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Source: Journal of Wildlife Diseases, 40(1) : 99-102

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.1.99

Journal of Wildlife Diseases, 40(1), 2004, pp. 99–102

Detection of Mustelid Herpesvirus-1 Infected European Badgers (*Meles meles*) in the British Isles

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ABSTRACT: The aim of this study was to assess the frequency of mustelid herpesvirus-1 (MusHV-1) infection in free-ranging badgers (*Meles meles*) in the British Isles. A polymerase chain reaction assay was developed that detected MusHV-1 DNA in 95% (18/19) and 100% (10/10) of anticoagulant-treated blood samples collected from free-ranging badgers sampled in the southwest of England and the Republic of Ireland, respectively. An indirect immunoassay was also developed to detect MusHV-1–specific immunoglobulin-G in serum samples. Using an arbitrary cutoff of twice the optical density obtained with a virus-negative preparation, 32.7% (36/110) of sera sampled from badgers were positive. The conclusion drawn from these data is that infection with MusHV-1 is common among free-ranging badgers in the British Isles.

Key words: Badger, herpesvirus, PCR, serology.

Mustelid herpesvirus-1 (MusHV-1) is a newly described herpesvirus isolated from a primary pulmonary cell culture derived from a European badger (*Meles meles*) in Cornwall, England (Banks et al., 2002). Phylogenetic analysis of sequence data demonstrates that MusHV-1 is a novel member of the *Rhadinovirus* genus within the *Gammaherpesvirinae* closely related to equine herpesvirus-2 and -5. The presence of this virus has not yet been associated with any particular lesion, gross pathology, or clinical signs in badgers. However, on the basis of biological properties of some closely related viruses, it is possible to speculate that infection can result in respiratory disease, immunosuppression, and adenopathy and could also play a role in the etiology of tumors (Agius and Studdert, 1994; Schlocker et al., 1995; Ablashi et al., 2002). There is scant data regarding the presence of viral agents in badgers in the British Isles. A previous survey failed to show any evidence of canine distemper virus in badgers in the United Kingdom (Delahay and Frölich, 2000). As an initial step to assess the potential effect of MusHV-1 on the ecology of badgers, the aim of this study was to use molecular tools and a serologic assay to assess the frequency of infection in badgers in the British Isles.

Venous blood samples were obtained from free-ranging badgers located at Woodchester Park ($n=19$), Gloucestershire, England (51°42′N, 2°16′W) or on Fota Island ($n=10$), Republic of Ireland (51°54′N, 8°21′W). Six captive badgers housed under Home Office A(SP)A licence regulations were also sampled. DNA was extracted from ethylenediaminetetraacetic acid (EDTA)–treated blood samples with a QIAamp DNA minikit (Qiagen, Crawley, UK) according to manufacturer's instructions. Negative controls consisting of nuclease-free water were also prepared in parallel to the badger samples. Additional samples were taken from an archive of material originally obtained from badgers killed as part of Statutory Badger Removal Operations performed between 1996 and 1997. These samples included lymphoid tissues (from six animals) and bronchial-alveolar lavage macrophage cultures (from a further three animals). In these cases, DNA was extracted by phenol/chloroform (Maniatis et al., 1989) before testing by polymerase chain reaction (PCR).

The presence of MusHV-1 genomic DNA in the blood samples was determined by PCR. This assay used sense 5′-TTT GAG TAG TGG TCC AGT ACA CTT GT-

