PCR-based investigation of the presence of herpesvirus in the peripheral vestibular system in cats and dogs

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For my mother and my sister

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Please note that the consecutive numbering of the figures and tables only applies to the scientific background. In papers 1 to 3 the layout of the text and the numbering of the figures and tables is in accordance with the editorial guidelines of the respective journals.

Abbreviations

bp	base pair
CHV-1	Canine herpesvirus 1
CN	cranial nerve
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ELMI	electron microscopy
FCV	Feline calicivirus
FHV-1	Feline herpesvirus 1
HSV-1	Herpes simplex virus type 1
IFN	interferon
IHC	immunohistochemistry
ISH	in-situ hybridization
kbp	kilo base pairs
LATs	latency-associated transcripts
LMU	Ludwig-Maximilians-Universität München
MLV	modified live virus vaccine
N.	nervus
NLV	non-live virus vaccine
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse-transcription PCR
TG	trigeminal ganglion
VG	vestibular ganglion
VL	vestibular labyrinth

1. Introduction

The vestibular labyrinth is the organ for sensation of equilibrium. Being part of the inner ear, it is located in the caudodorsal aspect of the temporal bone [83; 151]. The peripheral compartment of the vestibular system consists of the vestibular labyrinth, the vestibular portion of the vestibulocochlear nerve and the vestibular ganglion [49; 144; 212]. Disruption of any part of this signal chain may cause peripheral vestibular dysfunction.

In humans, morphological alterations of vestibular inner ear structures suggestive of herpesvirus infections were demonstrated in patients suffering from various vestibular diseases [68; 70-72]. Moreover, herpesvirus infections in the human vestibular system have been detected by using different molecular tools [9; 10; 61; 210; 230]. Members of the herpesvirus family display a marked neurotropism and have the ability to establish lifelong latency in the nervous system [21; 55; 64]. Distress and immunosuppression may cause virus reactivation and replication with subsequent host cell damage, potentially leading to clinical deficits [21; 64; 107]. Suchlike reactivation within the vestibular ganglion is discussed to cause various recurrent human vestibulopathies [61; 65; 68].

Even though, vestibular diseases in cats and dogs are common [144; 212] and herpesvirus infections show a high prevalence in these species [50; 75], vestibular inner ear structures have not been investigated for a possible herpesvirus infection so far.

Comparable to humans [140], vestibular inner ear structures in cats [116] and dogs [36; 41] are very difficult to access and technical processing is challenging and time consuming. Therefore, only few morphological [6; 39; 62; 103; 143; 175; 187] and immunohistochemical [41] investigations have been performed so far while molecular analyses of the vestibular labyrinth and ganglion in cats and dogs have not been reported.

The aim of the present study was to evaluate, if vestibular inner ear structures of dogs and cats can be infected by herpesviruses. For this purpose, first a method for preparation of vestibular inner ear samples from cats and dogs had to be established, allowing for subsequent performance of PCR-based analyses.

2. Scientific background

2.1 Alpha-herpesviruses

2.1.1 General view

2.1.1.1 Architecture of the herpesviruses

Based on their architecture, herpesviruses can readily be distinguished morphologically from all other viruses [47; 181]. A typical herpesvirion ranges from 120 to nearly 300 nm in size, consists of a core with a linear, double-stranded DNA genome of 125-290 kbp, an icosahedral capsid, a tegument and an envelope [47; 180-182] (Fig. 1). The capsid approximates 100 to 110 nm in diameter and includes 162 (150 hexameric and 12 pentameric) capsomeres [55; 180; 181]. The tegument consists of proteinacous matrix and is enclosed within the envelope from which numerous spikes, resembling virus specific glycoproteins, project outwards [47; 180; 181].

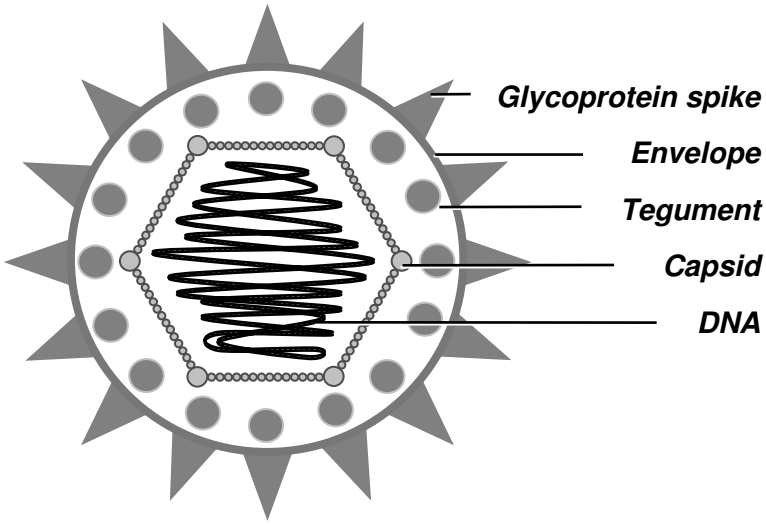


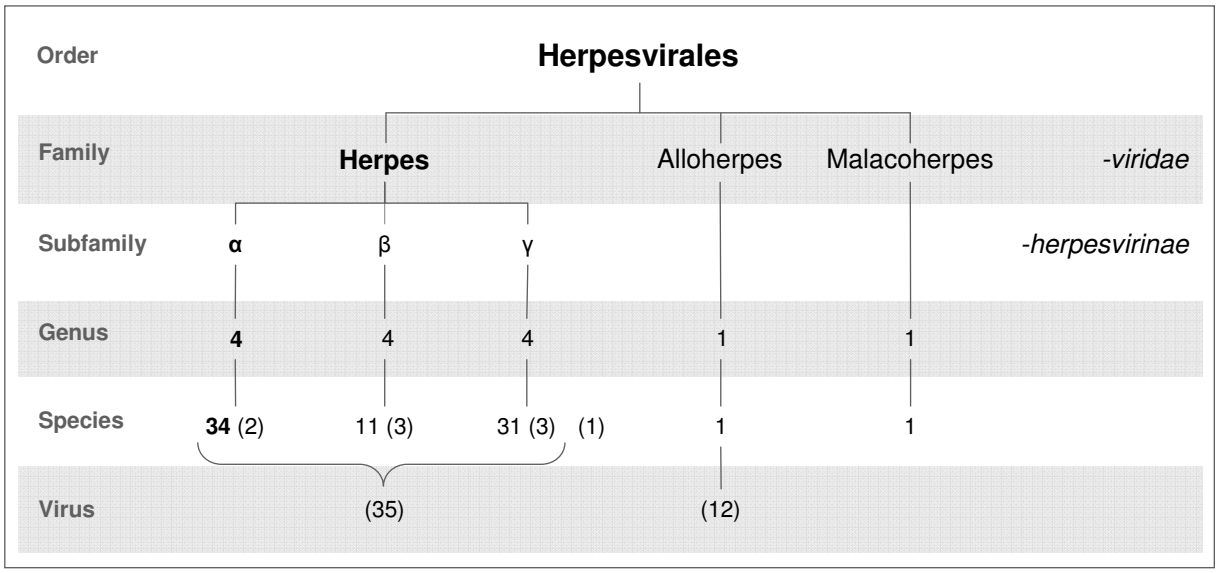
Figure 1.
Structural characteristics of a herpes virion.
A typical herpes virion consists of an envelope with different glycoproteins, a capsid and a double-stranded DNA genome. The tegument contacts both the envelope and the capsid *[by courtesy of Dr. A. Blutke]*.

2.1.1.2 Herpesvirus taxonomy

According to the latest update by the International Committee on Taxonomy of Viruses, the former family *Herpesviridae* consists of three families, which constitute the new order *Herpesvirales* [47]. The family *Herpesviridae* includes mammal, avian and reptile viruses whereas the new families *Allo- and Malacoherpesviridae* comprise fish and frog, and a bivalve virus, respectively [47] (Table 1).

By means of biologic properties and genetic background, the family *Herpesviridae* is grouped into three subfamilies (α, β, γ) which contain several genera [47; 108; 180; 181] (Table 2). Since feline and canine herpesvirus are alpha-herpesviruses [47], this subfamily will be discussed in the following.

Table 1: Current taxonomy of the order *Herpesvirales*.



[Data according to Davison et al. (2009), Arch Virol **154:**171-177]. Numbers given in brackets indicate yet unassigned species and viruses in the subfamily and family.

Table 2: Current taxonomy of the subfamily *Alphaherpesvirinae*.

Subfamily	Alphaherpesvirinae			
Genus	**Varicellovirus**	Simplexvirus	Mardivirus	Iltovirus
Species	Bovine herpesvirus 1,5	Ateline herpesvirus 1	Columbid herpesvirus 1	Gallid herpesvirus 1
	Bubaline herpesvirus 1	Bovine herpesvirus 2	Gallid herpesvirus 2,3	Psittacid herpesvirus 1
	Canid herpesvirus 1	Cercopithecine herpesvirus 2	Meleagrid herpesvirus 1	
	Caprine herpesvirus 1	Human herpesvirus 1,2		
	Cercopithecine herpesvirus 9	Macacine herpesvirus 1		
	Cervid herpesvirus 1,2	Macropodid herpesvirus 1,2		
	Equid herpesvirus 1,3,4,6,8,9	Papiine herpesvirus 2		
	Felid herpesvirus 1	Saimiriine herpesvirus 1		
	Human herpesvirus 3			
	Phocid herpesvirus 1			
	Suid herpesvirus 1			

[Data according to Davison et al. (2009), Arch Virol **154:**171-177]. Chelonid herpesvirus 5 and 6 are yet unassigned species in the subfamily.

2.1.1.3 Alpha-herpesvirus characteristics

Alpha-herpesviruses are widespread amongst humans, domestic and wild animals with seroprevalences greater than 90% in some species [47; 55; 68; 107; 176]. They display a variable host and cell range with a wide or narrow spectrum, respectively [33; 182]. The pseudorabies virus, for example, has a wide host range and infects pigs, ruminants, dogs, cats, etc., whereas Feline herpesvirus-1 (FHV-1) and Canine herpesvirus-1 (CHV-1) are restricted to the *Felidae* or *Canidae* family [50; 75; 182]. Alpha-herpesviruses are characterized by a relatively short productive cycle, rapid spread in culture with destruction of infected cells and the ability to establish lifelong latency primarily, but not exclusively, in sensory ganglia [107; 180; 181]. Alpha-herpesviruses are neurotropic viruses with affinity for neural structures [139]. They may cause subclinical or severe generalized disease and diseases confined to the nervous system [21; 50; 64; 75; 117; 182]. Humoral, and notably cell-mediated immunity are crucial to prevent extensive virus dissemination and lethality [85; 106; 117; 165; 182; 195].

A variety of murine models, thus, demonstrated the importance of various T-cells in the clearance of infectious virus from peripheral and neural sites [84; 85; 106; 194; 195].
After primary infection of the mucosal epithelium, virus may enter nerve terminals therein and translocate to sensory ganglia at a rate of ~1.8 mm/h using the retrograde axonal transport [21; 35; 64; 106; 142; 195; 210]. A productive cycle with lysis of infected ganglion cells does not necessarily occur; instead, latency may follow infection [21; 150].

