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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Polar Bear Encephalitis: Establishment of a Comprehensive Next-generation Pathogen Analysis Pipeline for Captive and Free-living Wildlife

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Summary

This report describes three possibly related incidences of encephalitis, two of them lethal, in captive polar bears (*Ursus maritimus*). Standard diagnostic methods failed to identify pathogens in any of these cases. A comprehensive, three-stage diagnostic 'pipeline' employing both standard serological methods and new DNA microarray and next generation sequencing-based diagnostics was developed, in part as a consequence of this initial failure. This pipeline approach illustrates the strengths, weaknesses and limitations of these tools in determining pathogen caused deaths in non-model organisms such as wildlife species and why the use of a limited number of diagnostic tools may fail to uncover important wildlife pathogens.

Keywords: diagnostic tests; encephalitis; polar bear; wildlife diseases

Introduction

Infectious diseases are suggested to be a potential key factor in the decline and extinction of populations and species of conservation concern (de Castro and Bolker, 2005; Hofer and East, 2012; MacPhee and Greenwood, 2013). In comparison with the knowledge accumulated for model organisms, livestock and domestic animals, diagnosing the source of mortality in non-model wildlife is a massive challenge that faces a number of problems, even when the species concerned are well known, charismatic and the clinical signs are obvious and life-threatening or fatal (de Castro and Bolker, 2005; Wyatt *et al.*, 2008;

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Hofer and East, 2012). Polar bears (*Ursus maritimus*) are illustrative of such problems associated with the study of wildlife diseases. Knowledge of diseases in this and other wildlife species is limited, which makes the identification of causative agents during disease outbreaks in captive wildlife particularly problematic (Aguirre *et al*., 2012). Non-specific clinical signs, such as seizures, may be caused by a wide range of causative agents, even when very conspicuous. Furthermore, the lack of knowledge of wildlife pathogens and their reservoirs may make it difficult in many cases to pinpoint the cause of fatalities.

When several deaths occur within a short time frame in small and threatened wildlife populations of conservation concern, and the affected individuals display similar clinical signs, the diagnostic challenge is considerable. The question arises as to whether this is an early warning of a disease outbreak that might deplete the population of an endangered species or the effect of inappropriate husbandry or management measures in captive or free-ranging situations, respectively, or perhaps a consequence of genetic predispositions that may emerge in small and highly structured populations with limited genetic variation.

Such concerns were raised in the veterinary and conservation communities, the zoo world, the general public and the press when two polar bears, Knut of Berlin Zoological Garden and Jerka of Wuppertal Zoological Garden, died unexpectedly after seizures, with no initial clinical signs to warn of a developing health problem, within a limited time frame of 2 years. The health of a third bear, Lars (also in Wuppertal Zoological Garden and the father of Knut) was severely compromised, but this animal recovered after successful veterinary intervention.

A detailed investigation into the death of both bears was undertaken and blood samples from the third bear were analyzed. Possible issues concerning husbandry were considered, but were excluded. The observed clinical signs (i.e. seizures) can be caused by a wide range of pathogens and so it was unclear whether the deaths were caused by the same agent. Initial screening for obvious pathogens failed to reveal a cause in the case of the two dead bears, at which point the cause of death would have normally been assigned as unknown. As a consequence of the initial failure, a three-stage diagnostic 'pipeline' employing both standard serological methods and new DNA microarray and next generation sequencing-based diagnostics was developed. In this report, we describe the different stages of this pipeline and discuss the strengths, weaknesses and limitations of these tools in determining pathogen-associated deaths in non-model organisms and why the use of a limited number of diagnostic tools may fail to uncover important wildlife pathogens.

Materials and Methods

Case Material

Knut was born in the Berlin Zoological Garden, Germany, in December 2006, to a wild-born female caught in Canada and a captive-born male, Lars (Blaszkiewitz, 2009). Knut gained worldwide attention when, as a cub, he was rejected by his mother and subsequently hand-reared by a zookeeper. The zookeeper's interactions with Knut were held in public and the bear therefore became the focus of much media attention throughout its life. As a result, many hours of professional and amateur film are widely available of Knut's life, including his sudden seizures followed by death by drowning in the moat of the polar bear enclosure at the Berlin Zoological Garden in March 2011. Medical files did not indicate health problems prior to these seizures or in any of the co-housed polar bears or other bear species in neighbouring enclosures.

Lars lived in Wuppertal Zoo from October 2009 to 2012 and never had direct contact with Knut. He lived with a female polar bear, Jerka, and both suffered seizures in 2010. With medical intervention Lars survived but Jerka died. The cause of death was identified as a recombinant equine herpesvirus (EHV)-1/EHV-9 derived from zebras (Greenwood *et al*., 2012). The same study found no evidence that Lars and Knut's illnesses were related, because recombinant EHVs were not detected by genetic or serological methods (Greenwood *et al*., 2012).

Pathological Examinations

Knut's body remained in the enclosure moat for approximately 48 h before submission of the carcass for necropsy examination. Cerebrum, brainstem, serum, blood, lung, liver, spleen, kidney, small and large intestine and faeces were collected from Jerka and serum, faeces and saliva were collected from Lars. Additional clinically healthy polar bear control samples were collected as follows: whole blood samples from four bears, brain samples from two bears and serum samples from six bears. The experiments undertaken in this project were approved by the Internal Ethics Committee of the Leibniz-Institute for Zoo and Wildlife Research (IZW), Approval No. 2012-04-01. Further descriptions of all polar bears studied can be found in Greenwood *et al*. (2012).

Prior to routine necropsy examination, samples of cerebrospinal fluid (CSF) were aspirated from Knut as described for dogs (Gwalter and Wegmann-Ehrenspenger, 2006) and smears were stained with methylene blue (Böck, 1989). Fresh tissue samples and samples fixed in 4% neutral buffered formalin

were taken for further investigation. Samples of cerebrum, cerebellum, brainstem, spinal cord, CSF, blood, lung, lymph nodes, thymus, spleen, liver, bile, small intestine, large intestine, kidneys, faeces, fat, muscles and bones were frozen at −80°C for further investigations. Samples from Jerka including cerebrum, brainstem, serum, blood, lung, liver, spleen, kidney, small intestine and large intestine, which had been stored at −80°C since her death, were obtained. After fixation, tissues were processed routinely and embedded in paraffin wax. Sections (3 μm) from all of the organs from Knut and of the samples from Jerka were stained with haematoxylin and eosin (HE). Sections from the brain were stained by Gram, periodic acid–Schiff and Grocott's silver stain (Böck, 1989; Stevens and Francis, 1996). Sections from spleen and lymph nodes were stained with Perl's Prussian blue (Böck, 1989).

For electron microscopical examination, frozen brain tissue and formalin-fixed tissues from Knut were fixed in 3% glutaraldehyde, washed in phosphate buffered saline (PBS, pH 7.2) and fixed in 2% osmium tetroxide. Dehydration was performed in ethanol and samples were embedded in EPON 812 before preparation of ultrathin sections and staining with uranyl acetate and lead citrate. Ultrathin sections were examined with a Tecnai Spirit™ transmission electron microscope (Fei, Eindhoven, The Netherlands).

