SHORT COMMUNICATIONS

Genital Tract Screening Finds Widespread Infection with Mustelid Gammaherpesvirus 1 in the European Badger (*Meles meles*)

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ABSTRACT: Sexually transmitted diseases (STDs) can be important drivers of population dynamics because of their negative effects on reproduction. However, screening for STDs, especially in wildlife populations, is widely neglected. Using the promiscuous, polygynandrous European badger (*Meles meles*) as a model, we investigated the presence and prevalence of herpesviruses (HVs) in a wild, high-density population and assessed potential differences in somatic fitness and female reproductive condition between infected and uninfected individuals. We collected $n=98$ genital swabs from 71 females (51 adults and 20 cubs) and 27 males (26 adults and 1 cub) during spring and summer 2015. Using a PCR specific for a mustelid α-HV, all genital-swab samples tested negative. In a panherpes PCR, a γ-HV was found in 55% (54/98; 39 adults and 15 cubs), identified as mustelid gammaherpesvirus 1 (MusGHV-1) using DNA sequencing. This contrasts with the results of a previous study, which reported MusGHV-1 in 98% (354/361) of blood samples taken from 218 badgers in the same population using PCR. The detection of MusHV-1 in the female reproductive tract strongly indicates the potential for a horizontal and, likely also a vertical, route of transmission. Our results suggest a potential linkage of genital HVs and impaired future reproductive success in females, but because reproductive failure can have many reasons in badgers, the causative link of this negative relationship remains to be investigated.

Key words: Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae, MusAHV-1, mustelid herpesvirus, sexually transmitted diseases, horizontal/vertical transmission, reproductive success.

Sexually transmitted diseases (STDs) have an important role in natural selection and can cause severe population declines, sometimes threatening species extinction (Knell and Webberly 2004). Understanding STD transmission dynamics, prevalence, and health implications is thus key to effective conservation management. Genital-tract herpesvirus (HV) infections can cause decreased host fecundity or sterility, affecting fitness (Lockhart et al. 1996) and can be transmitted horizontally or vertically (Widén et al. 2012).

Little empirical research has examined the prevalence and effects of sexually transmitted HVs in wildlife populations. Among terrestrial carnivores, α-HVs (AHVs) are common, and canid AHV-1 has been linked to genital ulcers, infertility, and abortion in domestic dogs (Lockhart et al. 1996). γ-HVs have been reported in six wild, terrestrial carnivores; four of which are mustelids infected by species-specific pathogens, with three species displaying clinical signs (Cabello et al. 2013). β-HVs have, thus far, not been reported in carnivores.

European badgers (*Meles meles*) provide an informative wildlife model to study HV epidemiology because they are group-living with a promiscuous, polygynandrous mating system but display low fecundity (Macdonald et al. 2015). Studies on badger population dynamics have typically discussed reproductive failure in terms of environmental or behavioral factors or genetic compatibility, and research into badger diseases has focused on nonvenereal infections, disregarding the potential involvement of STDs (Macdonald et al. 2015). An α-HV, tentatively named *Mustelid alphaherpesvirus* 1 (MusAHV-1), was recently identified from lymph nodes, salivary glands, and liver of French badgers (GenBank no. AF376034), and a γ-HV, currently classified as *Mustelid gammaherpesvirus 1* (MusGHV-1; GenBank no. AF376034; Banks et al. 2002) is highly prevalent in the blood of

badgers throughout the UK (King et al. 2004), but their pathogenicities are unknown.

We screened a high-density badger population for α-, β-, and γ-HVs, relating genital-infection status to body condition and female reproductive fitness. Because of likely vertical transmission, we expected a proportion of cubs to acquire MusAHV-1 and MusGHV-1 before sexual maturity.

Samples were collected between 25 May 2015 and 5 June 2015, after cubs were fully weaned, from a high-density badger population (mean=44.55, SE=5.37 badgers/km; Macdonald et al. 2015) in Wytham Woods, Oxfordshire, UK (51°46′26″N, 1°19′19″W). Following protocols in Macdonald et al. (2015), badgers were caught in steel-mesh box-traps and transported to a central facility.

Individuals were identified from unique tattoos, given at first capture (usually as cubs). Age (categorized as cub: <1 yr; yearling: <2 yr, sexually immature; adult: ≥2 yr), social group, body condition (categorized as 1=emaciated to 5=very good condition), and female reproductive status (classified as estrus: vulva swollen and moist; nonestrus: vulva flat and dry) were recorded. Reproductive success was inferred from teat size and recent lactation (Dugdale et al. 2011), corroborated by genetic pedigree for the study year, enabling assessment of current and subsequent reproductive success in the following breeding season.