2.1.1.4 Latency

Latency is described as a reversible non-productive infection of a cell by a replication-competent virus which can be reactivated following different types of distress [21; 64; 107]. During latency, viral DNA exists in an unintegrated and circular form primarily within the nerve cell nuclei [21; 33; 107; 155]. While Herpes simplex virus type-1 (HSV-1) [21; 33; 196], FHV-1 [155] and CHV-1 [142] have been demonstrated in peripheral ganglion cells, Varicella-zoster virus genome was also detected in satellite cells during latency [21; 33; 120; 230].
Estimates both in humans and animals propose that 0.01%-10% of ganglion cells may be latently infected, with 10-1000 copies of virus genome per neuron [21; 33; 60; 142; 155; 193; 196]. The number of infected ganglion cells and the copies of virus genome therein has been suggested to correlate with the probability and the frequency of reactivation [21].
During latency, transcription seems to be restricted to latency-associated transcripts (LATs) which therefore provide a molecular marker for latency [21; 107; 213]. Detection of viral DNA via polymerase chain reaction (PCR) does not permit a differentiation between infectious virus and latent infection [21]. Demonstration of LATs, using reverse-transcription PCR (RT-PCR) [21; 210; 213] or in-situ hybridization (ISH) [21; 155; 210], however, indicate true latency. Consequently, even low-level persistent infection may be distinguished from latent infection [21].
Moreover, latent virus may be detected with explant and co-cultivation techniques [21; 77; 146], whereas common virus isolation [11; 24; 136; 204] and immunohistochemistry (IHC) [24; 136; 142], for detection of viral antigen, can be used for identification of infectious virus.

2.1.1.5 Herpesvirus reactivation

Once latency has been established, reactivation of latent virus may occur following different types of distress or immunosuppression [21; 75]. Reactivated virus travels from its site of latency back to its entrance zone resulting in further virus replication, shedding and transmission to susceptible hosts, as well as destruction of infected cells with possible recrudescence of clinical signs [21; 64; 75; 182].

Herpesvirus reactivation in humans is implicated in a great variety of diseases including recurrent vestibulopathies, (e.g. vestibular neuritis [65; 68; 72], benign paroxysmal positional vertigo [65; 68; 71], Ménière's disease [65; 68; 70; 220], cochleovestibular signs in Ramsay-Hunt-Syndrome [61; 130; 156]), herpes simplex labialis [182], conjunctivitis [182], keratitis [182], shingles [33; 182], Bell's palsy [69; 189], trigeminal neuralgia [182] and encephalitis [32; 33]. FHV-1 reactivation from the trigeminal ganglion is thought to cause conjunctivitis, keratitis and rhinitis in cats [75; 211] and CHV-1 reactivation corneal ulcers in dogs [125].

2.1.1.6 Antiviral therapy

Antiviral therapy against alpha-herpesviruses has been used in humans for some decades now. Even though clinical signs and virus shedding is reduced and recrudescence of clinical signs may be suppressed, neither the establishment of latency can be prevented with early antiviral therapy nor eradication of herpesvirus genome from latently infected cells will be achieved with today's antiviral compounds [55; 211].

To date, the key protein target for antiviral therapy against alpha-herpesviruses is mainly the virus encoded DNA-polymerase [55; 99]. During latency, however, the virus does not express this gene. Thus conventional nucleoside analogues do not affect latent virus [55]. Development of successful antiviral therapies in human medicine is largely based on experimental work, using laboratory animals as in-vivo infection models. However, our domestic animals had little benefit from these experiments so far [55].

In cats, different nucleoside analogues (e.g. trifluridine, idoxuridine, ganciclovir, acyclovir, penciclovir, cidofovir, valaciclovir and famciclovir), feline IFN-ω, human IFN-α, L-lysine and lactoferrin have been proposed for the treatment of FHV-1-associated ocular disease [57; 75; 100; 137; 211]. While in vitro efficacy has mostly been established, in vivo trials are still missing for the majority of these drugs [211].

Besides, systemic application of nucleoside analogues in cats has been problematic so far in terms of poor in-vivo-efficacy against FHV-1, poor bioavailability or toxic side effects such as bone marrow suppression, renal and liver necrosis [5; 55; 57].

The efficacy of lactoferrin has been tested in-vitro, and it is thought to protect against CHV-1 infection in-vivo. Apart from lactoferrin, there is no other antiviral therapy, which has been tested in dogs [55; 208].

2.1.2 Survey on FHV-1 and CHV-1

2.1.2.1 FHV-1

2.1.2.1.1 Taxonomy and prevalence

FHV-1 is a member of the Varicellovirus genus of the herpesvirus subfamily *Alphaherpesvirinae* and is closely related, genetically as well as antigenically, to CHV-1 [47; 75; 186]. FHV-1 infects felids and it is widespread amongst cat populations [76; 171; 199]. All isolates of FHV-1 belong to one single serotype and are genetically fairly homogenous [75; 97; 231].

2.1.2.1.2 Epidemiology and pathogenesis

Virus is shed in ocular, nasal and oral secretions and is mainly transmitted by close contact with an infected cat [18; 81; 171; 199]. Since virus is short-lived in the environment and is inactivated by common disinfectants, indirect transmission is not thought to play a major role in virus transmission [75; 171; 199] .

Nasal, oral and conjunctival mucous membranes are natural sites for infection, whereas vaginal and transplacental infections have only been shown experimentally [19; 75; 96]. After infection, virus replication primarily takes place in the mucosae of the nasal septum, the turbinates, nasopharynx and tonsils, however, the conjunctivae, mandibular lymph nodes and upper trachea are often involved, too [75; 80]. Viremia and generalized disease may occur in neonatal kittens or in debilitated animals. Nevertheless it appears to be rare, since viral replication preferentially occurs at lower body temperatures like in the respiratory tract [75; 197].

After infection, more than 80% of the diseased and recovered cats become latent virus carriers [79; 80; 221]. FHV-1 is thought to be transported retrograde from peripheral infectious sites to neurones of the trigeminal ganglion (TG) [155; 223]. Even though viral DNA has been detected in a wide range of tissues, true latency has only been suggested for the TG so far [77; 146; 155; 213].

Viral reactivation with or without recrudescence of clinical signs may occur spontaneously or within a three-week period following a stressful event such as re-housing, pregnancy, lactation or corticosteroid treatment, thus, transmitting virus to cats in a new environment or from the queen to their kittens [75; 78; 79; 134].

2.1.2.1.3 Clinical signs

Infection with FHV-1 causes severe upper respiratory tract disease in susceptible animals [44; 75]. Early clinical signs of disease may include depression, marked sneezing, inappetence, pyrexia and excessive salivation with drooling [44; 75]. Conjunctivitis, corneal ulcers, serous to mucopurulent ocular and nasal discharge as well as dyspnoea and coughing in severely affected cats may follow [44; 75; 171]. Oral ulceration may occur with FHV-1 infection, but is more commonly caused by feline calicivirus (FCV) [170; 171]. Generalized infection and primary viral pneumonia are occasionally encountered especially in young or debilitated animals, whereas neurological signs as a sequel to infection are rare [75; 76; 82].

Although in-utero transmission, abortion and congenitally-infected kittens have been reported after experimental FHV-1 infection of pregnant queens, reproductive disease does not seem to be clinically relevant under natural conditions [75; 76; 218]. Moreover, FHV-1 is thought to be involved in different recurrent and/or chronic conditions, such as conjunctivitis, ulcerative and stromal keratitis, corneal sequestrae, keratoconjunctivitis sicca [5; 134; 145; 147-149; 200; 201], ulcerative dermatitis, gingivostomatitis and rhinitis [88; 95; 174].

2.1.2.1.4 Vaccination

Vaccination against FHV-1 has been used for a number of years to control disease [17; 161]. FHV-1 vaccines are used to reduce the severity of disease, however, they neither prevent infection nor the development of a carrier state [119; 199]. There are modified live (MLV) and non-live (adjuvanted inactivated) virus vaccines (NLV), both invariably given in association with FCV [75; 122; 199]. MLV and NLV are usually administered parenterally and intranasal MLV are also licensed in some countries [75; 122; 199]. FHV-1 vaccines are generally safe, however, transient signs like fever, sneezing, nasal and ocular discharge, conjunctivitis and ulceration of the nasal philtrum may occur in some cats [75; 122; 178]. Intranasally applied MLV can become latent [75; 221], whereas the situation with parenteral MLV is still unclear [75; 203].

Despite possible respiratory signs, intranasal MLV vaccine is advocated for use in young kittens and in conjunction with parenteral vaccine on entry into shelters since it induces rapid protection [75; 122]. On the contrary, conventional MLV inadvertently administered via the oro-nasal route induces disease [75; 119].

After primary immunization and the first annual booster, annual vaccination is generally recommended in the EU and triannually in the USA, however, with individual risk-benefit assessment for a particular animal [48; 75; 211].

2.1.2.1.5 Diagnosis

The diagnosis of primary FHV-1 infection in kittens is commonly based on characteristic clinical signs, whereas chronic or recurrent conditions in older cats possibly related to FHV-1 infection are more difficult to interpret [134; 199]. Since direct and indirect diagnostic tests only aid in the diagnosis of FHV-1 associated disease due to the occurrence of healthy carriers and the widespread use of FHV-1 vaccines, test results should be critically questioned and always evaluated in conjunction with clinical signs [17; 24; 75; 199; 204].

Serum antibody tests are positive in most cats with varying titers as a consequence of the widespread use of vaccines and possible previous infection and hence are not significant [136; 199]. Given that there are no commercially available marker FHV-1 vaccines, positive PCR results for example could imply vaccine or field-virus [75; 135; 203; 221]. Furthermore, the detection of viral DNA could resemble FHV-1 as a causative agent of the disease, a consequential or coincidental finding [24; 134; 135]. Negative results of direct diagnostic test, on the other hand, either demonstrate the absence of FHV-1 or the limitations of some tests. Additionally, under chronic conditions, viral antigens may be bound by secreted antibodies which impede the binding of the primary IHC antibody and, therefore, may lead to false negative results [134].

2.1.2.2 CHV-1

2.1.2.2.1 Taxonomy and prevalence

CHV-1 belongs to the Varicellovirus genus of the alpha-herpesvirus subfamily and infects canids [47; 50]. Amongst dogs CHV-1 is distributed worldwide with seroprevalences ranging as high as 94% [1; 16; 118; 138; 152; 176; 179; 184; 206].

2.1.2.2.2 Epidemiology and pathogenesis

Since CHV-1 is quickly inactivated in the environment, direct transmission via oronasal and veneral secretions are the main routes of infection [43; 50; 158; 183]. In contrast to FHV-1, in utero-transmission and reproductive disorder is common and poses a severe problem with high economic losses to breeding units [75; 90; 92; 101; 172; 185]. Infection of pregnant bitches with CHV-1 may cause abortion, resorption or mumification of the fetus, stillbirth or weak pups which seem normal at parturition but will die within a few days [90-92; 168; 185]. Dams develop protective immunity after infection, and pass maternal antibodies on to the litter thus protecting the following generation of pups from fatal CHV-1 related disease [50; 101]. Neonatal pups may also be infected with CHV-1 during passage through the birth canal or via infected oronasal secretions of other dogs [43; 129].

Several studies demonstrate that virus grows best at temperatures between 35°C and 37°C, resembling the normal body temperature of pups during their first two weeks of life, whereas virus formation is largely depressed at the average temperature of 39°C of adult dogs [11; 27; 28; 101; 132]. The inability of neonates to adequately regulate their body temperature and their naïve immune system benefits viral growth and favors severe virus replication and viremia [28; 101; 165]. Thus, initial replication of CHV-1 in the oropharyngeal mucosa is followed by hematogenous virus dissemination to the liver, kidneys, lymphatic tissues, lungs and CNS [43; 101; 226]. If maternally derived antibodies are missing litter mortality may reach 100% in neonatal puppies, whereas puppies older than two weeks of age usually remain asymptomatic after CHV-1 infection [50; 101].