Immunohistochemistry

Immunohistochemistry (IHC) was performed to detect 11 different viruses, the apicomplexan parasite *Toxoplasma gondii*, as well as the lymphoid markers CD3 and CD79α using embedded brain tissue from both bears (Table 1 and Supplementary Table S3). Briefly, following dewaxing, tissue sections were immersed in H_2O_2 0.5% in methanol for 20 min. Non-specific primary antibody binding was blocked by incubation with inactivated normal serum diluted 1 in 5 in PBS (pH 7.1). Primary antibody diluted in PBS was applied and tissue sections were incubated in a humid chamber at 4°C overnight. After washing, the sections were incubated with biotinylated secondary antibody. The antibody against influenza A virus was directed against the nucleoprotein of the virus (Nicholls *et al.*, 2012). For rabies, the secondary antibody was directed against fluorescein isothiocyanate (FITC), which is coupled to the primary antibody. The detection system used was the avidin–biotin–peroxidase complex method (Vector Laboratories, Burlingame, California, USA). The chromogenic solution consisted of 0.05% 3,3′-diaminobenzidine tetrahydrochloride with 0.03% H_2O_2 as the substrate in 0.1 M Tris-buffered saline (pH 7.6). Tissue sections were counterstained with Mayer's haematoxylin. As negative controls, tissue sections from a polar bear without encephalitic lesions were used. In addition, the primary antibody was replaced by ascites fluid from non-immunized BALB/cJ mice (Cedarlane, Ontario, Canada) or rabbit serum

Table 1
PCR and immunohistochemistry screening

Pathogens	*IHC results*	*PCR results*
Viruses		
Adenoviruses	Negative	Negative
Bornaviruses	Negative†	Negative
Canine distemper virus (CDV)*	Negative	Negative
Cytomegalovirus*	Not tested	Negative
Encephalomyocarditis virus	Not tested	Negative
Enterovirus (ENTC)*	Not tested	Negative
Equine herpesvirus	Negative†	Negative
Feline herpesvirus*	Negative†	Negative
Feline leukaemia virus (FeLV)	Negative	Not tested
Hanta virus	Not tested	Negative
Influenza	Not tested	Negative
Inkoo virus	Not tested	Negative
Pan flavivirus*	Not tested	Negative
Pan herpes	Not tested	Negative
Pan morbillivirus	Not tested	Negative
Parvovirus	Negative†	Negative
Pseudorabies	Not tested	Negative
Poxviridae	Not tested	Negative
Rabies*	Negative	Negative
Schmallenberg virus	Not tested	Negative
Sindbis virus	Not tested	Negative
Tahyna virus	Not tested	Negative
Tettnang virus	Not tested	Negative
Tick borne encephalitis*	Negative†	Negative
Venezuelan equine encephalitis virus (VEEV)	Not tested	Negative
Western equine encephalitis virus (WEEV)*	Not tested	Negative
West Nile virus (WNV)	Negative†	Negative
Parasites		
Acanthamoeba	Not tested	Negative
Naegleria fowleri	Not tested	Negative
Pan-*Plasmodium**	Not tested	Negative
*Toxoplasma gondii**	Negative	Negative
Fungi		
Coccidioides spp.*	Not tested	Negative
*Cryptococcus neoformans**	Not tested	Negative
*Histoplasma**	Not tested	Negative
Bacteria		
Borrelia spp.	Not tested	Negative
Listeria	Not tested	Negative
Streptococcus spp.	Not tested	Negative

*Primers produce correct size band, but sequencing results indicated that the sequence amplified product was either polar bear or other carnivore related sequence.
†The sample was tested by IHC for Jerka and Knut.

(Sigma–Aldrich, Taufkirchen, Germany). As positive controls, tissues sections from confirmed cases of infection with the respective agents were used.

Bacteriology and Parasitology

Bacteriological investigations were only performed on samples from Knut. Swabs from the frontal sinus, brain surface, cerebrum, CSF and alveolar socket of a canine tooth were taken. Samples were cultured on Columbia agar with 5% sheep blood, Gassner's agar, chocolate agar (all from Fisher Scientific GmbH, Schwerte, Germany) and CHROMagar Orientation (Mast Diagnostica GmbH, Reinfeld, Germany) under aerobic, capnophilic (5% CO_2) and anaerobic conditions. Dependent on the results of the Gram stain, as well as the catalase and oxidase tests (BD, Heidelberg, Germany), biochemical identification of the bacteria was performed by using the API® Identification System (bioMérieux Deutschland GmbH, Nürtingen, Germany) (Böck, 1989). Parasitological analysis, primarily to exclude trichinellosis, was performed in the Institute for Food, Drugs and Epizootics according to European act No. 2075/2005 (Gylstorff, 1987; European Commission, 2005).

Serology

Serum samples from Lars and six control polar bears and whole blood samples from Knut were examined for the presence of antibodies against influenza virus type A nucleoprotein (ID Screen® Influenza A Antibody Competition ELISA kit, ID-vet, Montpellier, France; IDEXX Influenza A AB test, Ludwigsburg, Germany) and against the N1 protein (ID Screen® Influenza N1 Antibody Competition ELISA kit, ID-vet). To verify positive results from the ELISA, haemagglutination inhibition (HI) was performed according to the OIE Manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2012a). Serum as well as whole blood samples tested against influenza A H1N1 pandemic strain 2009 and an H5N1 strain reacted non-specifically. Material for HI after pre-treatment (e.g. with receptor destroying enzyme) was not available. Blood from Knut and another control bear (Anton) and serum from the latter were also examined for the presence of neutralizing antibodies to EHV-1, EHV-4 and equine arteritis virus (EAV) using microneutralization assays as previously described (OIE, 2012b, c). A canine distemper virus (CDV) serum neutralization test (SNT) was performed on the same samples following the protocol described by de Vries *et al.* (1988), with the modification of CDV strain used for VNT. The Onderstepoort strain of CDV (GB number: AF014953), kindly provided by V. von Messling (INRS-Institut Armand-Frappier, Laval, Quebec, Canada), was used.

Polymerase Chain Reaction

Molecular analysis was performed using brain (telencephalon caudal to the right olfactory bulb, lateral hemisphere of the cerebellum and brainstem), liver, spleen and lung from Knut and brain (cerebrum and cerebellum) and liver from Jerka. The polar bear tissue samples were homogenized and extracted using the Qiamp DNA Mini kit and RNeasy lipid tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Polymerase chain reactions (PCRs) performed on DNA- and RNA-based pathogens were analyzed by PCR or reverse transcriptase (RT)-PCR, respectively. Primer sequences are described in Supplementary Table S1. PCRs and RT-PCRs were performed using My-taq (Bioline GmbH, Luckenwalde, Germany), HiFi supermix (Invitrogen, Darmstadt, Germany), Superscript III (Invitrogen) and Superscript III one-step RT-PCR according to the manufacturers' protocols. All PCRs were performed at least in duplicate. PCR products were visualized after electrophoresis in 1% agarose gels using GelRed nucleic acid gel stain (Biotium Inc., San Francisco, California, USA). Amplification products with correct sizes, when detected, were sequenced using appropriate forward and reverse primers (StarSeq GmbH, Mainz, Germany). When multiple bands were generated, PCR products were cloned using the pGEM vector system (Promega, Madison, Wisconsin, USA). Positive colonies were identified by colony PCR and cleaned of reagents and primers using MSB Spin PCRapace (Stratek Molecular GmbH, Berlin, Germany). Multiple clones were sequenced (Starseq) using M13 forward and reverse primers. All sequences were aligned against GenBank sequences using BLASTn to search for homology to pathogens deposited in the database (www.ncbi.nlm.nih.gov/).