Rectal temperature and clinical signs of infection, such as vaginal/penile abnormalities or discharge were recorded. The vulval or penile genital mucosa was swabbed with sterile, cotton-tip swabs (TS/6-H, Technical Services Consultants Ltd., Heywood, Lancashire, UK), carefully avoiding contamination with blood (e.g., from suppurating infections). All samples were frozen immediately at −20 C. After recovery from sedation (≥3 h), all animals were released at their site of capture.

We extracted DNA from 98 genital swabs (71 females: 51 adults, 20 cubs; 27 males: 26 adults, 1 cub) using QIAamp DNA Mini Kits (QIAGEN Ltd., Manchester, UK), following the manufacturer's protocol. Swabs were placed in 2 mL phosphate-buffered saline (Oxoid) containing Pimaricin (Sigma, Poole, UK) at 5 µg/mL for 1 h. Resulting samples were spun at $5,000 \times G$ for 5 min. To lyse gram-positive bacteria, pellets were resuspended in 180 µL of enzymatic lysis buffer containing 20 mg/mL of lysozyme and 200 µg/mL lysostaphin and incubated at 37 C for 30 min in a shaking incubator. After adding 20 µL of proteinase K, samples were incubated at 57 C for 1 h, followed by 15 min at 95 C. The lysate was then purified according to the QIAamp kit protocol and stored at −20 C.

The DNA extracts were screened for α-, β-, or γ-HVs using a generic, nested panherpes (panHV)–PCR analysis (Ehlers et al. 1999), amplifying the 160 base pair (bp) to 181 bp of the HV-DPOL gene (primer-binding sites excluded). The first PCR round used primers 285 sense DAF: 5′-GAYTTYGC[N/I]AGYYT[N/I]TAYCC-3′, 285 sense ILK: 5′-TCCTGGACAAGCAGCAR[N/I]YSGC[N/I]MT[N/I]AA-3′, and 285 antisense KG1: 5′-GTCTTGCTCACCAG[N/I]TC[N/I]AC[N/I]CCYTT)-3′; then primer 286 sense TGV 5′-TGTAACTCGGTGTAYGG[N/I]TTYAC[N/I]GG[N/I]GT-3′ and 286 antisense IYG: 5′-CACAGAGTCCGTRTC[N/I]CCRTA[N/I]AT-3′) in the second round. The PCR reaction mix (25 µL) included 1 µM of each PCR primer (Metabion, Martinsried, Germany), 200 µM of each deoxynucleotide triphosphate, 1 unit of DNA Polymerase AmpliTaq Gold, and 2.5 µL GeneAmp 10× PCR buffer with 2 µM $MgCl_2$ (all from Applied Biosystems GmbH, Darmstadt, Germany), and 5% dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The PCRs were run on a TgradientS thermocycler (Biometra, Göttingen, Germany). In both PCR rounds, the reaction mixes were maintained at 95 C for 12 min for polymerase activation, and then cycled 45 times with 20 s of denaturation at 95 C, 30 s of annealing at 46 C, and 30 s of strand extension at 72 C, followed by a final extension step at 72 C for 10 min. All PCRs used 10 µL of genital swab DNA as a template, and DNA extracts of MusAHV-1 and MusGHV-1 positive organs were used as positive controls. All products from the panHV PCR of expected

length were purified and sequenced with the Big Dye terminator cycle sequence kit (Applied Biosystems, Warrington, UK) on a 377 automated DNA sequencer (Applied Biosystems, UK). We estimated the detection limit of the panHV PCR for MusGHV-1 sequence to be 100 copies/PCR reaction, by generating a MusGHV-1 PCR product that spanned all primer-binding sites of the panherpes PCR in MusGHV-1 DNA polymerase coding sequence, serially diluted 10-fold in HV-negative mammalian DNA (5 ng/PCR reaction). The dilution series (with 10,000 to 0.001 fragment copies per PCR reaction) was tested with a pan-HV PCR.