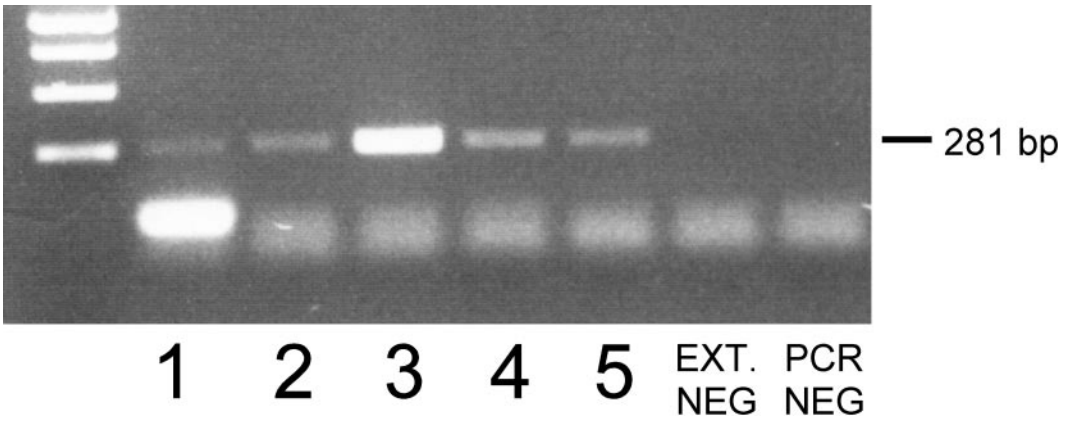

FIGURE 1. Agarose gel (1.2%) showing the PCR amplification of the MusHV-1 DNA polymerase fragment in representative EDTA–blood samples (5) from free-ranging badgers sampled at Woodchester Park, Gloucestershire, UK. Extraction-negative and PCR-negative control wells are shown.

3′ and antisense 5′-TGG ACT TCT CCA ACA TGC GTC GCC CTT C-3′ primers to target a 281–base pair fragment of the DNA polymerase gene of MusHV-1 (GenBank accession number AF376034). Fifty microliters of PCR reaction solution contained 2 mM $MgCl_2$(Promega, Madison, Wisconsin, USA), 50 mM KCl, 10 mM Tris-HCl, pH 8.0 (Thermo buffer, Promega), 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 units *Taq*DNA polymerase (Promega), and 5 μl of the prepared badger DNA template. Amplification conditions (Perkin-Elmer 9700, Applied Biosystems, Foster City, California, USA) used for 35 cycles were denaturation at 94 C for 45 sec, primer annealing at 60 C for 45 sec, followed by chain elongation at 72 C for 45 sec. Polymerase chain reaction products were visualized under ultraviolet (UV) light with 1.2% agarose gels stained with ethidium bromide (Fig. 1). Sequencing of a single PCR product was performed to confirm the identity of the amplicon produced. With the use of this assay, 97% (34/35) of the EDTA–blood samples tested were positive for MusHV-1 DNA. There was no amplification of a product in any of the extraction-negative controls or PCR-negative controls. In addition to the blood samples, positive results were also obtained with DNA extracted from spleen (5/6), mesenteric lymph node (2/2), bronchial alveolar macrophage culture (3/3), and tonsil (1/1). These additional tissue samples represented material from nine individuals.

A preliminary assessment of the seroprevalence of MusHV-1 in free-ranging badgers was performed by an indirect enzyme-linked immunosorbent assay (ELISA). The antigen for this assay was prepared from MusHV-1–infected NBL-7 (fetal mink lung cell line) cultures exhibiting advanced cytopathic effect (CPE). MusHV-1 was purified by ultracentrifugation (L8-55 Beckman, High Wycombe, UK) at 85,000 ×G through a 30% (w/v) sucrose cushion from clarified supernatants generated from disrupted NBL-7 cells. A negative control preparation consisting of material from uninfected NBL-7 cells was also prepared. The viral and control antigen pellets were resuspended in STE (150 mM NaCl, 10 mM EDTA, 20 mM Tris, pH 8.3), and the protein content was assessed by $^{D}_{C}$ protein assay (Bio-RAD, Hemel, Hempstead, UK). Optimum conditions for the ELISA were established by checkerboard titrations. Briefly, 96-well microtiter plates (Probind, Falcon Franklin Lakes, New Jersey, USA) were coated overnight with 10 μg/ml MusHV-1 or control antigen at 4 C. After washing the wells with PBS-T (145 mM NaCl, 7.5 mM