DNA Microarrays

For virochip analysis RNA was first extracted from tissue samples using Qiagen RNeasy Lipid tissue mini kit. For enrichment, total RNA extractions were further processed following the manufacturers' protocols with Terminator exonuclease (Epicentre Biotechnologies, Madison, Wisconsin, USA) to remove rRNA or with oligotex mRNA mini kit for poly-A mRNA isolation (Qiagen). RNA was reverse transcribed using Superscript III (Invitrogen) with primer 5′-CGC-TCT-TCC-GAT-CTN-NNN-NN-

3′. Following reverse transcription, cDNA was synthesized using T7 DNA polymerase (Fermentas, Waltham, Massachusetts, USA) and the same primer to synthesize products tagged at both ends. These were amplified by PCR using primer: 5′-CTG-TCT-GGC-TCT-TCC-GAT-CT-3′ and GoTaq DNA polymerase (Promega). Thermocycling conditions were: 95°C for 2 min; three cycles of 95°C for 30 sec, 40°C for 30 sec and 72°C for 1 min, then 25 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with a final extension of 72°C for 5 min.

To fluorescently label libraries for virochip hybridization, a portion of each library was amplified by PCR as above with a dNTP mixture including 75% of 5-(3-aminoallyl)-dUTP (Ambion, Carlsbad, California, USA) instead of dTTP, which is normally in the mixture. PCRs were purified using a DNA Clean and Concentrator-5 column (Zymo Research, Irvine, California, USA). Eluate was heated at 95°C for 2 min, cooled on ice, then labelled in reactions containing 100 mM sodium bicarbonate pH 9.0, 10% dimethyl sulphoxide and 667 μM Cy3 mono-NHS ester (GE Healthcare, Little Chalfont, UK) for 1 h at 25°C. Labelled DNA was purified as above and added to hybridization reactions containing 3 × SSC, 25 mM HEPES pH 7.4 and 0.25% sodium dodecyl sulphate (SDS). Hybridization mixtures were heated at 95°C for 2 min, then added to microarrays and hybridized overnight at 65°C. Arrays were washed twice in 0.57 × SSC and 0.028% SDS and twice in 0.057 × SSC, then scanned on an Agilent XYZ scanner. Three tools were used to analyse array data: E-predict (Urisman *et al.*, 2005), Z-score analysis (Chiu *et al.*, 2006) and cluster analysis (Eisen *et al.*, 1998).

For a second microarray screening using a different chip, the pan-viral DNA-microarray platform Biochip 5.1 for the detection of approximately 2,000 different viruses was used (Gurrala *et al.*, 2009). Isolated RNA and DNA from brain and liver of Knut and Jerka were tested. The genetic material from Jerka was used as a control in the investigation. For sample preparation, an established procedure involving a separate cDNA and second-strand synthesis of RNA and DNA was used. Prior to labelling, the second-strand synthesis products of RNA and DNA were pooled and the labelled DNA was hybridized onto the array. The microarrays were scanned with an Axon GenePix 4300A (Molecular Devices, Sunnyvale, California, USA) and the raw data were extracted with GenePixPro7 software (Molecular Devices).

Next-generation Sequencing

Extracted genetic material from brain and liver samples from Knut and Jerka was used as input material. Library preparation was performed using the SPRIworks System II (Beckman Coulter Fullerton, California, USA) for Roche GS FLX DNA Sequencer (Roche, Basel, Switzerland). The resulting libraries were then prepared for 454 sequencing according the manufacturer's instructions.

Illumina sequencing was performed on 100 ng of rRNA-depleted RNA that was fragmented. RNA-seq library preparation was carried out as described previously (R Development Core Team, 2009). RNA-seq was performed on a HiSeq 2000 sequencing platform with 1 × 100 cycles of sequencing, following the manufacturer's instructions (Illumina, San Diego, California, USA).

Bioinformatics

Statistical analysis of the array data was performed in R Development Core Team (2009). An R-package for the standard microarray evaluation method 'Limma' (Smyth and Speed, 2003; Wettenhall and Smyth, 2004; Smyth, 2005; Smyth *et al.*, 2005; Ritchie *et al.*, 2007) and the data normalization method 'ZScore Transformation' (Cheadle *et al.*, 2003) were used. Polar bear-derived Illumina sequences were removed in several steps of increasing computational complexity. Firstly, sequences were searched against a custom database of bear ribosomal and mitochondrial sequences using the BLASTn algorithm (Altschul *et al.*, 1997). Sequences producing alignments of ≥95% identity over 90 nucleotides were removed. Next, sequences were searched against a draft assembly of the polar bear genome (Stenglein *et al.*, 2012) using BLASTn. Sequences producing alignments of ≥90% identity over 90 nucleotides were removed. Next, low complexity sequences were identified by compression with the Lempel–Ziv–Welch algorithm (Welch, 1984). Sequences whose compressed length was less than 0.49 × the size of their uncompressed length were filtered. Remaining sequences were searched against the NCBI non-redundant nucleotide (nt) database using BLASTn. Sequences with hits to database sequences with *E*-values $\leq 1 \times 10^{-8}$ were removed from further analysis. Sequences were also searched against custom databases of viral protein sequences using the BLASTx algorithm.

Reads that hit any viral sequence with an *E*-value ≤0.001 were assigned to the database entry with the lowest *E*-value. For each of the latter hits, a species was determined and a *P* value was computed. The resulting dataset was then analyzed to find species that had multiple non-overlapping hits to different parts of its genome. To estimate an overall probability measure for each occurring species, the *P* values of non-

overlapping hits were considered independent and thus multiplied. For each group of overlapping hits, the smallest *P* value was used, as overlapping hits cannot be considered independent and the minimum *P* value gives an upper bound for the combined *P* value of the group.

In a second approach to analyse the Illumina shotgun data, the reads were assembled into contigs after filtering out reads matching to rRNA, 7S RNA and polar bear mitochondrial DNA. The assembly was carried out with Velvet (version 1.2.03; European Bioinformatics Institute, Cambridge, UK) using standard parameters and a hash length of 23. Out of the above results, contigs longer than 200 base pairs (bp; average length 254.64 bp, minimum 200 bp, maximum 528 bp, average coverage 6.77, minimum 1.33, maximum 65.35) were selected and BLASTn searches against the NCBI nucleotide database were performed. Each contig was assigned to a species by its best BLASTn hit. In contrast to the Illumina contig coverage, the GS FLX sequence coverage was an average of 2.13, a minimum of 1 and a maximum of 15 reads.