Infection with CHV-1 is thought to be followed by a latent carrier state [26; 50; 142; 157; 158]. Latency of different alpha-herpesviruses has been subjected to advanced research for many years, whereas data on CHV-1 latency is still rudimentary [22; 32; 73; 84; 87; 150; 155; 193; 196; 209; 213; 229].

Burr and coworkers investigated twelve key sites associated with latency for other herpesviruses and detected CHV-1 DNA in nine out of twelve dogs with no history of exposure or illness from CHV-1, using PCR [26]. Tissues most commonly affected included lumbo-sacral ganglia, tonsils, parotid salivary glands and liver [26]. Miyoshi and coworkers aimed to determine sites of latency in convalescent dogs that had been experimentally infected with CHV-1 by the intravaginal, intranasal and/or intravenous inoculation route using PCR, ISH, and IHC [142]. PCR showed that TG and retropharngeal lymph nodes were most frequently infected with CHV-1 and ISH demonstrated virus genome in the nuclei of ganglion cells and in lymphocytes [142]. Antigen on the other hand could not be detected in any tissue sections via IHC [142]. Even though the studies of Burr and Miyoshi suggest CHV-1 latency in neural and non-neural tissues of dogs, molecular markers are still missing to confirm true latency. Latent carriers are epidemiologically important, since they may experience episodic viral reactivation with subsequent viral shedding with or without recrudescence of clinical signs, thus, transmitting virus to susceptible hosts [50; 157; 158].

2.1.2.2.3 Clinical signs

Based on the biology of CHV-1, clinical manifestation of an infection mainly concerns puppies. Fatal CHV-1 infection in neonates may cause vocalization, anorexia, dyspnea, abdominal pain, incoordination, soft feces, serous/hemorrhagic nasal discharge and petechial hemorrhage on the mucous membranes [30; 43; 50]. Puppies older than two weeks of age at the time of infection present with mild upper respiratory disease whereas CHV-1 infection in adult dogs is usually subclinical [7; 27; 76]. Nevertheless, CHV-1 is further associated with reproductive disorders, genital lesions, respiratory disease, and different ocular diseases such as corneal ulcers and retinal dysplasia in adult dogs [3; 50; 52; 89; 91; 94; 109; 125; 168; 172; 185].

2.1.2.2.4 Vaccination

Since 2001, there is a commercially available subunit vaccine (Eurican Herpes 205®, Merial, France) for active immunization of pregnant dams in order to prevent fatal hemorrhagic disease in neonatal puppies which is authorized in the European Union by the European Medicines Agency (EMEA).

Detailed information on this vaccine is available under: http://www.emea.europa.eu/vetdocs/PDFs/EPAR/euricanherpes/V-059-PI-de.pdf and http://www.emea.europa.eu/vetdocs/vets/Epar/euricanherpes/euricanherpes.htm.
The vaccine contains antigen of the CHV-1 glycoprotein gene B and is first administered subcutaneously either during the dam's season or within seven to ten days after mating. Since the virus is a poor immunogen and antibody titers decrease rapidly, a second injection one to two weeks before birth is necessary for optimal protection of the puppies [26; 169; 183; 185]. Thus, puppies with passive immunity derived from neutralizing antibodies in the colostrum of vaccinated dams were protected, whereas 62% of the puppies from unvaccinated dams died of fatal hemorrhagic disease when challenged with a virulent strain of CHV-1 [169].

2.1.2.2.5 Diagnosis

Diagnosis of neonatal CHV-1 infection may be based on distinct pathologic findings and include multifocal necrosis and hemorrhage in most organs (lungs, liver, brain, kidneys, and intestines), meningoencephalitis, intranuclear inclusion bodies (Cowdry Type A), and enlarged lymph nodes and spleen [30; 166; 225-227]. Even though PCR, ISH, IHC, electron microscopy (ELMI) and virus isolation may strengthen the diagnosis in puppies with classical hemorrhagic disease, the interpretation of test results in adult dogs is more challenging, since CHV-1 has been detected in a variety of tissues from healthy and diseased adult dogs [26; 29; 52; 111; 142; 190; 202]. Thus, positive test results must be interpreted in conjunction with clinical signs and carefully in terms of their significance. Future research on transcriptional level hopefully will aid in identifying the importance of these findings. Detection of antibody titers has been used in determining seroprevalences in dog populations and the presence of CHV-1 in kennels [50; 118; 138; 152; 176; 179; 184; 206]. Assessment of individual antibody titers, however, is difficult. Thus, a positive result may indicate CHV-1 infection or vaccination in breeding bitches, whereas a negative test could indicate the absence of CHV-1 infection, an early phase of infection or previous infection with a decline of antibody titres below detection limits [118; 185]. Even though titer height in paired samples gives information about current CHV-1 infection, it does not necessarily correlate with the possible presence or absence of reproductive disorders [183; 185].

2.2 Functional neuroanatomy of the vestibular system

2.2.1 Anatomy

The inner ear comprises two different special sense organs, namely the cochlea within the rostroventral and the vestibular labyrinth in the caudodorsal aspect of the temporal bone [83; 151] (Fig. 2). The cochlea detects sound waves transmitted to the inner ear thus serving as the organ for hearing, whereas the vestibular labyrinth is responsible for sensation of equilibrium [34; 86; 167].

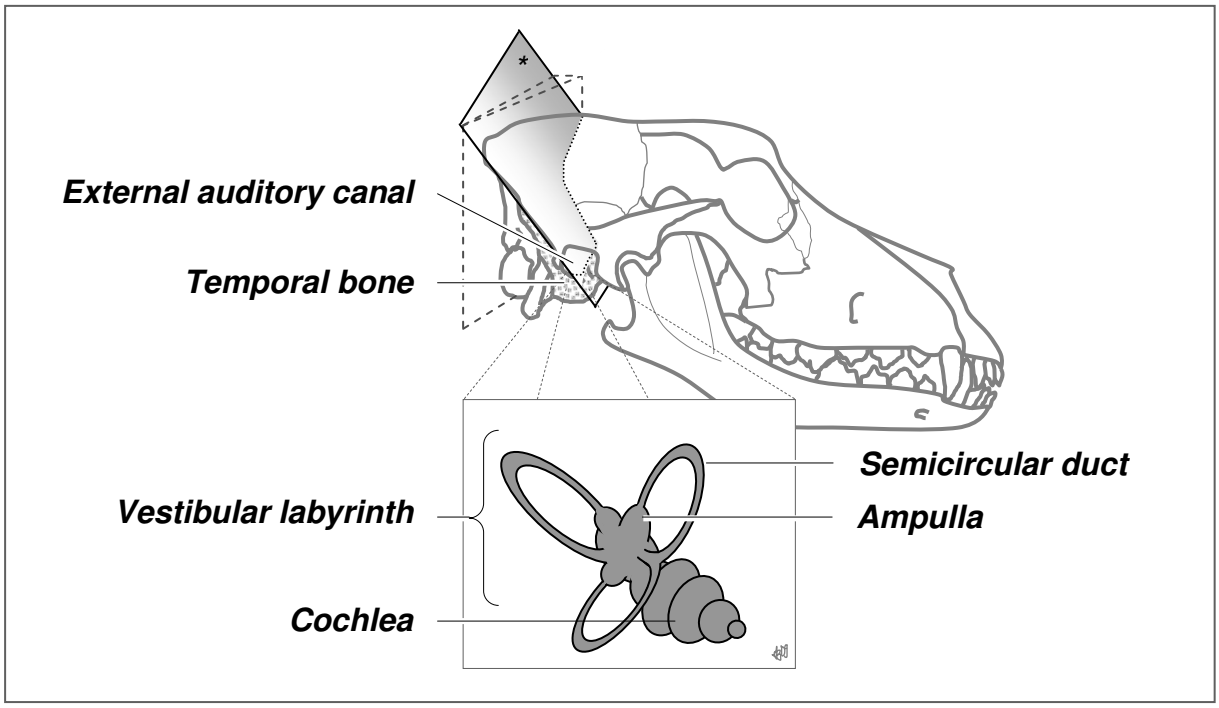


Figure 2. Location of the membranous labyrinth within a dog's skull. The asterisk indicates the sectional plane through the skull and therewith the position of the membranous labyrinth. *[by courtesy of Dr. A. Blutke]*

The inner ear is composed of a membranous labyrinth filled with endolymph, surrounded by perilymph, and enclosed within the compact cortical bone of the osseous labyrinth [83; 86; 151] (Fig. 3).

The endolymph, produced by the stria vascularis of the cochlea and by vestibular dark cells, is high on potassium and low on sodium ions and resembles intracellular fluid, whereas the perilymph has a contrary electrolyte composition and is similar to extracellular or cerebrospinal fluid [110; 114; 124; 198]. This converse electrolyte composition of endo- and perilymphatic fluid thus causes an electrochemical gradient essential for impulse generation within the labyrinth [86; 167].

Endolymph drains into the venous sinuses of the dura mater through the endolymphatic duct whereas the perilymphatic duct is connected to the cerebrospinal fluid of the subarachnoid space [124; 151; 191] (Fig. 3).

The vestibular labyrinth includes the saccule, the utricle and three ampulla with their corresponding semicircular ducts [49; 86; 127; 151] (Fig. 3). The three semicircular ducts (anterior, posterior and lateral) are perpendicular to one another and are linked with both their ends (ampullary and non-ampullary) to the utricle [127; 151]. The ampullary end of each semicircular duct consists of an enlargement which contains a crista [49; 151] (Fig. 6). Analogous to the crista ampullaris there is a thickening within the utricle as well as the saccule wall named macula utriculi/sacculi [49; 127; 151] (Fig. 5).

