Veterinary Microbiology 175 (2015) 211–217

Contents lists available at ScienceDirect

Veterinary Microbiology

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

The use of quantitative PCR to detect *Felis catus* papillomavirus type 2 DNA from a high proportion of queens and their kittens

N.A. Thomson *, M. Dunowska, J.S. Munday

Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North 4472, New Zealand

ARTICLE INFO

Article history:
Received 24 February 2014
Received in revised form 7 November 2014
Accepted 16 November 2014

Keywords:
Feline
Papillomavirus
Cutaneous squamous cell carcinoma
FcaPV-2
qPCR

ABSTRACT

Squamous cell carcinomas are common feline skin cancers that have been associated with infection with *Felis catus* papillomavirus type 2 (FcaPV-2). Currently, little is known about the epidemiology of FcaPV-2 infection. The aim of this study was to develop a real-time PCR assay to quantify FcaPV-2 DNA in plucked hairs and skin swabs from 11 healthy breeding queens and their kittens. Samples were taken prior to kittening and then 2, 7 and 28 days after kittening to determine the age at which the kittens were first exposed to the virus. FcaPV-2 DNA was amplified from all of the queens and from 91% of the kittens at 2 days of age. There was a wide range in the quantity of FcaPV-2 DNA detected, from 1 to 92,520 copies per swab, and from 0.01 to 234 copies per copy of reference gene DNA in the hair plucks. The quantity of FcaPV-2 DNA detected in samples collected from the kittens was strongly correlated to that of their respective queens and the mean viral DNA load was similar for cats within a household but varied significantly between households. This is the first time that quantitative PCR has been used to detect FcaPV-2 DNA and the results suggest that the virus is ubiquitous but there is a wide variation of viral DNA loads. Kittens appear to be exposed to FcaPV-2 early in life, presumably from direct contact with their queen. These results are important when determining if FcaPV-2 infection of cats is preventable.

1. Introduction

Papillomaviruses (PVs) are small, non-enveloped, double stranded DNA viruses that infect stratified epithelia. Over 280 PV types have been identified, each infecting a specific anatomic site in a particular species (de Villiers, 2013; Rector and Van Ranst, 2013). While most PV infections are asymptomatic, a small number of PV types cause cancer including the well-known examples of cervical cancer in women caused by human PV type 16 and cutaneous squamous cell carcinomas (SCCs) in rabbits induced by *Sylvilagus floridanus* PV type 1 (Durst et al., 1983; Rous and Beard, 1935). To date, four PVs from the domestic cat have been fully sequenced and classified (Dunowska et al., 2014; Lange et al., 2009; Munday et al., 2013a; Tachezy et al., 2002; Terai and Burk, 2002). Of these, *Felis catus* papillomavirus type 2 (FcaPV-2) has been consistently associated with several pre-neoplastic and neoplastic skin lesions including feline viral plaques, Bowenoid in situ carcinomas and cutaneous SCCs, particularly those occurring in UV protected skin (Munday and Aberdein, 2012; Munday et al., 2011a,b; Munday et al., 2008; Munday and Peters-Kennedy, 2010). PV-associated feline cutaneous SCCs contain FcaPV-2 DNA that is amplifiable by polymerase chain reaction (PCR) and demonstrate immunostaining for p16 which is used as a biomarker of PV aetiology in human oral SCCs (Smeets et al., 2007). Further supporting a causal role of FcaPV-2 in

* Corresponding author at: Pathobiology, Institute of Veterinary, Animal and Biomedical Sciences, Massey University, University Avenue, Palmerston North 4472, New Zealand. Tel.: +64 6 356 9099x85798; fax: +64 6 350 5714.
E-mail address: N.Thomson1@massey.ac.nz (N.A. Thomson).

http://dx.doi.org/10.1016/j.vetmic.2014.11.028
0378-1135/

cancer development, PV-associated SCCs on the nasal planum have been shown to have a different clinical behaviour to non-PV-associated nasal planum SCCs (Munday et al., 2013b).

While evidence is accumulating for a role of FcaPV-2 in feline skin cancer (Munday, 2014), there has only been one study in which the prevalence of infection has been investigated (Munday and Witham, 2010). In this study Munday and Witham (2010) detected FcaPV-2 DNA in skin swabs from 23 of 44 adult cats using conventional PCR, suggesting that asymptomatic FcaPV-2 infection is common among cats. There have been no investigations however to determine the age at which kittens become infected with FcaPV-2 or the source of this infection. This information is crucial to determine whether preventing infection with FcaPV-2 could be a feasible method of reducing the incidence of SCCs in cats. Additionally, as all previous studies have used conventional PCR, the quantity of viral DNA has not been determined. Quantifying the load of FcaPV-2 DNA present within the skin of infected cats is important as wide variations in viral copy number may explain why FcaPV-2 infection remains asymptomatic in some cats, but causes neoplasia in others.