Results

Overview of the Analysis Pipeline

Fig. 1 outlines the pipeline employed to examine samples from Knut. In all cases, negative captive polar bear controls representing living individuals or those who had died of causes differing from Knut (i.e. non-encephalitic disease), were included for comparative purposes. In all experiments, tissue samples from Jerka, who had died from infection with a zebra-derived recombinant EHV-1/EHV-9 (Greenwood *et al.*, 2012), served as a positive control for similar

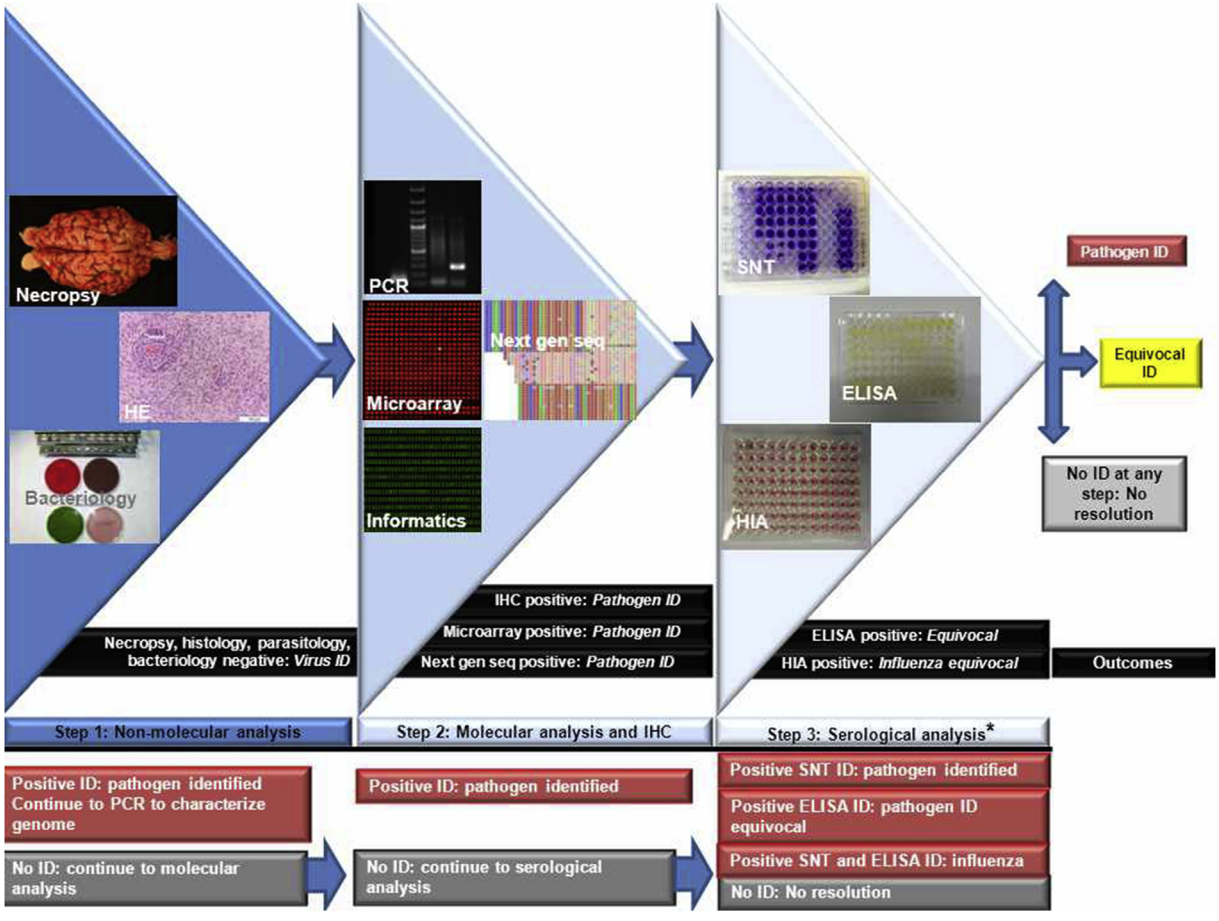

Fig. 1. The research pipeline. The three steps of the pipeline are shown with the possible outcomes at each stage. For each step, pathogen identification (ID) is a possibility. When not identified at each individual stage, the sum of the three stages will yield an ID or an equivocal ID, such as with serological evidence, but lack of genomic evidence for a pathogen. No ID remains a possibility throughout. Black boxes indicate positive or equivocal ID for each sub-stage. The red boxes below indicate positive outcomes of each stage. Grey boxes indicate negative ID at each stage. The three possible outcomes post pipeline analysis are shown at the far right of the figure. HE, haematoxylin and eosin histopathology; IHC, immunohistochemistry; SNT, serum neutralization test; ELISA, enzyme linked immunosorbent assay; HIA, haemagglutination inhibition assay.

tissue samples from Knut and Lars. Tissues from Jerka that tested negative for a range of pathogens were included as negative controls for these pathogens in the pipeline analysis. As PCR and quantitative PCR (qPCR) showed that Jerka was infected with recombinant zebra EHV-1/EHV-9, we expected that she should differ in her pathogen profile from Knut and be positive for EHV-1 by non-target specific genetic screens.

Necropsy Examination

Knut weighed 305 kg and was in good overall condition. The cerebrum showed mild asymmetry of the olfactory bulb region. Caudal to the right olfactory bulb was a small convex area. The meningeal vessels and capillaries were congested (Fig. 2). After fixation of the brain, transverse sections were performed, but no detectable asymmetry or dilation of the lateral ventricles was visible. Consistent with drowning, large quantities of water were present in the respiratory tract and paranasal sinuses. The deciduous maxillary canine teeth were present and both were fractured. On the right side this was associated with purulent alveolitis. The first deciduous maxillary premolar and both deciduous mandibular canine teeth were also present. The lung showed marked congestion, alveolar oedema and moderate alveolar emphysema. Paltauf spots were not detected. The stomach was filled with water and the remains of a meal. In contrast, Jerka weighed 245 kg and was in excellent condition. The only relevant gross findings were congested meningeal vessels.

Histopathology and Electron Microscopy

Examination of Knut's brain revealed severe multifocal lymphoplasmacytic and eosinophilic panmeningoencephalomyelitis. The cellular infiltration in the cerebrum was perivascular and perineuronal (Fig. 2). Moderate multifocal nodular microglial proliferation was detected. Moderate cortical neuronal necrosis could be seen with an eosinophilic appearance of the neurons with central chromatolysis and partly peripheral nuclei. In the cerebral cortex, lymphocytic and eosinophilic inflammation of the choroid plexus with focal perivascular haemorrhages was identified. Cerebellar changes comprised only perivascular inflammation. The inflammation in the dura mater, meninges and spinal cord was highly variable and affected areas presented with mild to severe inflammation. Generally, inflammation was more severe in the cerebrum and brainstem than in the cerebellum, spinal cord and meninges. However, inflammatory lesions did not show a predilection for a specific cerebral region, although the mild asymmetry at the olfactory bulb would suggest this. Myelitis decreased from cranial to caudal levels. Inclusion