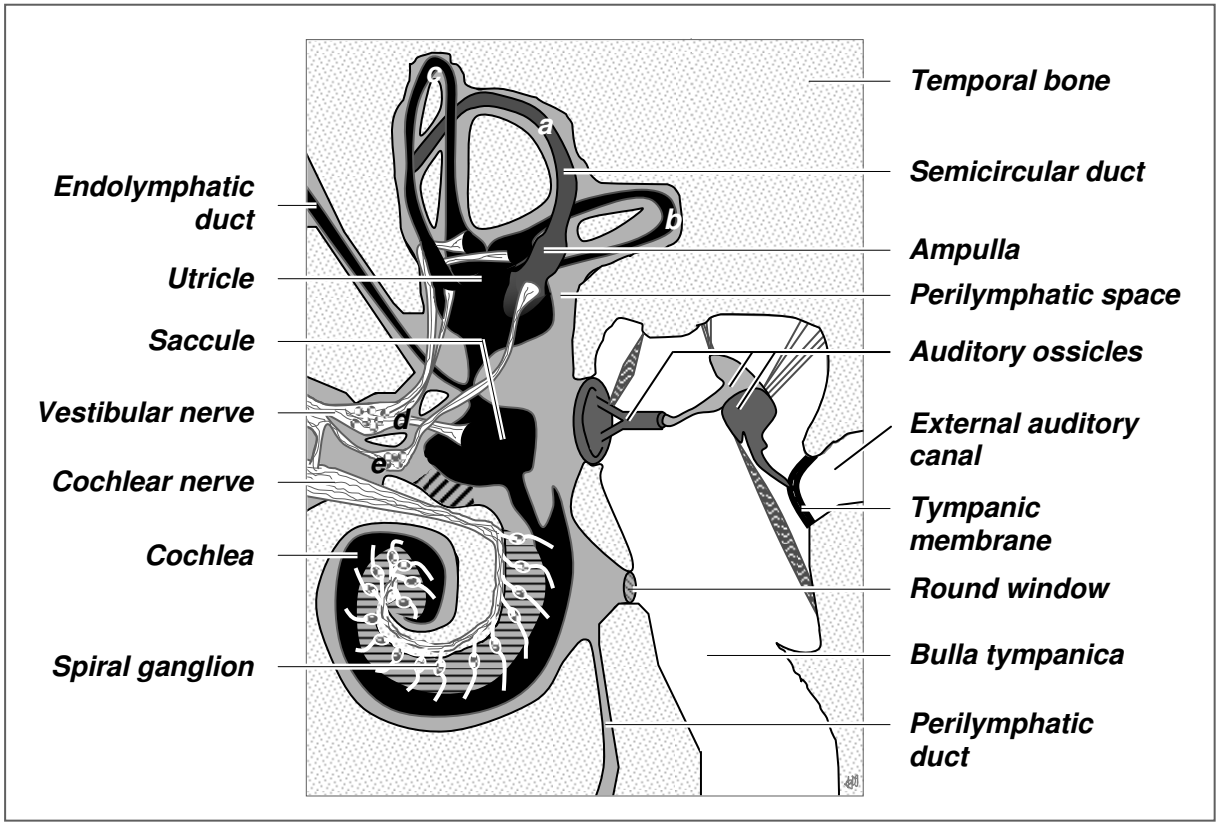


Figure 3. View upon the three different compartments of the ear. Overview of the external, middle and inner ear after sectioning of the skull as indicated in Fig. 2. The external auditory canal ends at the tympanic membrane which is connected to the auditory ossicles (malleus, incus and stapes) of the middle ear. The footplate of the stapes sits on the membrane of the oval window (fenestra vestibuli) thus contacting the inner ear. The three semicircular ducts (a: posterior, b: lateral, c: anterior) are perpendicular to one another. Superior (d) and inferior (e) portion of the vestibular ganglion. *[by courtesy of Dr. A. Blutke]*

2.2.2 Physiology

The hair cells within the macula organs and the cristae ampullares are the actual receptors of the vestibular labyrinth for detection of gravity or linear and angular acceleration/deceleration [49; 86; 123; 124; 151].

Each vestibular hair cell contains 50–110 stereocilia and one kinocilium on top [86; 127] (Fig. 4). The geometrical arrangement of this ciliary bundle and its interconnections are essential for impulse modification [86; 127; 207]. Deflection of stereocilia towards the kinocilium causes depolarization of the hair cell with subsequent increase in the impulse rate of the afferent nerve fibre while deviation away from the kinocilium causes opposite effects [51; 86; 127].

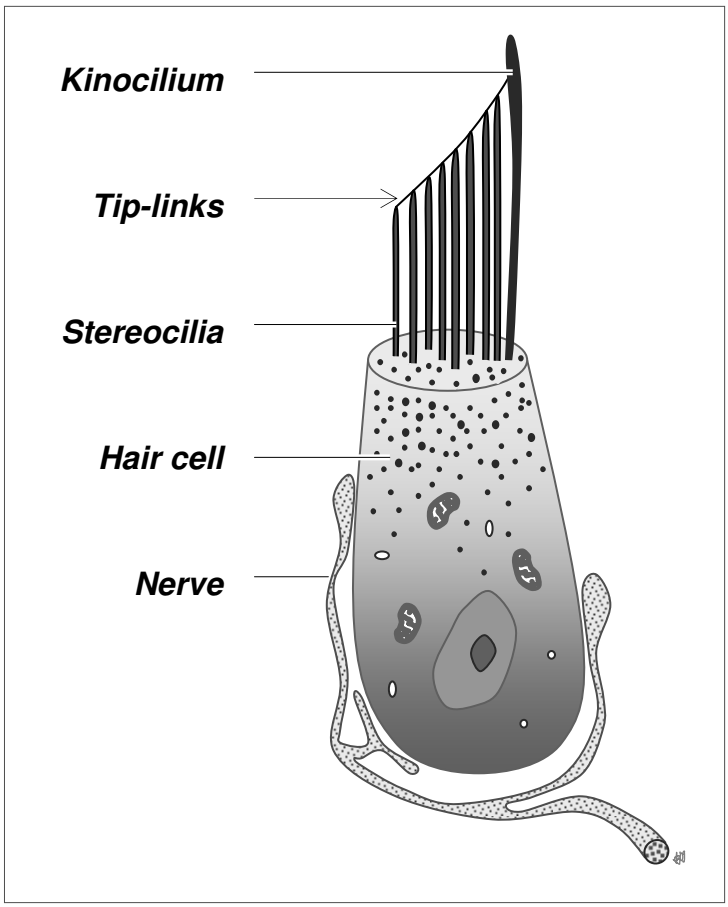


Figure 4. Vestibular hair cell. Each vestibular hair cell contains 50-110 stereocilia and one kinocilium on its top. The geometrical arrangement of this ciliary bundle is achieved by means of side- and tip-links. This is essential for the function of the hair cell and hence for the modification of the impulses. *[by courtesy of Dr. A. Blutke]*

The neuroepithelium of the macula organs is covered by a gelatinous substance in which calcium carbonate crystals are embedded [49; 151] (Fig. 5). This statoconial membrane, which displays a higher specific gravity compared to the surrounding tissue and fluid, stays behind when the head is displaced thus causing shear stress on the ciliary bundle and in turn de- or hyperpolarisation of the hair cells [86; 191].

Since the macula organs within the utricle and saccule are perpendicular to one another and the hair cells therein are orientated in all directions [127], any head displacement, linear acceleration/deceleration or the constant force of gravity may be detected [86; 191; 212].

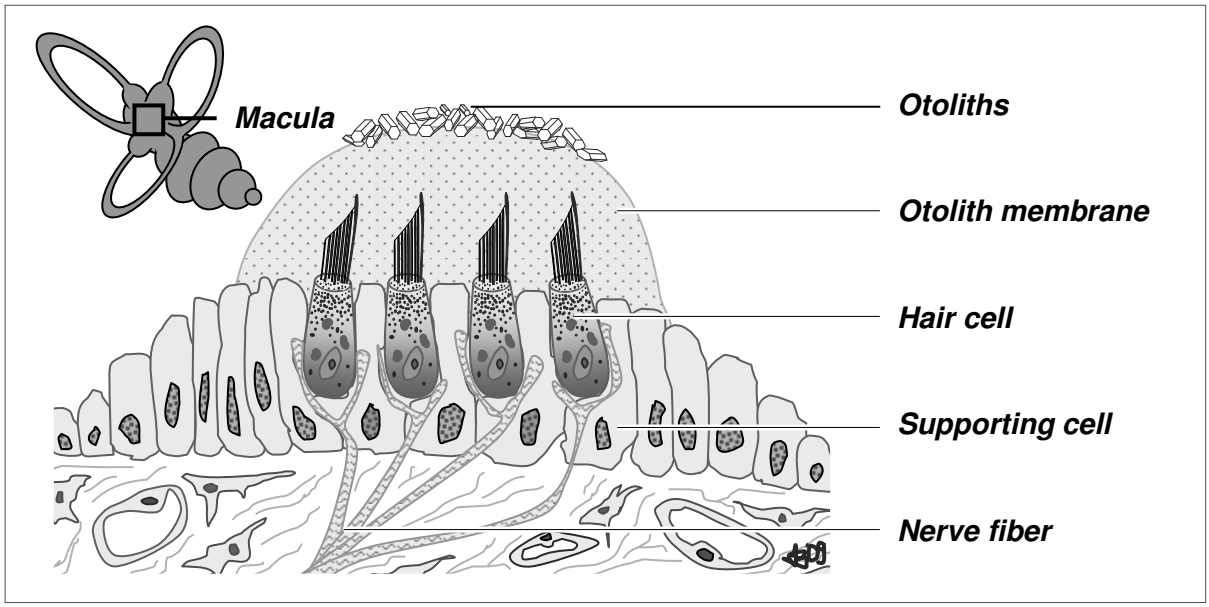


Figure 5. Macula utriculi/sacculi. There is a thickening in the utricle and saccule wall called the macula uriculi/sacculi. Each macula consists of a neuroepithelium with hair and supporting cells, covered by a proteinaceous mass with calcium carbonate crystals, named the otolith or statoconial membrane. *[by courtesy of Dr. A. Blutke]*

Sensory cells of the crista again are embedded in a gelatinous mass called cupula which extends from the neuroepithelial surface to the ampulla roof [49; 151] (Fig. 6). Rotation of the head thus causes deflection of the cupula and therefore shear stress on the ciliary bundle, due to the inertia of the endolymph [86; 191; 212]. Since hair cells within the crista ampullaris are morphologically and functionally polarized, all the kinocilia within a crista are either directed towards the semicircular duct or towards the utricle [127]. Opposite polarization of hair cells in the ampulles of the right and left ear as well as different orientation within the three ampulles enables the detection of angular (rotational) acceleration/deceleration [86; 127; 191; 212].

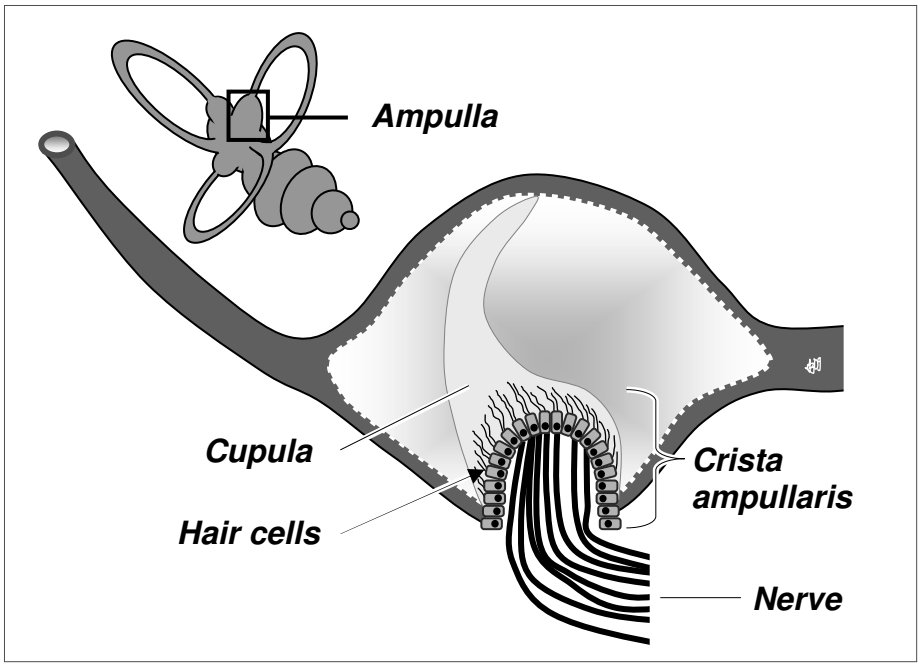


Figure 6. Crista ampullaris. Each semicircular duct has an enlargement on the utricle side called an ampulla with a crista ampullaris. The crista contains the neuroepithelium, which is covered by the gelatinous cupula reaching up to the ampulla roof.
[by courtesy of Dr. A. Blutke]

2.2.3 Nervous pathways

Generated impulses are transmitted from the N. utricularis, N. saccularis and Nn. ampullares to the superior and inferior division of the vestibular ganglion (VG) [151]. Vestibular fibres from the VG then join the cochlear nerve, and along with the facial nerve, exit the temporal bone within a common dura sheath [151]. The vestibulocochlear nerve (cranial nerve (CN) VIII) travels to the rhombencephalon where it divides into a radix vestibularis and cochlearis before entering the rostral medulla [151]. Fibres of the vestibular division synapse to the four vestibular nuclei (rostral, medial, lateral and caudal) and some directly enter the cerebellum [49; 86; 115; 123; 144; 151; 191]. The vestibular nuclei are connected to multiple parts of the nervous system (cerebellum, spinal cord, motor nuclei of CN III, IV, VI, reticular formation, thalamus, cortex and vestibular labyrinth) in order to maintain equilibrium [34; 49; 67; 123; 124; 144; 151; 191]. An appropriate relationship of eye, trunk, limb and head position during posture and locomotion is achieved by means of vestibulospinal and vestibuloocular reflexes [63; 67; 123; 124; 212].