Although the generic PCR used here can detect α-, β-, and γ-HV (e.g., Ehlers et al. 1999, 2008), we detected only γ-HVs. To verify our detection sensitivity, we also tested specifically for MusAHV-1 (GenBank no. MF042164) using a nested PCR with the primers 7207 sense 5′-GGTTTATTACC TTGTCTACACATAGCT-3′ and 7207 antisense 5′-TTTACATAGTAAGATTTGTTCG GAACG-3′ in the first round (amplification product: 170 bp, without primer binding sites) and 7489 sense 5′-ACCTTGTCTACACAT AGCTGCA-3′ and 7489 antisense 5′-GGAACGGCTATGGATGAGGC-3′ in the second round (amplification product: 141 bp, without primer binding sites). These PCRs were carried out as described for the panHV PCR, with annealing temperatures of 62 C and 61 C. A DNA extract of a MusAHV-1 PCR-positive organ was used as a positive control. The detection limit was determined as mentioned earlier, using a MusAHV-1 PCR that spanned all primer binding sites of the MusAHV-1–specific PCR in MusAHV-1 DNA polymerase coding sequence and was estimated as 100 MusAHV-1 copies/PCR reaction.

Statistical analyses were carried out using R software (version 3.2.3; R Development Core Team 2014). Wilcoxon rank-sum test and Fisher's exact test were used to investigate the effects of HV infection on body and reproductive condition. χ^2 tests with the Yates continuity correction were used to test for differences in current and subsequent reproductive success based on infection status.

Venereal MusGHV-1 infection was widespread in this population. All 98 swabs tested negative for α- and β-HVs, both in the pan-HV generic PCR as well as the MusAHV-1–specific PCR. However, 55% (54/98) of those samples tested with pan-HV generic PCR were positive for a γ-HV, identified as MusGHV-1 by sequencing. The positive samples were from 43% (22/51) of adult females, 65% (17/26) of adult males, and 75% (15/20) of female cubs.

Across the 18 social groups in which more than one badger was tested, MusGHV-1 infected and uninfected badgers were detected in 83% (15/18); all individuals tested (three adult females and one adult male) were positive in two social groups (11%, 2/18); and infection was not detected in only one group (6%, 1/18). Cubs (n=12 females) were infected, inferring vertical transmission of MusGHV-1.

Infection with MusGHV-1 had no effect on female (Wilcoxon rank-sum test: W=144.5, P=0.173) or male body condition (W=144.5, P=0.173). Female reproductive condition was unaffected (Fisher's exact test, P=1), with near equal ratios of infected (32%, 7/22) and uninfected females (31%, 9/29) being in estrus, compared with nonestrus (infected= 68%, 15/22; uninfected=69%, 20/29), and current female reproductive success did not relate to infection status (χ^2=0.005, df=1, P=0.942). Subsequent reproductive success, however, tended to be lower among infected females, with only 20% (3/15) of infected females displaying signs of recent lactation the following year, compared with 50% (7/14) of uninfected females (χ^2=1.709, df=1, P=0.191). No infected individuals exhibited fever or genital discharge.

This first systematic investigation of venereal HV in any wild terrestrial carnivore confirmed the γ-HV MusGHV-1 in 55% of adults and in 75% of female cubs, inferring horizontal as well as vertical transmission. This is consistent with blood screening using PCR to detect nearly 100% exposure to MusGHV-1 in this study population (Sin et

al. 2014) and across the UK and Ireland (King et al. 2004). Importantly, body condition and fever did not correlate with venereal infection, whereas intensity of hematologic MusGHV-1 infection was linked to impaired body condition (Sin et al. 2014), highlighting that clinical population screening must also include apparently healthy individuals. We did not detect any α- or β-HVs, although we are confident that our assay sensitivity was sufficient (Ehlers et al. 2008), implying a true venereal absence, although that does not exclude the possibility of latent infections (Widén et al. 2012).

Promiscuity is often linked to high STD prevalence and virulence (Kokko et al. 2002). Despite all social group members typically mating together (Macdonald et al. 2015), not all individuals exhibited venereal infection, implying differences in immune-genetic or behavioral susceptibility. Infection with HV typically suppresses immune responses, with potential damage to the reproductive tract and subsequent infertility (Widén et al. 2012). Although we found no evidence of venereal MusGHV-1 infection correlating with current female reproductive success, results indicated potential impairment of subsequent reproductive success. Venereal MusGHV-1 infection could thus contribute to only 45% of females in this population producing cubs each year (Macdonald et al. 2015). Therefore, females may need to balance the risks of acquiring active HV-infection against maximizing mating opportunities, restricting mate choice. Similarly, actively infected males may suffer reduced mating opportunity. In conclusion, we show that venereal MusGHV-1 infection may affect breeding systems and reproductive success of wild-living carnivores and that population-wide STD screening must be integrated into conservation strategies.

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