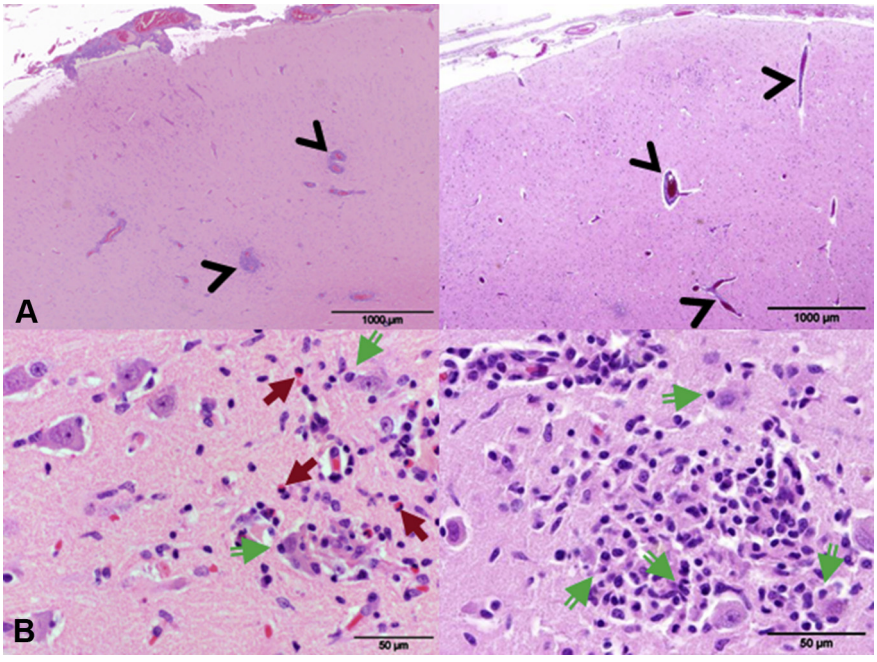

Fig. 2. Neuropathological findings in Knut (left) and Jerka (right). Panel A shows histological images with HE staining. All show an overview of the cerebral cortex with perivascular non-suppurative inflammation located around blood vessels in both animals (black arrows) and meningeal vessels in Knut only. Panel B shows a higher magnification of grey matter inflammation with perineuronal microglial infiltration (green arrows) and few eosinophils (red arrows) in Knut.

bodies, parasites, fungi, bacteria or other possible agents were not detected.

In contrast to the brain, only minor changes unrelated to the encephalitis were detected in other organs. Associated with the right deciduous maxillary canine tooth was a focal, mild, purulent and lymphohistiocytic alveolitis. Within the submandibular, popliteal and inguinal lymph nodes and the thymus, haemorrhages and isolated macrophages containing haemosiderin were noted. Despite congestion, the spleen showed isolated macrophages with haemosiderin deposition, but no evidence of follicular hyperplasia. Pulmonary lymph nodes had mild anthracosis. Mild, lymphohistiocytic rhinitis was present. The lung was filled with eosinophilic fluid and foreign bodies (e.g. small parts of plants and sand-like particles).

Electron microscopical inspection of frozen brain tissue was inconclusive due to the poor condition of the samples. Several fixed brain tissue samples were also examined by electron microscopy, but there was no evidence for viral particles in the brain. The results were, however, consistent as no inclusion bodies were identified.

Histological examination of Jerka's brain revealed multifocal mild to severe non-suppurative meningoencephalitis with gliosis and mostly perivascular lymphohistiocytic infiltrates. There was no evidence of inclusion bodies, parasites, fungi, bacteria or other possible agents. The other organs showed no abnormalities.

Bacteriology and Parasitology

Bacteriological examination of swabs from Knut's alveolar socket and frontal sinus revealed a diverse, but non-specific aerobic and anaerobic flora in varying combinations (Fig. 3). As the histopathological results revealed no signs of bacterial infection or intoxication, no further bacteriological diagnostic procedures were performed. All identified bacteria were either components of the normal flora of the respiratory tract mucosa and the alveolar socket or likely originated from the inhaled water from the enclosure moat, including faecal contamination (Uchida *et al.*, 1995; Schuster, 1999). Bacteria isolated and characterized from the brain most likely spread to the cranial cavity during decomposition over the 48 h elapsing between death and necropsy examination, while Knut's carcass was floating in the moat. These results were supported by the IHC findings (see below). Jerka's brain was not subjected to bacteriological examination.

No ectoparasites or endoparasites, including *Trichinella* spp., were identified grossly in Knut. Parasites or parasite eggs were not detected in any histological sections. Similarly, no parasitic infection was identified in Jerka.

Immunohistochemistry and Polymerase Chain Reaction

A broad panel of viral and non-viral encephalitis-causing pathogens, representing DNA and RNA viruses, bacteria, fungi and parasites were tested for by PCR and IHC (Table 1, Supplementary Table S1). All IHC results were negative. Approximately 30% (and up to 50%) of the perivascular and perineuronal inflammatory cells were $CD3^+$ T lymphocytes. Labelling for $CD79\alpha^+$ B lymphocytes and plasma cells was more variable (approximately 10% of cells were clearly labelled with 40–50% of cells showing a weak reaction that was likely non-specific).

Although PCR products were obtained in some pathogen-specific reactions, the product sequences did not originate from pathogens (Table 1). None of the 37 tested viral, bacterial and parasite pathogens were found to be associated with the encephalitis. Sensitivity is likely to be an issue where primer mismatches exist between known and unknown or novel strains, particularly for viruses, and this could yield false-negative results. However, the majority of the assays were 'pan-pathogen' PCRs designed to avoid such limitations (Supplementary Table S1). The same PCR assays were negative for all pathogens in brain samples from Jerka, except that a zebra EHV-1 strain was identified as described in Greenwood *et al.* (2012) using pan-herpesvirus primers (Table 1, Supplementary Table S1).

Screening by DNA Microarray

Two microarray platforms were employed for pathogen identification. The first, the Virochip, has usually been applied to human cases of disease (Wang *et al.*, 2002) and has also been successfully used to identify novel pathogens from non-human animals (Kistler *et al.*, 2008; Stenglein *et al.*, 2012). It contains sequences from approximately 3,000 mostly animal viruses. Recently, however, it has been demonstrated that its sensitivity is lower than that of shotgun sequencing from purified viral particles from a study of human serum (Yozwiak *et al.*, 2012). The second microarray, Biochip 5.1, was developed in a project within the EU Network of Excellence for Epizootic Disease Diagnostic and Control (EPIZONE) (Gurrala *et al.*, 2009) and is a diagnostic tool for the detection of approximately 2,000 viruses at the genus, species and genotype level. Given the methodological similarity between both virus chips, sensitivity is likely to be similar for both arrays.