2.3 Vestibular dysfunction

The vestibular system consists of a peripheral (vestibular labyrinth within the inner ear, vestibular portion of CN VIII) and a central component (vestibular nuclei, vestibulocerebellum, pathways) [49; 144; 212].

Vestibular system disorders, which are relatively common, both in man and in domestic mammals, cause varying degrees of loss of equilibrium manifesting as vestibular ataxia, falling, circling, rolling, head tilt, and nystagmus [49; 115; 124; 144; 191; 212].

Vestibular signs may be unilateral or bilateral. Distinction of central from peripheral vestibular disease is achieved by recognition of clinical signs caused by the dysfunction of other systems located in the brainstem or cerebellum [34; 49; 144; 212; 214].

While neoplasia and infection/inflammation are the most common causes of central vestibular disease in cats and dogs [144; 212], otitis media/interna and idiopathic vestibular disease are the major etiologies for peripheral [144; 191; 192; 212] vestibular disorders. Besides, thiamine deficiency [123; 191; 217], metronidazole intoxication [123; 212; 217], head trauma [191; 217], hypothyroidism [93; 219], anomalies [144], cerebrovascular [144] and degenerative diseases [144; 191] may cause central vestibular dysfunction. Tumors of or involving the middle/inner ear [123; 191; 212; 217], head trauma resulting in a fractured temporal bone or fractured bulla tympanica [123; 191; 217], hypothyroidism [105; 212; 219], congenital diseases [14; 58; 123; 126; 191; 217], and application of ototoxic drugs [123; 212; 217] may induce peripheral vestibular disease.

Apart from vestibular disorders with known etiology, idiopathic vestibular diseases play a major role in cats and dogs. Idiopathic vestibular disease affects geriatric dogs and cats at all ages and manifests as a peracute onset of severe peripheral vestibular dysfunction resolving without therapy within two weeks [25; 123; 144; 191; 212]. The etiology of the disease is still unknown and diagnosis is largely based on the exclusion of other causes for vestibular disease and the resolution of clinical signs [25; 49; 123; 144; 191; 212]. Even though herpesvirus infections are widespread amongst cats [75] and dogs [50] and infection of vestibular inner ear structures by FHV-1 or CHV-1 may be a potential cause of idiopathic vestibular disease, such investigations have not been reported yet. In humans on the other hand, the possible association of vestibular herpesvirus infections with vestibulopathies has been subject of research for many years. Thus, a variety of herpesviruses have been located in different compartments of the vestibular system [8-10; 61; 230] and are discussed to cause various recurrent vestibulopathies [65; 159; 173].

2.4 Processing of the canine and feline vestibular labyrinth

Due to the inaccessibility and the complex anatomy of the membranous labyrinth, technical processing of these structures for further investigations is very challenging and tedious and, therefore, currently not a routine procedure in cats and dogs [46; 49; 214]. Established standard protocols, e.g. for histological investigation, include long-lasting fixation and decalcification of temporal bones [36]. Depending on the chemicals and temporal bone size, decalcification following fixation, takes approximately 40 days in cats [13] and up to 225 days in dogs [36].

Consequently, only few reports about morphological [6; 39; 62; 103; 143; 175; 187] and immunohistochemical [41] investigation of canine and feline vestibular inner ear structures exist.

Molecular analyses, as PCR-based investigations, performed on isolated structures of the vestibular inner ear, have not been reported in cats and dogs so far. This might probably be due to the absence of protocols suitable for molecular analyses following standard vestibular inner ear preparation procedures.

3. Own scientific experiments

3.1 A rapid approach to ultrastructural evaluation and DNA analysis of the vestibular labyrinth and ganglion in dogs and cats

Birgit Parzefall, Wolfgang Schmahl, Andreas Blutke, Kerstin Baiker, Kaspar Matiasek

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A rapid approach to ultrastructural evaluation and DNA analysis of the vestibular labyrinth and ganglion in dogs and cats*

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Figures: 13

Tables: 2

* Parts of this study were presented as a poster presentation at the 21st annual symposium of the European Society of Veterinary Neurology (ESVN) in Rhodes, Greece (2008) and as an oral presentation at the 52nd annual meeting of the Fachgruppe Pathologie of the Deutsche Veterinärmedizinische Gesellschaft (DVG) in Fulda, Germany (2009).
The abstract has been published in the Deutsche Tierärztliche Wochenschrift: Parzefall, B., Schmahl, W., Blutke, A., Baiker, K., Matiasek, K., 2009. Peri-/endolymphatisches Tracing zur bimodalen Untersuchung des vestibulären Labyrinths und Ganglions von Hund und Katze. Dtsch Tierarztl Wochenschr. 116 (7), 279.

Abstract

The vestibular labyrinth is the organ for sensation of equilibrium. It is part of the inner ear and located in the caudodorsal aspect of the temporal bone which makes it very difficult to access. This study evaluated a preparation technique in cats and dogs for morphological and DNA analysis.

The study included 44 temporal bones of 14 cats and 11 dogs collected within 48h after death. Preparation was performed after peri-/endolymphatic injection of Fast Green FCF through the fenestra vestibuli to visualize the membranous labyrinth. The vestibular nerve, including its ganglion, and the vestibular labyrinth were exposed by drilling and cracking of the petrous temporal bone along the meatus acusticus internus. The posterior ampulla was collected for histology and transmission electron microscopy whereas the vestibular nerve, the utriculus, sacculus, and the lateral and anterior ampullae were harvested for subsequent DNA analysis.

Histology and electron microscopy showed well-preserved cells. A total DNA amount of 4753 ± 1502 ng in cats and 5865 ± 2911 ng in dogs was retrieved from the ganglion, and 2390 ± 561 ng in cats and 2544 ± 1277 ng in dogs, respectively, from membranous vestibular organs. Polymerase chain reaction of a 229 base pair product of the Gapdh-gene proved for presence of amplifiable DNA.

Taken together, mechanical bone removal after Fast Green FCF injection allows for reliable gross, microscopic and ultrastructural examination of the feline and canine vestibular labyrinth, and it does not interfere with DNA analysis via PCR. This technique is feasible for multimodal investigation of the vestibular labyrinth retrieved from individual necropsy cases.

Keywords: Labyrinth, Crista ampullaris**,** Ganglion**,** Vestibular, Canine, Feline, PCR**,** Fast Green**,** Electron microscopy.

1. Introduction

The vestibular labyrinth contains the organs for sensation of equilibrium. It is part of the inner ear and located in the caudodorsal aspect of the temporal bone [19; 35]. It consists of a membranous labyrinth that is surrounded by compact cortical bone which makes it very difficult to access.

In order to investigate the vestibular labyrinth, temporal bones used to be fixed in aldehydes, decalcified for months and finally embedded in either paraffin [8-9; 12; 36], or celloidin [7; 22; 32-33]. These procedures take 40 days in cats [4] and up to 225 days in dogs [8] until a morphological investigation becomes possible and they effectively impede further molecular analyses on the tissue.

In particular, studies on inner ear stem cells, hair cell biology and neurotransmission employ investigational techniques that rely on optimal preservation of intracellular proteins and nucleic acids. Other areas of advanced vestibular research focus neurotropic viruses that may infect the vestibular labyrinth [3] and/or ganglion [2; 18; 40-41] by using polymerase chain reaction (PCR).

This investigation was aimed to assess the influence of peri- and/or endolymphatic tracer injection on the ultrastructure of the crista ampullaris, and DNA-preservation within the vestibular labyrinth and vestibular nerve ganglion.

2. Materials and methods

2.1 Animals and tissues

This study enrolled 44 temporal bones from 14 cats and 11 dogs (Table 1), presented for routine post-mortem examination. All samples were processed within 48 hours after death.

Table 1: Numbers, ages and gender of investigated animals.

	Number of animals	Number of investigated temporal bones	Age		Gender	
			Adult > 1 year	Juvenile < 1 year	Female	Male
Cats	14	24	12	2	5	9
Dogs	11	20	8	3	5	6

2.2 Preparation of the vestibular labyrinth

2.2.1 Gross preparation

On necropsy, the head was obtained after removal of skin and neck muscles with subsequent exarticulation in the atlanto-occipital joint. The skin, the two pinnae and muscles were removed from the ventral part of the head to expose the bony part of the external auditory canal and the ventral surface of the bulla tympanica. In the next step, the skull was split sagitally in the midline with a band saw. After removal of the brain from the caudal cranial fossa, the yellow-white tinged temporal bone, which is slightly paler than the surrounding bone became visible (Fig. 1). The bulla tympanica was opened from the ventrolateral aspect (Fig. 2). The auditory ossicles, the M. tensor tympani and all bulla parts were removed in order to expose the fenestra cochleae and fenestra vestibuli (Fig. 3).

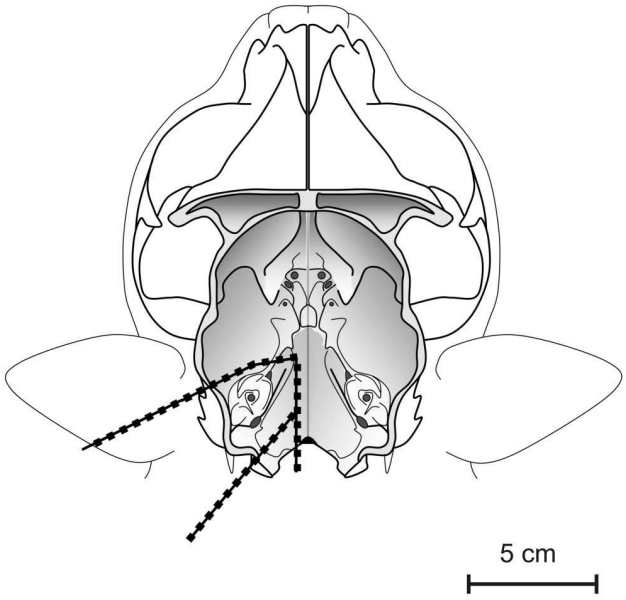

Figure 1: Topographic anatomy of the cat's temporal bone (TB) visible after calvarial removal. The cutting lines for preparation are indicated. Scale bar = 0.5 cm.

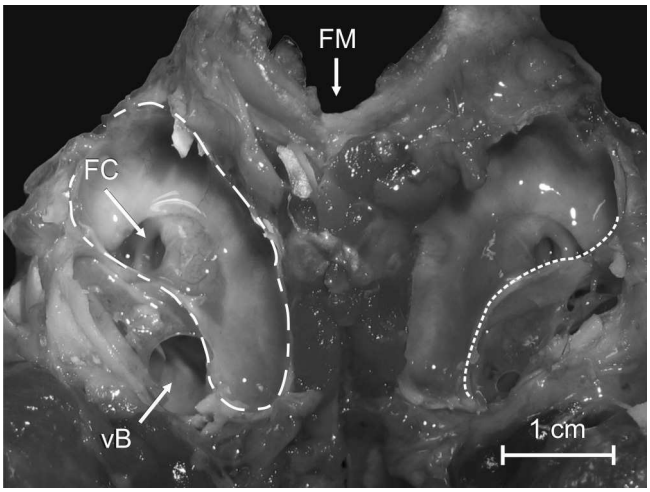

Figure 2: Ventral view on the opened bullae tympanicae (BT) of an unsplit cat's skull which are divided into a rostromedial and a caudolateral portion (dashed lines) through an incomplete bony septum (dotted lines).
FM = foramen magnum, FC = fenestra cochleae, vB = ventral BT.
Scale bar = 1 cm.

