cross-contamination, has now largely been replaced by real-time PCR, which is performed in a closed system using stringent quality controls (Cai et al., 2014).

In this study, the major objective was to compare a commercially available real-time PCR panel for the diagnosis of feline URTD infectious agents with reference laboratory methods. A secondary objective was to determine the best combination of anatomic sites to use when collecting swab specimens for PCR detection of URTD pathogens in cats.

Materials and methods

Animals and specimen collection

Specimens were collected from 18 cats that were originally sourced from a large municipal shelter where they had been admitted as strays or surrendered by their owners. Cats were vaccinated once at intake with a combination modified-live feline panleukopenia, FCV, and FHV-1 vaccine (Fel-O-Guard Plus 3, Fort Dodge) administered SC. After at least 5 days at the municipal shelter, they were transferred to a large adoption-guarantee shelter where the study was performed.

On intake at the adoption-guarantee shelter, the cats were examined by a veterinarian, who documented clinical signs of upper respiratory tract infection, such as ocular and/or nasal discharge and sneezing. Vaccination was repeated and serological tests were performed for anti-feline immunodeficiency virus (FIV) antibodies and feline leukemia virus (FeLV) antigen (SNAP FIV/FeLV Combo test, IDEXX Laboratories). Conjunctival and nasal swabs were collected from each cat and submitted for PCR (Feline Upper Respiratory Disease RealPCR Panel, IDEXX Reference Laboratories), virus isolation, and bacterial culture for *Bb* and *Mycoplasma felis* (*Mf*). Oropharyngeal swabs were submitted for PCR and VI but not for culture. Multiple sterile cotton tipped swabs were used at each anatomical site and submitted dry in plastic culture tubes for PCR and in transport medium for bacteriology (BBL culture swab Collection and Transport system, BD Diagnostics) and VI (BD Universal viral transport for viruses, Chlamydiae, Mycoplasmas and Ureaplasmas, BD Diagnostics).

Specimens for bacteriology were collected from each site first, followed by swabs for PCR and VI, alternating the order of collection of specimens for PCR and VI between cats at each anatomical site. Specimens for bacteriology and VI were stored at –80 °C until the assays were performed and those for PCR were assayed within 2 days of collection.

Permission to perform this study was obtained by the Purdue University Animal Care and Use Committee (PACUC No. 09-020).

Real-time TaqMan PCR assays

Real-time PCR assays targeting five infectious agents that contribute to feline URTD were used (IDEXX Laboratories, Feline Upper Respiratory Disease RealPCR Panel). The infectious agents were *Bb*, *Cf*, FHV-1 (Vögtlin et al., 2002), FCV and *Mf*. All assays were designed and validated according to industry standards[1]. Target genes for each application were: *Bb*, hemagglutinin fusion protein gene (*FhaB*), AF140678; *Cf*, outer membrane protein A (*OmpA*), AP006861; FHV-1, glycoprotein B (*gB*); FCV, ORF 1, AF109465; *Mf*, simple sequence repeats (ssr) RNA – ITS-1, AF443608.

Specimen preparation and real-time PCR analysis

Conjunctival, nasal and oropharyngeal swabs were processed separately with protocols adapted as previously published (Mapes et al., 2008). Briefly, swabs were submerged in lysis solution and incubated for 10 min. Lysates were extracted using Whatman binding plates in a Corbett X-Tractor platform (Qiagen). Nucleic acids were eluted into 150 μL of PCR-grade nuclease-free water (Fisher Scientific) and 5 μL was amplified in subsequent real-time PCR reactions. For FCV and the pre-analytical RNA control, 20 μL of total nucleic acid was used to reverse transcribe into cDNA using random hexamer primers and SuperScript III (Invitrogen) in a final volume of 40 μL.

Five microliters of a diluted cDNA solution (100 μL final volume after the reverse transcription [RT]-step) was used for real-time PCR using the FCV-specific assay. Analysis was performed using an automated analyzer (Roche LightCycler 480, Roche Applied Science) and raw data were analyzed using the second derivative maximum method to generate crossing points. Real-time PCR was run with seven quality controls including: (1) PCR-positive controls; (2) PCR-negative controls; (3) negative extraction controls; (4) DNA pre-analytical quality control targeting the host ssr rRNA (18S rRNA) gene complex; (5) RNA pre-analytical quality control targeting the host ssr rRNA gene complex; (6) an internal positive control spiked into the lysis solution, and (7) an environmental contamination monitoring control.