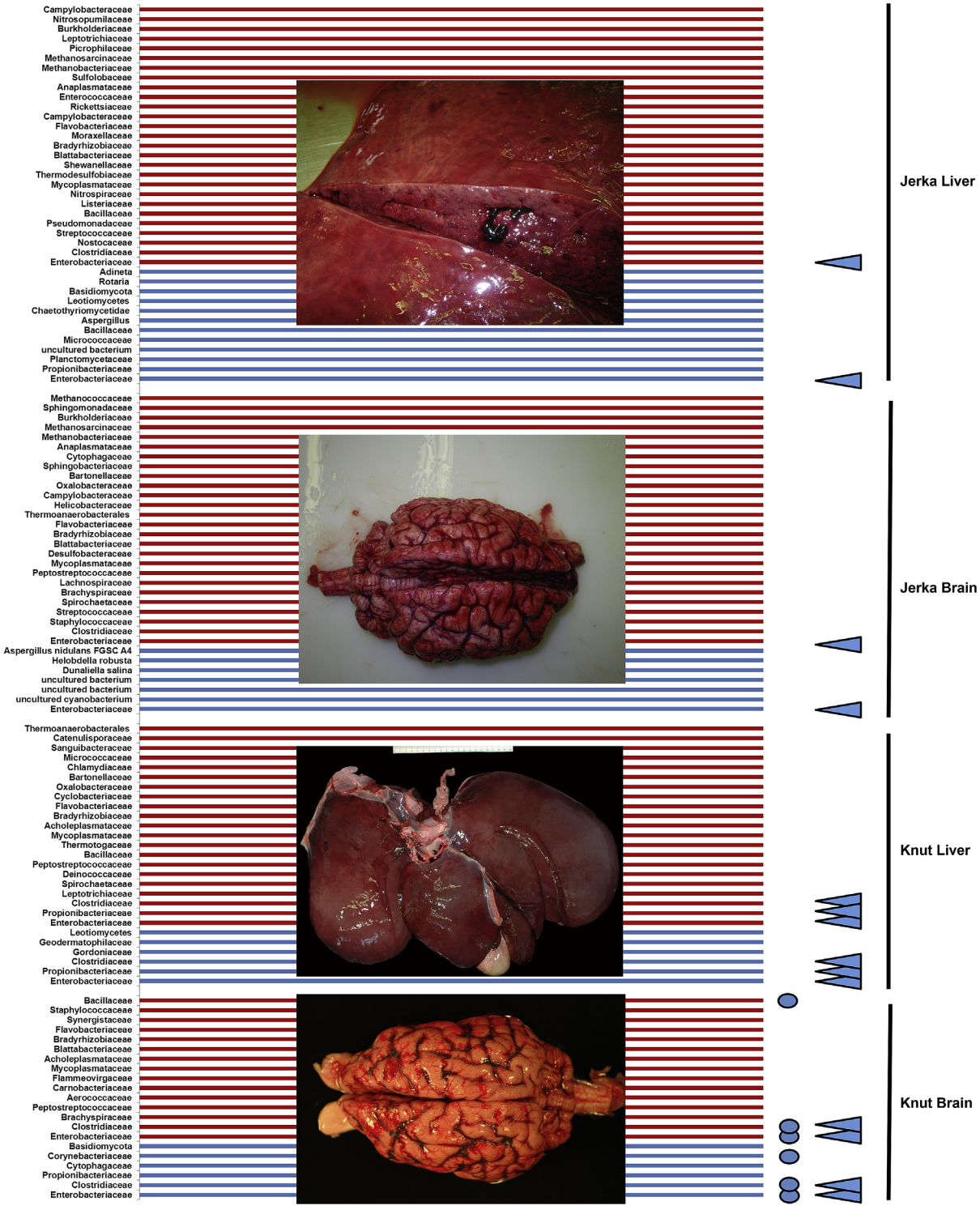

Fig. 3. Bacteria and fungi identified by shotgun sequencing and bacterial culture. Identified bacterial families are shown on the left. Tissues of origin are indicated on the right and shown by respective bear within the figure. Blue lines indicate bacteria identified from multiple hits to different parts of the genome by Illumina shotgun sequences. Red lines indicate bacterial sequences identified by GS FLX. Bacterial families found by sequencing and culture are indicated for Knut by a blue circle. Bacteria found by more than one method are indicated by a blue triangle. Details about bacterial identification are shown in Supplementary Table S2.

Both arrays were hybridized with cDNA and DNA from brain and liver from Knut and Jerka. As reported in Greenwood *et al.* (2012), the Virochip did not produce any signals above background for any of the samples tested from either polar bear. Thus, it did not detect EHV-1 DNA, which was easily retrieved by PCR from Jerka. An identical result was obtained for Biochip 5.1.

Next-generation Sequencing

DNA libraries prepared from RNA and DNA isolated from the brains and livers of both polar bears were sequenced on a GS FLX platform and yielded 83,760 reads from Knut's brain and 66,353 from Knut's liver and 66,952 reads from Jerka's brain and 97,973 from Jerka's liver. Given that necropsy examination of Knut occurred 48 h after death, viral enrichment was not performed as it was considered likely that host and viral degradation would have reached a point where removal of host RNA and DNA would also remove viral nucleic acids. Data analysis was performed by a workflow, which is currently under development. The workflow combines various alignment programs and reads are classified into separate taxa based on sequence similarities. The results are documented in Figs. 3 and 4. Of note, no viral hits were observed for Knut's brain with the exception of *Cafeteria roenbergensis*, a virus that is known to infect marine invertebrates. Jerka's brain displayed reads with homology to avian retroviruses and herpesviruses and further reads