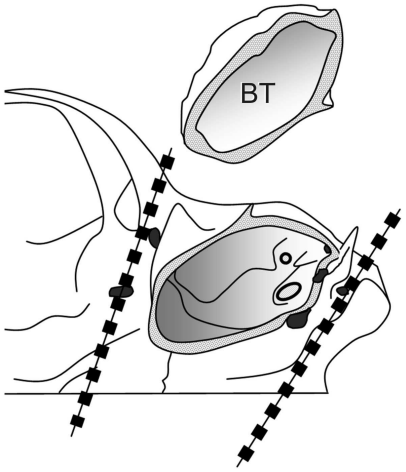

Figure 3: Lateroventral view on the TB after removal of the bony parts of the BT. The fenestra vestibuli and cochleae are the most important landmarks for orientation during the preparation procedure.

2.2.2 Micropreparation

All the following preparations were performed using a dissection microscope (Stemi DV4, Carl Zeiss AG, Jena, Germany) with the temporal bone placed in a Petri dish filled with Aqua bidest. in order to allow preparation through perpetual removal of bone graft and avoidance of exsiccation and extensive heat production during the burr procedure (see below).

Throughout all further steps of preparation, the fenestra cochleae and vestibuli were the most important landmarks (Fig. 4).

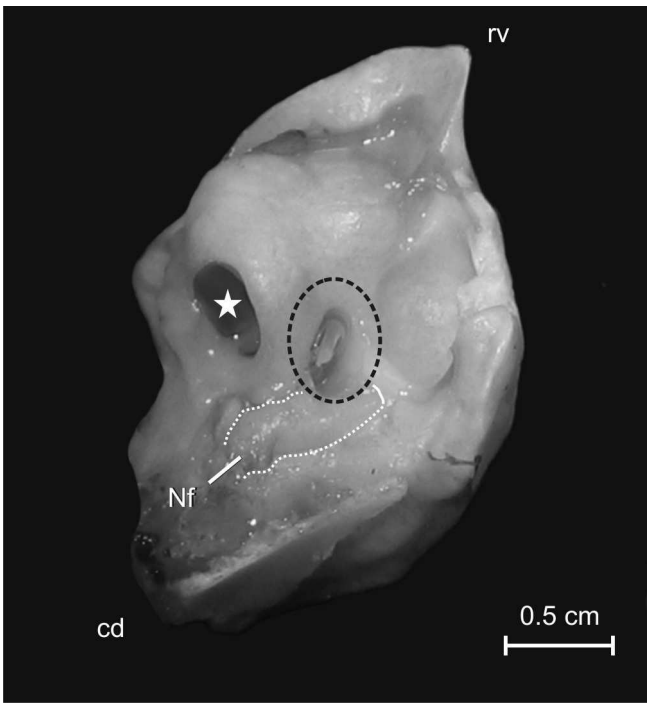

Figure 4: Closer view on the left TB.
The asterisk is placed on the fenestra cochleae. The fenestra vestibuli (surrounded by the dashed line) still contains the footplate of stapes sitting on its membrane. The facial nerve (Nf; dotted line) emerges from the facial canal and, over a short distance, is exposed to the lumen of the middle ear cavity.
cd = caudodorsal, rv = rostroventral. Scale bar = 0.5 cm.

The caudodorsal aspect of the petrous temporal bone (PTB) was directed towards, the rostroventral apex of the cochlea positioned away from the right-handed preparator. For the left PTB bone, the medial side of the PTB, containing the meatus acusticus internus, was oriented to the right, while the lateral aspect was directed to the left, with the fenestra vestibuli on top of the fenestra cochleae. For the right PTB the medial side was positioned to the left, while the lateral part was on the right side, respectively.

The membrane of the fenestra cochleae was fenestrated with a sterile 23 gauge needle and between 0.05 ml and 0.1 ml of 1% Fast Green FCF [1; 13] were peri-/endolymphatically injected into the vestibulum through the pierced membrane of the fenestra vestibuli (Fig. 5). Thereby the dye merged with the peri/endolymphatic fluid and visualized the almost transparent membranous labyrinth. The injection was performed slowly, over a period of at least 3 s until the dark-green discoloration of the cochlear parts gleamed through the overlying bone.

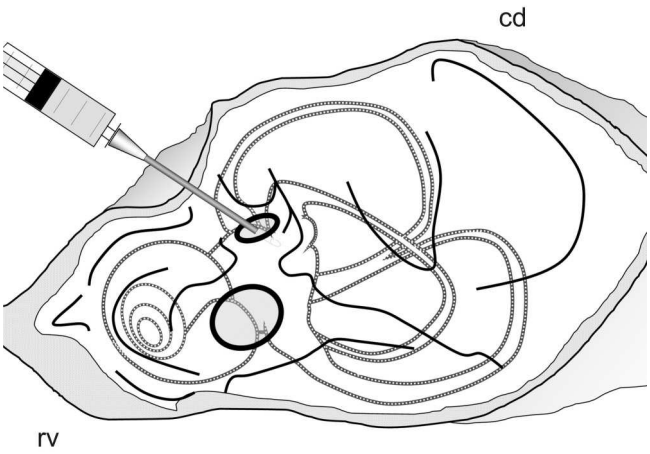

Figure 5: Peri-/endolymphatic injection of the dye. In order to visualize the membranous vestibular labyrinth Fast Green FCF is injected via the fenestra vestibuli after puncture of the membrane of the fenestra cochleae for pressure relief. rv = rostroventral, cd = caudodorsal.

To gain access to the ampullae, the facial canal was opened by a burr (GG 12, Proxxon, Niersbach, Germany) and the facial nerve was removed. Its rostral aspect, however was spared, in order to guarantee an intact superior division of the vestibular nerve (own unpublished observation) that anastomoses with the facial nerve [17; 35].

At first, the lateral ampulla was identified, followed by the anterior ampulla. From there, the course of the lateral semicircular canal was followed to the posterior ampulle, that is located caudolateral to the fenestra cochleae. For unambiguous identification of the ampullae, the bony labyrinth surrounding the ampullae and the beginning of the corresponding semicircular ducts was carefully drilled, leaving nothing but a very thin bone lamella intact (Fig. 6). Thereafter, the PTB was split along the internal auditory canal (Fig. 7) into two parts containing the rostroventral cochlea and the caudodorsal vestibular labyrinth (Fig. 8), respectively.

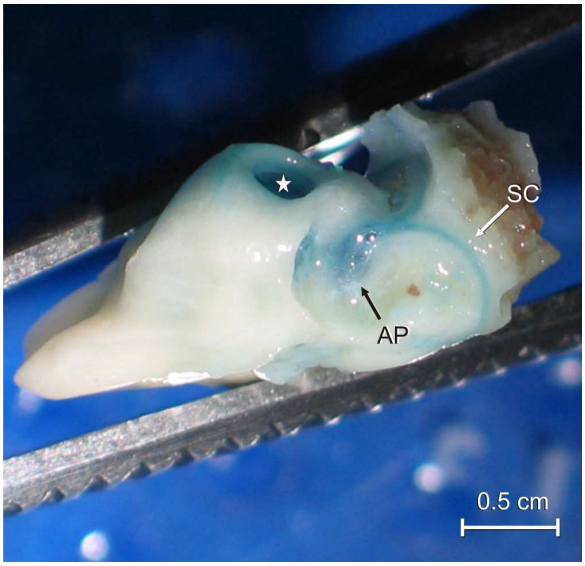

Figure 6: Left feline TB after stepwise removal of the superficial bone laminae overlying the vestibular labyrinth. Through injection of Fast Green FCF the posterior semicircular canal (SC) and ampulla (AP) became clearly visible. Asterisk = fenestra cochleae.
Scale bar = 0.5 cm.

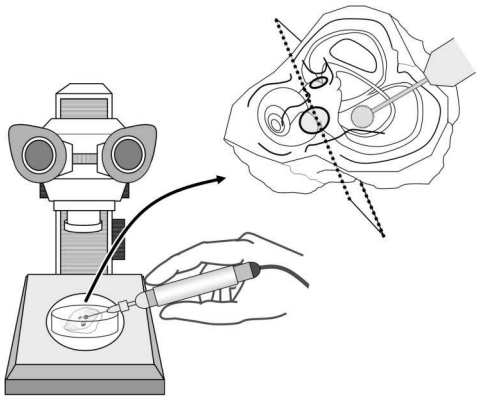

Figure 7: Further preparation of the TB. In order to expose the ampullae and the beginning of the semicircular ducts the TB is drilled with a burr followed by cracking (plane indicated through dotted lines) along the meatus acusticus internus.

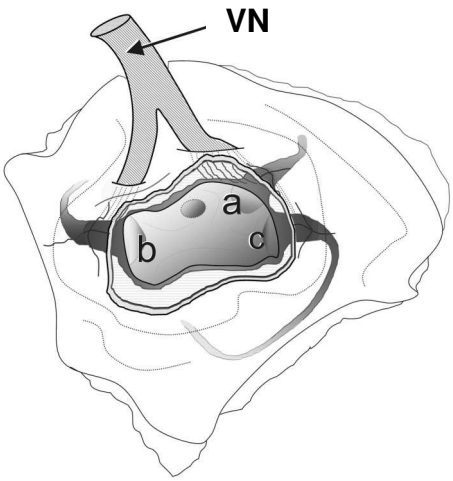

Figure 8: View of the vestibular labyrinth. After cracking of the petrous temporal bone, the vestibular labyrinth which includes the utriculus, sacculus, ampulla anterior (a), posterior (b) and lateralis (c) become visible in the osseous vestibulum. The vestibular nerve (VN) with its ganglion superior and inferior can now be harvested.

The vestibular nerve, including the superior and inferior vestibular ganglia, was carefully separated from the cochlear and facial nerve by microforceps, and collected for further sample processing.

In order to remove the utriculus, the sacculus, the ampullae and semicircular ducts, the thin bone layer covering the ampullae and semicircular ducts was removed. In close proximity to the posterior ampulla, the utriculus, the crus commune (resembling the non-ampullary aspect of the posterior and anterior semicircular ducts) as well as the non-ampullary region of the lateral semicircular duct and the ampullary part of the posterior semicircular duct were transsected with microscissors. Then, the posterior ampulla was harvested for histotechnical processing, while the utriculus, the sacculus and the lateral and anterior ampulla were collected for DNA isolation.

2.3 Sample processing for histology

The posterior ampulla was prepared for histological evaluation using standard techniques for epoxy embedding [31]. In short, the posterior ampulla was immersed in 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate puffer (pH 7.4) for 1-2 hrs at room temperature, then rinsed with Sorensen's buffer, post-fixed in 2% OsO_4 for 2 hours, followed by repeated buffer rinses and a graded alcohol series. The posterior ampulla was then embedded in epoxy resin in such a way that the ampullary nerve was perpendicular to the sectioned surface (Fig. 9). Semithin sections of 0.5 µm thickness were mounted on triethoxysilane-coated glass slides and stained with azur II methylene blue-safranin.