The first aim of this study was to develop a real-time PCR assay to detect and quantify FcaPV-2 DNA in skin swabs and hair plucks. The second aim was to use this assay to determine the proportion of the pregnant queens infected, the range of viral loads, and the age at which kittens were exposed to the virus. Furthermore, by quantifying the FcaPV-2 DNA present on infected cats, the viral loads could be compared between queens and kittens, and between the different litters and households.

This is the first time that FcaPV-2 has been quantified and the first time that the presence of this virus has been investigated in very young cats.

2. Materials and methods

2.1. Sample collection

Eleven pregnant queens were recruited for the study. Each queen was sampled on three separate occasions: once within the 3 weeks prior to kittening, and then 7 and 28 days after kittening. Samples were also collected from two kittens from each litter when the kittens were 2, 7 and 28 days of age. Four samples were collected at each time point including hair plucks from the head, dorsal midline and umbilical region, and a pooled sample of skin swabs taken from the same regions. Each hair pluck consisted of 10–20 hairs which were placed in 500 μL sterile saline (0.9% NaCl, pH 5.4). The cotton tipped swabs (Protec Solutions Ltd, Wellington, New Zealand) were first moistened in sterile saline then drawn across a 3 cm × 3 cm area of skin five times. The three swab heads were collected into 500 μL sterile saline in one 5 mL universal container (Sarstedt AG and Co, Nümbrecht, Germany). The samples were transported to the laboratory within 1 h of collection. The hair pluck samples were then centrifuged at 10,000 × *g* for 1 min and the supernatant removed leaving 100 μL of saline with the hairs. The pooled swab samples were vortexed, then the swab heads squeezed out and removed. The remaining solution and the hair plucks were kept at −20 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from both the hair plucks and skin swabs using the Roche High Pure PCR template preparation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions, with the exception that the proteinase K digestion step was extended from the recommended 1 h to overnight. The presence of amplifiable DNA was confirmed by qPCR for *Felis catus* 28s ribosomal RNA gene.

2.3. Generation of recombinant plasmids as standards for qPCR

Forward E7SF (TGGCACGACACCTACCATTAAAGACA) and reverse E7SR (GCCTCCACCTCAACCTCGATCTC) primers were designed to amplify a 111 bp fragment of the FcaPV-2 E7 gene. Conventional PCR was used to amplify this fragment from a swab sample of a viral plaque previously shown to be FcaPV-2 positive. The PCR was performed using TopTaq Master Mix (Qiagen, Hilden, Germany), a final concentration of 0.6 μM of each primer and 4 μL of template DNA in a total volume of 50 μL. The cycling conditions consisted of an initial 3 min denaturation step at 94 °C followed by 35 cycles of denaturation (94 °C for 20 s), annealing (60 °C for 20 s) and elongation (72 °C for 90 s), with a final extension step (72 °C for 7 min). The gel-purified PCR product was cloned into a commercial plasmid vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA) using One Shot Chemically competent *Escherichia coli*, according to the manufacturer's recommendations. Six clones were analysed by PCR and all contained the 111 bp insert on gel electrophoresis. Plasmids were isolated from two clones using the commercially available Roche High Pure Plasmid Isolation Kit (Roche Applied Science) according to the manufacturer's recommendations and sequenced to confirm the identity of the insert. The quantity and quality of isolated plasmid DNA was assessed using a Nanodrop spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). The E7 plasmid was then linearised with Pst 1 restriction enzyme (Thermo Scientific Inc.) in a total volume of 100 μL for 2 h at 37 °C. The reaction was stopped with 4 μL of 0.5 M EDTA. The resulting preparation comprised a stock solution containing the equivalent of 10^9 copies/μL of the target DNA sequence. The same protocol was used to create a recombinant plasmid for the feline 28S ribosomal RNA gene using previously published primers (Helps et al., 2003). Serial dilutions in water of these recombinant plasmids were used to derive standard curves for absolute quantification.

2.4. Quantitative PCR assay

Quantitative PCR assays were run using an Eco real time instrument (Illumina Inc., San Diego, CA, USA). The PCR was performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), a final concentration of 0.4 μM of E7SF primer, 0.3 μM of E7SR primer and either 2 μL of E7 plasmid DNA or 4.5 μL of sample DNA in a total