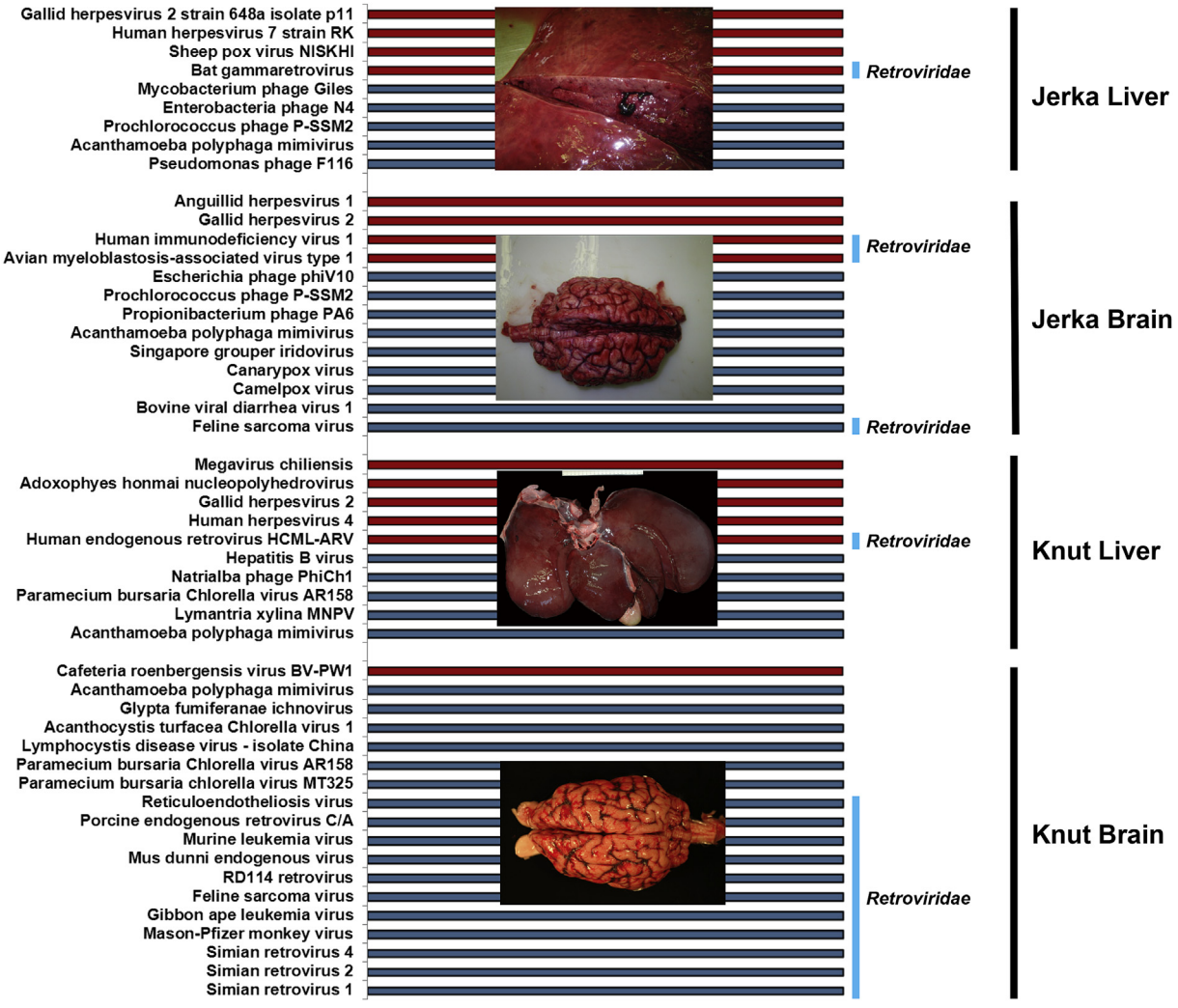

Fig. 4. Viruses identified by shotgun sequencing. Identified viral groups are shown on the left. Tissues of origin are indicated on the right and shown by respective bear within the figure. Blue lines indicate viruses identified from multiple hits to different parts of the genome by Illumina shotgun sequences. Red lines indicate viral sequences identified by 454 FLX. Retroviruses, in contrast to other viral groups, were identified consistently and are indicated next to their respective lines. Details of viral identification are shown in Supplementary Table S2.

with homology to herpesviruses were also detected in Jerka and Knut's livers. Various reads with limited homology to several viruses (*Megavirus chilensis*, human endogenous retrovirus, avian myeloblastosis-associated virus, human immunodeficiency virus and a few gallid herpesviruses) were identified in the liver reads from Knut. All reads showing homologies to viral sequences were BLASTn searched against a sequencing database generated from all previously performed 454 sequencing runs. As no significant homologies were found, contamination during library preparation, emulsion PCR and sequencing are unlikely to be the source and the sequences therefore very likely originate from nucleic acids derived from the bears.

A second approach was shotgun Illumina (HiSeq 2000) sequencing of cDNA from the same tissues after partially normalizing the libraries for ribosomal RNA, which makes up the largest fraction of all expressed RNAs. In contrast to the GS-FLX, the Illumina reads are much shorter (100 bp), but coverage is much higher than with the GS-FLX (hundreds of millions of sequences as opposed to tens of thousands). The resulting data were curated by subtracting the draft genome sequence of the polar bear (Li *et al.*, 2011). Reads were aligned using the BLASTn algorithm and sequences aligning with ≥90% identity over 90 bases were subtracted. Sequences were similarly filtered using databases of bear ribosomal RNA and mitochondrial sequences. Low complexity sequences were also removed from further analysis (Welch, 1984). The numbers of reads remaining after filtering were 4,552,863 and 5,156,929 for Knut's brain and liver and 4,312,537 and 2,833,574 for Jerka's brain and liver, respectively. Several methods were employed to search for possible pathogen-derived sequences. Firstly, the filtered reads were searched against the NCBI nucleotide (nt) database using the BLASTn algorithm and an *E*-value cut off of 1×10^{-8} (Fig. 4). No virus sequences were identified; however, the relatively short read length (100 bp) may have limited the sensitivity of this strategy. Secondly, the reads were searched against databases of viral protein sequences using the BLASTx algorithm. Matches with an *E*-value <0.001 were further analyzed. Each read was assigned to a virus species based on its best match and viruses with multiple non-overlapping hits were determined. Several putative viral hits were identified (Fig. 4).

Given the possibility that short single reads hamper pathogen identification by BLASTn methods, a de-novo assembly of the reads was performed. Reads that were not excluded by filtering for polar bear genomic DNA were assembled into contigs with Velvet (Zerbino and Birney, 2008). The assembly resulted in 3,079,082 and 210,374 contigs for the samples from Knut's brain and liver, respectively, and 919,464 and 83,602 contigs for the samples from Jerka's brain and liver, respectively, with an N50 score of 132, 17, 100 and 15, respectively. Contigs longer than 200 bp were selected and a BLASTn search against the NCBI nucleotide (nt) database was performed. Most of the contigs gave matches to sequences from mammals and therefore most likely originated from the polar bears. Microorganism sequences identified by this approach are summarized in Fig. 3.

The different sequencing methods overlapped poorly in the viral hits identified with a few exceptions at the viral group level. Hits to various gammaretroviruses and betaretroviruses were observed in the tissues from both polar bears, particularly with Knut's brain sample, which had a somewhat better enrichment in the shotgun sequencing. Obtaining longer sequences of the betaretrovirus by PCR and querying the draft polar bear genome revealed that a novel polar bear endogenous retrovirus had been identified. A similar result was obtained for the betaretrovirus identified in Jerka's liver. The betaretrovirus has been described in detail as a newly discovered bear retrovirus elsewhere (Mayer *et al.*, 2013). These results reveal that viral sequences were detected from the polar bear samples by next generation sequencing methods that were of bear origin. In terms of overall virus similarity, retroviruses and herpesviruses were identified in both datasets even though the specific matches differed. None were specific to the brain samples and none to Knut's brain. These overlapping viral groups are likely to represent species-specific viruses of polar bears. Notably, none of the methods retrieved the recombinant EHV-1 that was identified by PCR from Jerka. Thus, sensitivity is a critical issue for samples that cannot be enriched for virus particles prior to analysis irrespective of the high throughput method applied.

The bacterial results obtained by different methods were more consistent than those for viruses as shown in Fig. 3. Enterobacteriaceae and Clostridiaceae were consistently found by both sequencing approaches and by bacteriological analysis. Similarly, Bacillaceae and Corynebacteriaceae were found by sequencing and by bacterial culture. However, the only bacterium exclusively identified in Knut's brain, but not Jerka's brain, was *Propionibacterium acnae*, a species usually associated with topical acne.

Serology and Defined Immunohistochemistry

Blood from an unrelated polar bear (Anton) was frozen and thawed to mimic the state of Knut's whole

blood. Serum from Anton was also available. Assay interference from whole blood versus serum could therefore be controlled in serological assays. Knut had been vaccinated against CDV at 1 year of age and thus had a low serum neutralizing antibody titre to this virus (not shown). All other tests were negative, except for the influenza ELISA (ID Screen® Influenza A Antibody Competition ELISA kit, ID-vet). This assay was run repeatedly and was positive for the influenza nucleoprotein (NP) in Knut's blood only. Whole blood samples from Anton and Jerka were negative. Thus, we concluded that the positive result from Knut was not caused by non-specific reactions of whole blood in the assay. In contrast, a second NP assay from a different company (IDEXX Influenza A AB test) was negative. Knut's blood also tested positive in an ELISA assay targeting influenza virus N1 (ID Screen® Influenza N1 Antibody Competition ELISA kit, ID-vet). Results from HI assays were inconclusive because all bear blood and serum samples showed non-specific inhibition of haemagglutination, suggesting that there may be a factor in bear blood and serum that generally interferes with such assays. A follow-up influenza-specific IHC antigen test on brain sections with particularly strong inflammation was negative (Nicholls *et al*., 2012). The test used a commercially available monoclonal antibody HB65 (European Veterinary Laboratory, Woerden, The Netherlands) specific for the NP protein of influenza A virus. The test was controlled with lung sections from pigs infected experimentally with influenza A virus, which invariably tested positive. In summary, evidence for the presence of influenza A antigen was consistently negative, although antibodies against influenza A NP and N1 were detected in assays using Knut's samples, but not in samples from the other polar bears tested.