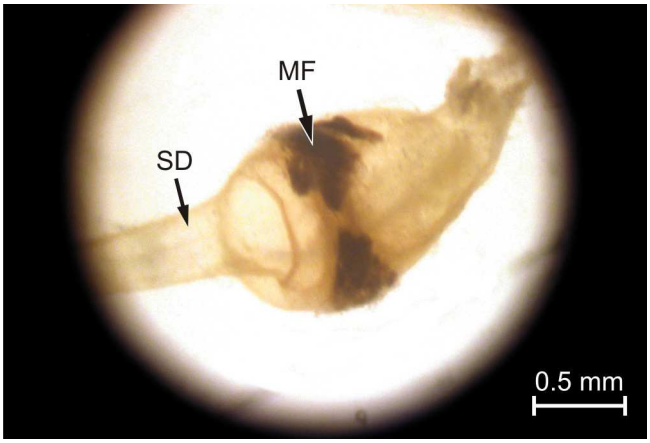

Figure 9: Extracted ampulla posterior, fixed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide. Osmium staining allows for detection of myelinated nerve fibres (MF) under the dissection microscope. This step facilitates the correct probe positioning during epoxy embedding. SD = semicircular duct. Scale bar = 0.5 mm.

2.4 Transmission electron microscopy

Ultrathin sections (~70 nm) of the crista ampullaris posterior were mounted on formvar-chloroform covered copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (EM 10, Carl Zeiss AG, Jena, Germany). Cells were identified according to the listed criteria (Table 2).

2.5 Polymerase chain reaction

Superior/inferior vestibular ganglion, utriculus, sacculus, anterior and lateral ampullae and their corresponding semicircular ducts were placed in two sterile 1.5 ml tubes, snap frozen in liquid nitrogen, and stored at -80°C. DNA extraction was performed with a commercially available kit (QIAmp DNA Micro-Kit, Qiagen, Hilden, Germany), according to the manufacturer's description, followed by spectrophotometric measurement (NanoDrop 1000, peQLab Biotechnologie GmbH, Erlangen, Germany) of DNA content.

To confirm the presence of amplifiable DNA in the nucleic acid preparations, published primers were used to amplify a 229 base pair segment of the glyceraldehyde-3-phosphate dehydrogenase gene (Gapdh) [20; 23]. Reaction products were electrophoresed through a 2% agarose gel with 0.5 x TAE buffer and stained with ethidium bromide.

Table 2: Ultrastructural cell characteristics.

Cell type	Shape	Cytoplasm	Membrane specifications	Cell-to-cell connections	Other
Haircell I (HC I)	Flask shaped	-Nucleus located in the bulbous area -Most organelles located supranuclear (endoplasmatic reticulum, Golgi complex, ribosomes, vesicles, mitochondria) -Cuticular plate	20-100 Sc, 1 kinocilium, Mv	TJ, AJ	Chalice-like nerve ending
Haircell II (HC II)	Irregular, cylindrical	-Nucleus varies through the middle one-third of the cell -Organelles appear more evenly distributed than in HC I	20-100 Sc, 1 kinocilium, Mv	TJ, AJ	Bouton-shaped nerve ending
Supporting cell (SC)	Tall	-Run from the epithelial surface down to the basal lamina -Nucleus located in the lower part of the cell near the basal lamina and below the nuclei of the HCs -Reticular lamina -Upper half to two-thirds of the SC is filled with large, densely packed vesicles (mitochondria, Golgi complexes, lysosomes, free ribosomes)	Mv	TJ, AJ, GJ	
Transitional cells (TCs)	Columnar-cuboidal	-Nucleus more central than in SC	Mv	GJ, AJ, JC	
Dark cells (DCs)	Tall-cuboidal-squamous	-Central located lobulated nucleus -Extensive interdigitation of the cytoplasm in the basal portion of the cell -Most organelles located in the upper two-thirds of the cell -Nucleus and cytoplasm stain darkly	Few Mv	TJ, AJ	Degenerating otoconia on cell surface, subepithelial melanocytes
Planum semilunatum (PSL) cells	Columnar-cuboidal	-Nucleus circular-oval, basal or centrally located	Few Mv	GJ, AJ, JC	Subepithelial reticular layer
Epithelium	Squamous	-Nucleus circular, centrally located		TJ, AJ	

Cell characteristics are extracted from specific literature [15; 21; 24-29; 38-39]. Mv = mikrovilli, TJ = tight junction, AJ = adherens junction, GJ = gap junction, JC = illdefined junctional complex.

3. Results

3.1 Preparation

In all included cases, peri-/endolymphatic injection of Fast Green FCF resulted in a discoloration of the membranous labyrinth and, hence, facilitated identification and preparation of the cavitary inner ear compartments. Preparation of both vestibular labyrinths and removal for further processing was achieved within 2 hours in cats and within 3 hours in dogs. There was no obvious difference in gross tissue preservation or its feasibility for preparation amongst specimens collected within 48 hours after death, whereas older samples, collected the same way, (not included in the study) became decomposed which impedes a clear identification of the vestibular nerve and a proper removal of the membranous vestibular parts. The position of the ampullae in relation to the fenestra vestibuli and cochleae, and their depth within the PTB was found to be individually variable. Ampulles in cats and and small dogs displayed a more superficial localization than in middle- and large-sized dogs and did not vary as much in position. In general, the anterior and lateral ampullae are easier to locate and isolate than the posterior ampulla. Preparation of feline temporal bones does not raise any special difficulties, whereas preparation of canine temporal bones is far more challenging due to the great variety of ampulla location and the increased fragility of the PTB in this species.

3.2 Histology

Mechanical removal of the bone enabled routine soft tissue processing without decalcification. Correct orientation of the specimens in the embedding procedure is crucial to obtain the same section plane. Some ampullae were displaced within the first hour of the embedding process and had to be put back into the right position. All histological sections of the ampulla revealed the crista ampullaris (Fig. 10), the epithelial lining of the ampulla, and sometimes parts of the semicircular duct. The same section plane was obtained throughout all samples, whereas the cutting level, with regard to the neighboring dark cell (DC) and planum semilunatum zones, sometimes differed and, therefore, slightly changed the histological picture. All sections revealed the stroma of the crista including myelinated nerve fibres and blood vessels as well as the sensorineural epithelium consisting of hair- and supporting cells. Transitional cells and DCs were obtained in deeper and planum semilunatum (PSL) cells in more shallow sections. The injection of Fast Green FCF did not interfere with the staining.

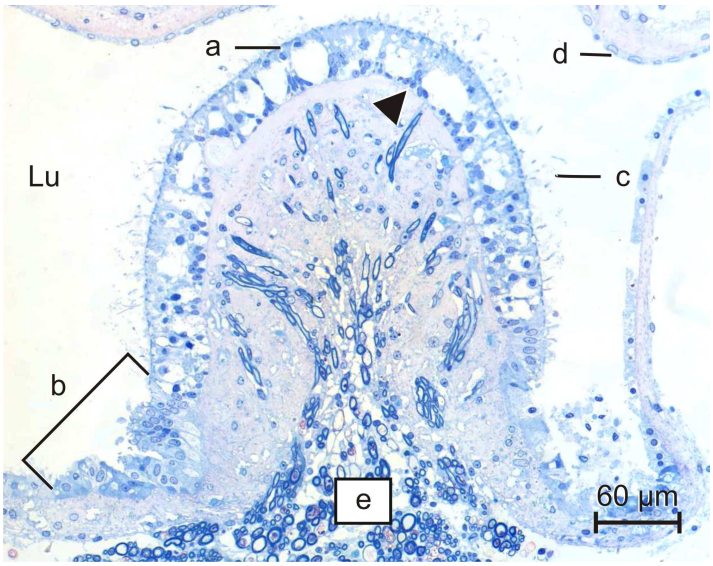

Figure 10: Histological appearance of the crista ampullaris posterior on semithin sections.
a = hair cell, b = transitional zone, c = stereocilia of the hair cells projecting into the lumen (Lu) of the ampulla, d = squamous ampulla epithel, e = myelinated fibres of the vestibular nerve (ampullaris).
Scale bar = 60 µm.

3.3 Transmission electron microscopy

Due to adequate preservation, all cells listed in Table 2 were readily identified on the basis of their ultrastructural characteristics. Numerous hair cells (HCs) type I (Fig. 11A) were seen along the crista ampullaris, whereas hair cells type II were only occasionally detected. HCs were in close contact with supporting cells (SCs) and displayed tight and adherens junctions (Fig. 11A). Stereocilia (Sc) were implanted in the cuticular plate (Fig. 11B) and their spatial relationship to the kinocilium was not always clearly discernible. However, the single kinocilium was found in some HCs. Cytoplasmic organelles were clearly visible. For many instances, and throughout all cells, the cisternal system appeared dilated. The nuclei of SCs were basally placed and their apical part was in close contact to the HCs where it displayed the typical reticular laminae (Fig. 11A). Cytoplasmic protrusions in the apical part of the SCs were frequently noticed. Some transitional cells (TCs) (Fig. 11C) were found at the base of the crista. They were contiguous with dark cells DCs. TCs were columnar with the nucleus located in the lower part of the cell. Their junctional complexes were clearly visible. DCs revealed darkly stained nuclei and also a rather electron-dense cytoplasm. Nuclei were lobulated and the cytoplasm showed characteristic basal interdigitations. Degenerating otoconia were often detected, confined to the DC surface. PSL were sometimes visible, depending on the cutting level. If present, PSL were found to be cuboidal with round central nuclei. Squamous epithelium was detected in the semicircular duct. The stroma of the crista ampullaris was well preserved showing myelinated nerve fibres (Fig. 11D) and blood vessels.