Quantitative FHV-1 real-time PCR

In order to enumerate viral particles per swab, a published protocol was used allowing the raw data of real-time PCR (Ct value) to be converted to a quantitative value (Burns et al., 2001). In brief, a standard curve generated with known FHV-1 DNA numbers served as the basis to convert Ct values in FHV-1 positive samples. By incorporating dilution factors during the extraction process, a quantitative particle number value could be generated per collected swab.

Virus isolation

Crandell-Rees feline kidney (CRFK) cells were grown in 48- and 96-well plates to approximately 90% in confluence. Swab samples were thawed and 100-μL sample in duplicate was added to the 48-well plates. The plates were incubated in 5% CO_2 at 37 °C for 5 days and examined daily for the presence of cytopathic effects. The 48-well plates were frozen and thawed and 50 μL of medium from each well was transferred to the 96-well plates with CRFK cells to incubate for 24–48 h prior to confirmation by immunofluorescent antibody staining. The 96-well plates were fixed with acetone for 10 min and air-dried at room temperature. A polyclonal FHV-1-specific antibody or a FCV monoclonal antibody was used to stain the designated wells that were examined for fluorescence under an inverted microscope (Slack et al., 2013).

Mycoplasma *culture*

Swab samples were rolled directly onto pleuropneumonia-like organism (PPLO; *Mycoplasma*) agar plates and incubated at 33–37 °C in 5% CO_2 for 24 h. If no growth appeared within 24 h, the PPLO plates were transferred to an anaerobic chamber and incubated at 33–37 °C. Plates were examined daily for up to 5 days before being reported as negative. Plates were examined using a microscope at 4× power; *Mycoplasma* colonies were identified as typical 'fried egg' colonies with opaque, granular central zones embedded in agar and flat translucent peripheral zones. Colonies were sub cultured to PPLO agar and incubated anaerobically to confirm identification.

Bb *culture*

Sheep blood and MacConkey agars were used for *Bb* culture. Plates were incubated aerobically at 33–37 °C for 24–48 h. Colonies were identified by their typical morphological appearance (very small, convex, smooth colonies with an entire edge after 24 h incubation), followed by Gram staining and biochemical testing to confirm small Gram-negative coccobacilli that were oxidase and catalase positive. Additionally, suspicious colonies were isolated and confirmed using an automated microbial identification system (VITEK-2, BioMérieux). Kirby Bauer susceptibility testing was performed by inoculating saline with colony specimens to make a 0.5 McFarland suspension. This was swabbed onto Mueller Hinton media and appropriate antibiotic disks were dropped onto plates.

Statistical analysis

After assessment of the data for normality, Mann–Whitney tests were used to compare quantitative PCR results for concordant and discordant specimens for FHV-1, *Bb* and *Mf*. Values of $P < 0.05$ were regarded as statistically significant.

Results

Prevalence data and diagnostic comparisons

Table 1 summarizes the prevalence and co-infection data for the 18 cats studied. Infectious agents were detected by PCR in all cats, but using VI and culture for diagnosis, none of the agents studied were identified in one cat. FHV-1 was the most prevalent infectious agent detected and *Cf* and FCV were the least prevalent. Co-infections were common and the two most common co-infections were (1) FHV-1 and *Mf* and (2) FHV-1, *Bb* and *Mf*.

Table 2 presents comparisons of diagnostic methods by infectious agent. While there was a very high level of agreement between diagnostic methods regarding FCV, this was almost entirely in specimens in which FCV was not detected by either method. FCV was identified in oropharyngeal swabs in one cat by both PCR and VI and in a second cat by PCR but not VI. The agreement between diagnostic methods for FHV-1 was 57.4%. The remaining discordant specimens were PCR-positive and VI-negative. Comparison of PCR

[1] See: Applied Biosystems, User Bulletin 3, http://tools.lifetechnologies.com/content/sfs/manuals/cms_041001.pdf (accessed 15 October 2014).