Discussion

During 2010 and 2011 there were three documented cases of encephalitis in captive polar bears in two zoological collections, including the related bears Lars and Knut. Additional cases of fatal encephalitis in polar and black bears have been reported previously (Schrenzel *et al*., 2008; Wohlsein *et al*., 2011), but definitive diagnosis in such cases is complicated by the extensive list of potential causative pathogens.

The extensive effort made to determine the cause of the observed encephalitis in these bears was required because of the gap in knowledge of polar bear diseases specifically and wildlife diseases in general. The analysis undertaken, however, revealed the strengths and weaknesses of current methodologies when applied to wildlife, but also helped us to establish a pipeline that could be successful in achieving a diagnosis, even in problematic cases. It became apparent that the current state of the art, advanced as it has become, is still not able to provide unequivocal diagnosis in all cases. Each stage of the pipeline was designed to identify one or more probable or suspected candidates for further study while ruling out others. Comparing the results for Jerka (infection with recombinant EHV-1) with those for Knut (no conclusive pathogen) is instructive for a prediction on how such a 'species-blind' pipeline would perform in other cases.

Initial necropsy examination and histopathological evaluation of tissues is a necessary step. In both cases, these studies lead to a likely diagnosis of viral encephalitis. Eosinophilic inflammation suggesting sodium chloride poisoning was observed, but such poisoning is associated with cerebral oedema and lamellar degeneration (Zachary, 2007), neither of which were detected. No parasites were identified, although parasites such as *Acanthamoeba* spp. could not be conclusively ruled out. Interestingly, mimiviruses specific to *Acanthamoeba* spp. were among the viral reads obtained for Knut, although PCR and shotgun sequencing were negative for the parasite. As Knut was in water for an extended period before pathological examination, the DNA could have been a contamination from the water. The pathology was inconsistent with a bacterial cause in the case of Knut. Additionally, bacteria isolated from the brain during bacterial investigation comprised only components of the normal flora and the inhaled water of the enclosure. Although Jerka's brain was not specifically investigated by bacteriological diagnosis, no subsequent evidence was found for a bacterial cause of death. Histology was negative for multiple pathogens. Thus, the end result of the first stage of the diagnostic procedure in both cases was an equivocal diagnosis of virus infection.

Molecular diagnostics have developed rapidly from PCR-based approaches to microarray-based detection to next-generation sequencing. Each method has benefits and drawbacks that have prevented any one from completely supplanting the others. PCR is extremely sensitive, but prone to false-negative results in the case of primer and target mismatch, a particularly likely situation for novel viruses or divergent viral strains. PCR is also prone to false-positive results where an appropriate product is detected, but on sequencing turns out to be an artefact. This was observed in 25% of the cases with PCR amplicons obtained from the samples from these polar bears. This increased the workload considerably as each false-positive band must be sequenced before it can be discarded.

In principle, microarrays suffer less from the possibility of false-positive and -negative results since the

longer oligonucleotides must be homologous over a longer stretch of nucleotides and, are therefore, less prone to mismatches. However, the conditions for hybridization are not easily optimized in practice and sensitivity may be substantially reduced. This has been observed when results from microarray analyses of enriched viral particles from multiple samples were compared with those of next generation sequencing. In such a comparison, the latter method detected viruses in 30% of the samples and the former in 8% of samples (Yozwiak *et al.*, 2012). The problem is likely to be exacerbated for solid tissue samples that cannot be enriched for viral particles, as was the case for tissues from both bears.

Next-generation shotgun sequencing either from enriched sources or directly from infected tissue can generate hundreds of millions to billions of individual sequence reads. The drawback of such sequencing without prior pathogen enrichment is that the majority of obtained sequences will be host derived (over 99% for Knut and Jerka). In the case of Jerka, both high-density microarray and next-generation sequencing-based methods failed to identify the recombinant EHV-1 eventually obtained with the help of PCR (Greenwood *et al.*, 2012). None of the three methods identified candidate pathogens in Knut, but interestingly, a divergent polar bear-derived betaretroviruses were identified in both Knut and Jerka (Mayer *et al.*, 2013), suggesting that the methods are able to identify previously unknown viral sequences. In combination, the three methods clearly are more powerful than any single method alone. In the case of Jerka, PCR, microarray and next-generation shotgun sequencing combined together identified the recombinant EHV-1 virus that was the likely cause of death, while excluding other potential viral, bacterial and parasite pathogens (Greenwood *et al.*, 2012). The uniform negative results for a candidate causal pathogen from Knut suggest that the genome of the pathogen was most likely no longer present. This may indicate either a 'hit-and-run' infection, post-mortem degradation of DNA/RNA or pathogen concentration below the sensitivity levels of all the methods employed.

Serum neutralization is one of the gold standards of pathogen diagnostics. However, it is limited by the fact that only a small number of viruses can be maintained in culture and is not sensitive in all cases. ELISA can be effective, but can lack both sensitivity and specificity, yielding false-positive and -negative results. For viruses that induce haemagglutination, HI assays can serve as confirmatory tests for SNT or ELISA results. However, as demonstrated in this study, polar bear blood and serum may interfere with this assay, rendering its outcome inconclusive. HI assays failed, regardless of viral strain (H1 and H5 tested) and Knut was serologically negative for all viruses that could be tested by SNT. It is unclear why HI assays failed in all of the polar bear samples, but the results again illustrate that there may be unknown properties of serum in specific wildlife species, which can interfere with assays that are standard for non-wildlife species.

The results of the diagnostic pipeline presented here resulted in the identification of a detectable antibody response against influenza A virus that was restricted to Knut and no other polar bear tested ($n = 6$), although no confirmatory genomic evidence was obtained. Influenza is known to instigate a cascade of cytokine effects that may persist and cause death after the virus has been cleared (Yamada, 1996; Jang *et al.*, 2009). Therefore, infection of Knut with influenza virus(es) is not unrealistic given the broad host range of influenza in general, which includes bears (Cretescu *et al.*, 1970) and the common occurrence of waterfowl, both migratory and resident, in zoos. Whether the infection was long ago, recent or coincident with the encephalitis is unclear.

In summary, the present study has shown that the diagnostic tools currently available are powerful, but also that there are still major technical problems in wildlife disease diagnostics, which cannot necessarily be overcome even if all existing techniques are applied. Promising new methods include solution hybridization capture-based methods coupled with next-generation sequencing, which allows for strong enrichment of specific target sequences, but without the introduced biases of PCR (Maricic *et al.*, 2010). Such methods have been highly effective for degraded samples (e.g. ancient DNA) and have permitted the identification of pathogens in samples that are hundreds of years old (Schuenemann *et al.*, 2011). However, their diagnostic potential in wildlife disease remains to be investigated.

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Conflict of Interest Statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcpa.2013.12.005.

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