Na_2HPO_4, 2.5 mM NaH_2PO_4, pH 7.2, containing 0.1% [v/v] Tween 80), wells were blocked with 1% (w/v) bovine serum albumin. Badger sera (n=110) collected from nine locations in southwest England were screened in duplicate at a dilution of 1 to 10 in PBS-T. Specific immunoglobulin-G (IgG) was detected by a horseradish peroxidase-conjugated anti-badger IgG monoclonal antibody (CF2: Goodger et al., 1994) followed by signal development with 3,3′,5,5′-tetramethylbenzidine substrate. For each serum, the optical density (OD) of the wells containing the viral antigen was read at 450 nm (UVmax, Molecular Devices, Winnersh Triangle, UK), and the ratio of the OD obtained with the corresponding control antigen well was calculated. With a cutoff of twice the OD of the control antigen, 32.7% (36/110) of these sera were positive for MusHV-1–specific IgG. The relationships between this ratio and age, sex, status of tuberculosis infection, and location of capture for 110 animals were analyzed by statistical software (SPSS 11.0, Chicago, Illinois, USA). These data were not normally distributed; therefore, nonparametric tests were used to compare the OD ratios between groups for each factor. Age was found to be a significant factor of variation (Mann-Whitney test, $P<0.05$) with higher ELISA OD ratios in the group of adults/yearlings (>1 yr old: n=76) than in cubs (<1 yr old: n=34) (Fig. 2). With a 2× background cutoff, 40% (30/76) of the animals more than 1 yr old were considered seropositive compared with only 18% (6/34) of cubs. Capture location (at nine locations) also had a significant influence on ELISA OD values (Kruskall-Wallis test, $P<0.05$; data not shown). The trend of higher serologic values in adults was generally preserved at these different locations. There were no significant differences in relative levels of MusHV-1–specific IgG or serostatus to MusHV-1 between tuberculous badgers confirmed by culture of *Mycobacterium bovis* (33% [17/52] seropositive) and non-infected badgers (33% [19/58] seropositive).

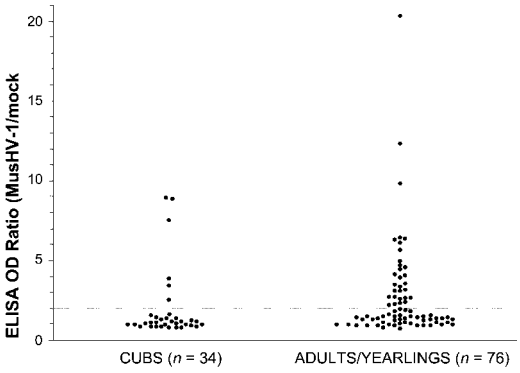

FIGURE 2. Badger herpesvirus–specific IgG detected by indirect ELISA (n=110 samples). Values shown represent the ratio of ELISA signal (OD 450 nm) obtained with the purified MusHV-1 compared with a control antigen preparation. Dotted line indicates positive/negative cutoff used for analysis on the basis of twice the background signal.

The aim of this study was to investigate aspects of the natural history of MusHV-1 among free-ranging badgers in the British Isles. At the time this virus was originally isolated and characterized, a similar CPE was observed in the primary cell cultures of another badger from elsewhere in the United Kingdom. This suggested that the occurrence of MusHV-1 was probably not uncommon among badgers. This hypothesis is further supported by the evidence presented here. With the use of molecular detection methods, a high percentage of free-ranging badgers sampled at two geographically distinct locations in the British Isles had evidence of infection by MusHV-1. Seroprevalence, although not as high as indicated by PCR, suggested that approximately one third of animals sampled in southwest England had detectable MusHV-1–specific IgG. Because different samples on separate occasions were collected for the respective molecular and serological analyses, it is impossible to compare the results from the two methods. Furthermore, because the assay used was not validated with known positive and negative sera, it is also possible the arbitrary seropositive/seronegative cutoff was too

high, thereby underestimating the number of animals with MusHV-1–specific IgG.

The significance of the results of this study indicating a high infection rate of badgers with MusHV-1 is not clear. The statistical analysis performed failed to reveal any significant link between the levels of MusHV-1–specific IgG and *M. bovis* infection. However, further studies are required to address the timing of MusHV-1 infection and to investigate whether this virus possesses immune-suppressive properties with the potential to affect the epidemiology of *M. bovis* in badgers.

The authors acknowledge the technical assistance of G. Heath, S. Grierson, and G. Hewinson for critical review of the manuscript. Samples from Ireland were kindly provided by the Badger Vaccination Group based in the Large Animal Clinical Studies Department, Faculty of Veterinary Medicine, UCD, Dublin. Badger samples from England were obtained through grants funded by DEFRA. British Crown copyright and crown user rights are reserved.

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Received for publication 22 February 2003.