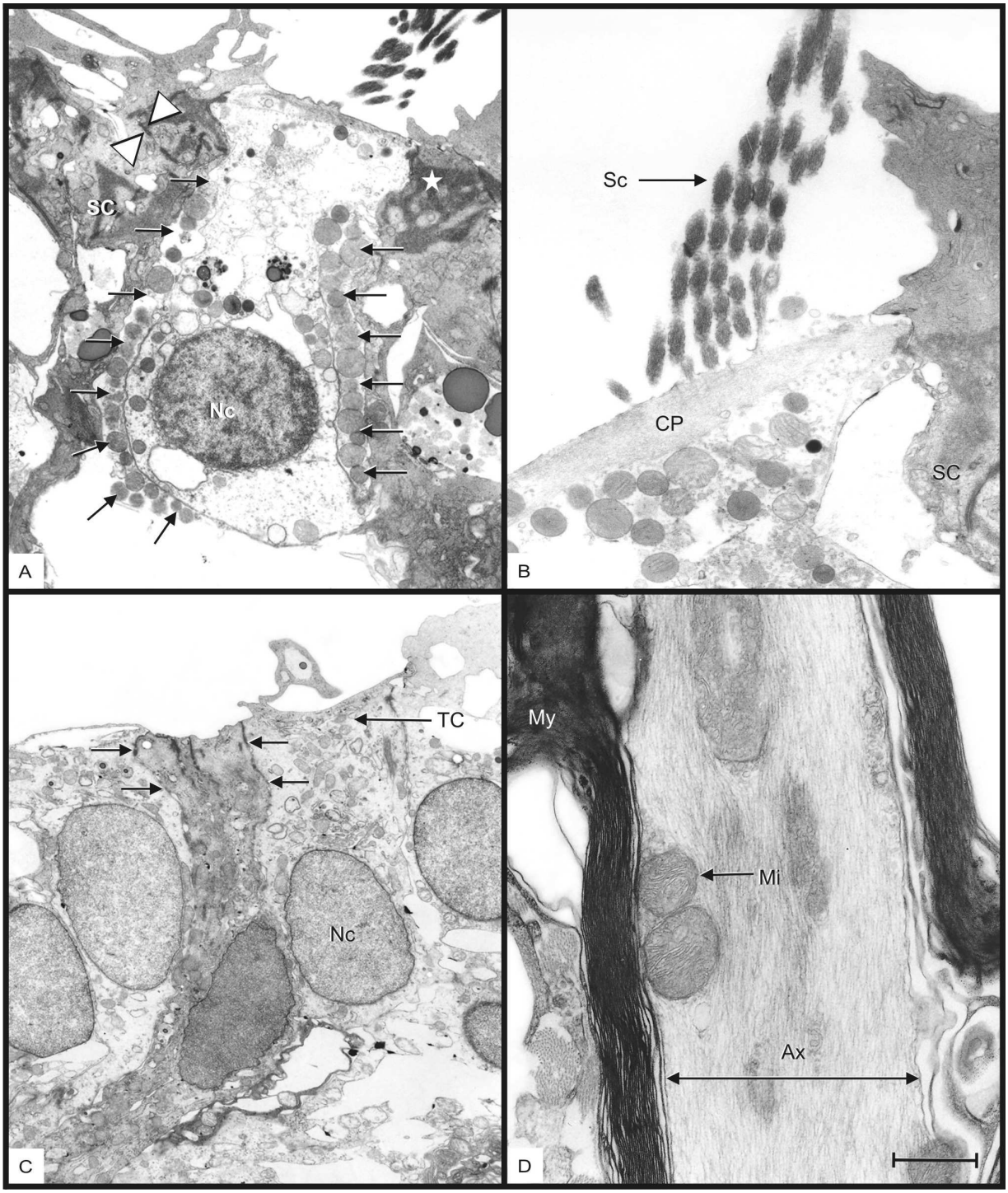

Figure 11: Well-preserved ultramorphology of the crista ampullaris posterior. A, type I hair cell with nerve calyx (arrows), showing numerous mitochondria. Neighboring supporting cells (SCs) with reticular lamina (asterisk) and desmosomes (arrowheads). B, apical part of a type I hair cell with stereocilia (Sc) implanted in the cuticular plate (CP), with numerous mitochondria in the infracuticular region. C, transitional cells (TCs). Junctional complexes are indicated by small arrows. D, myelinated nerve fibre with well-preserved compacted myelin (My), axon (Ax) and axoplasmic mitochondria (Mi). Nc = nucleus.

Scale bar: A = 1.4 µm, B = 1.1 µm, C = 2.0 µm, and D = 0.6 µm.

3.4 Integrity and amount of isolated DNA

DNA could be extracted from all samples with a mean amount of 4753 ng (±1502) for the feline and 5865 ng (±2911) for the canine vestibular ganglion.

The average amount of isolated DNA from the utriculus/sacculus, the lateral and anterior ampullae with their corresponding semicircular ducts were 2390 ng (±561) in cats and 2544 ng (±1277) in dogs, respectively (Fig. 12).

The integrity of DNA of all samples was confirmed by amplification of a 229 base pair product of Gapdh (Fig. 13).

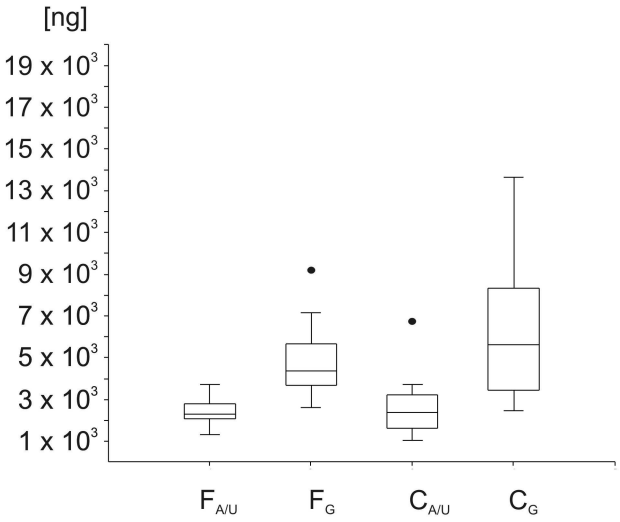

Figure 12: Box and whisker plot displaying the total amount of isolated DNA in nanogram (ng).
F = feline, C = canine,
A/U = ampulla/utriculus,
G = vestibular ganglion.

Figure 13: Amplification of the housekeeping gene Gapdh by PCR.
M: Fragment size marker. Visible marker bands indicate fragment sizes of 300, 250 and 200 base pairs from top to bottom. Ø: spacing lane.
NTC: no template control. DNA samples (approx. 100 ng) from cats' (F-) and dogs' (C-) ampullae (A/U) and vestibular ganglia (G). All templates show a specific PCR-product of 229 base pairs length (arrow).

4. Discussion

Previous studies performed in human cadavers have employed physical preparation techniques to collect the vestibular labyrinth for further DNA analysis [3]. To date, studies of the canine and feline inner ear have concentrated on morphological [6; 16; 30; 34] and histochemical [10-11] investigations while the preparation of the vestibular labyrinth for further DNA analysis has not been described. This study employed a histologic dye [1; 13] in order to visualize the membranous labyrinth which facilitates the orientation for the preparator. This is the first report that proves that Fast Green FCF is feasible for bimodal analyses at ultrastructural and DNA level. This method is reasonably fast and, therefore, advantageous compared to anatomical standard techniques which require time-consuming decalcification steps. The published standard procedures also have a negative effect on the integrity of DNA in terms of cross-linking and degradation which interferes with PCR analysis [5; 14; 37].

In addition, stepwise preparation of the vestibular labyrinth under the dissection microscope also allows for thorough gross examination which would be impossible if the entire temporal bone is fixed, decalcified and embedded [33]. Even though autolysis in the membranous labyrinth starts early and can cause difficulties in cadaver studies, the 48-h time frame still enables the preparator to perform the described investigations.

Taking the methodological aspects of this study into account, gross, microscopic and ultrastructural examination of the feline and canine vestibular labyrinth, as well as performance of PCR, e.g. for epidemiologic studies, can conveniently be performed with the described protocol herewith, even if just one single temporal bone is available.

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3.2 Evidence of feline herpesvirus-1 DNA in the vestibular ganglion of domestic cats

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Evidence of feline herpesvirus-1 DNA in the vestibular ganglion of domestic cats*

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* Parts of this study were presented as a poster presentation at the 21st annual symposium of the European Society of Veterinary Neurology (ESVN) in Rhodes, Greece (2008) and as an oral presentation at the 52nd annual meeting of the Fachgruppe Pathologie of the Deutsche Veterinärmedizinische Gesellschaft (DVG) in Fulda, Germany (2009).

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Parzefall, B., Schmahl, W., Fischer, A., Truyen, U., Matiasek, K., 2009. Untersuchung zum Vorkommen von FHV-1 DNA im vestibulären Labyrinth und Ganglion von Hauskatzen. Dtsch Tierarztl Wochenschr. 116 (9), 341.

Abstract

In humans, herpes simplex virus type-1 has recently been detected in the vestibular ganglion (VG) and labyrinth (VL) and may be associated with vestibular signs. Feline herpesvirus-1 (FHV-1) is widespread amongst cat populations and affects many different tissues. The aim of this pilot study was to investigate the presence of FHV-1 DNA in the VG and VL of randomly selected domestic cats using PCR. FHV-1 DNA was detected in the VG of 14% of the cats. There was no detectable FHV-1 DNA in the VL of any cat. None of the infected cats had vestibular signs related to the VG infection.

Keywords: FHV-1, Feline herpesvirus, Vestibular ganglion, Labyrinth, PCR.

Body of manuscript

Feline herpesvirus-1 (FHV-1) is a widespread alpha-herpesvirus causing acute upper respiratory tract and ocular disease in cats. More than 80% of cats become latent carriers after infection despite a specific immune response. The primary site of latency is considered to be the trigeminal ganglion (TG). Reactivation and shedding of virus occur spontaneously or after stressful stimuli [3]. Herpes simplex virus type-1 (HSV-1) has been detected in the vestibular ganglion (VG) and labyrinth (VL) in humans. Reactivation of HSV-1 in the VG is assumed to cause vestibular neuritis, and benign paroxysmal positional vertigo could be a sequel of viral labyrinthitis [1]. To date, none of these structures has been investigated for the presence of FHV-1 in healthy or diseased cats. This pilot study was launched to screen for FHV-1 DNA in the VG and VL of randomly selected domestic cats using PCR.

Tissue specimens were obtained from 50 cats (31 males, 19 females) presented to the Department of Small Animal Medicine, Ludwig-Maximilians University, Munich. All cats underwent post-mortem examination between 2007 and 2008. Their age ranged from 4 weeks to 17 years (mean ± standard deviation [SD]: 6.82 ± 5.39 years). Altogether, the VL and the VG of 97 temporal bones, and 60 TG were harvested as described elsewhere [5]. All samples were collected within 48 h after death, snap frozen in liquid nitrogen, and stored at -80 °C. DNA extraction was performed with a commercial kit (DNA Micro Kit, Qiagen) followed by spectrophotometric measurement of total DNA content (NanoDrop 1000, peQLab). The presence of amplifiable DNA was confirmed by PCR of a 229 bp segment of the Gapdh gene [4]. For PCR-based detection of FHV-1, 0.3 µg of total DNA was used to amplify a 383 bp fragment of the FHV-1-thymidine-kinase-gene [6]. DNA extracted from FHV-1 infected cultured cells was used as positive and water as negative target template in each PCR run. All clinical and pathological records were screened for neurological and non-neurological diseases and the cat owners were questioned about the vaccination status.

A mean DNA content of 2.8 µg (± 0.9) was obtained from the VL, 4.5 µg (± 1.1) from the VG, and 4.1 µg (± 1.2) from the extracted portion of the TG. FHV-1 DNA was detected in the VG of 14% of the cats with a unilateral distribution in six and bilateral affection in one of the cats. Thirty-two per cent of the examined cats harbored FHV-1 DNA in the TG.

All bilaterally investigated TGs were affected on both sides (Table 1). All available TG from VG-infected animals were positive for FHV-1. Twenty-two per cent of the TG positive temporal bones also exhibited FHV-1 in the VG. No FHV-1 DNA was detected in the VL of any cat. FHV-1 positive cats showed no sex predilection and ranged from 2.5 to 16 years of age (mean ± SD: 9.29 ± 4.64). All infected cats presented with systemic underlying diseases. The vaccination status is depicted in Table 2. The presence of viral DNA in the VG was not accompanied by related vestibular deficits.

Table 1: FHV-1 infected tissues.

Cat number	VG left	VG right	TG left	TG right	VL left	VL right
8	+	—	n/a	n/a	—	—
13	+	—	n/a	n/a	—	—
16	—	—	+	+	—	—
19	—	—	+	+	—	—
21	+	+	+	n/a	—	—
24	—	+	+	n/a	—	—
27	+	—	+	+	—	—
29	—	+	+	+	—	—
35	—	—	+	+	—	—
37	—	—	+	+	—	—
40	+	—	+	+	—	—
48	—	—	+	+	—	—

TG: trigeminal ganglion; VG: vestibular ganglion; VL: vestibular labyrinth; n/a: not available.

Table 2: Summary of vaccination status of investigated cats.

Vaccination status	Investigated cats	Cats with infected VG	Cats with infected TG
Yes	33	7	9
No	11	0	1
Data not available	6	0	0
Total	50	7	10

VG: vestibular ganglion; TG: trigeminal ganglion.

To date, the presence of FHV-1 in the VG of domestic cats has not been documented in literature. In the present study, we identified FHV-1 in 14% of randomly collected cats; this incidence ranged slightly below infection rates that we and others have observed in the TG. Notably, in all cats for which the complete data set was obtainable, infection of VG was accompanied by involvement of the TG. On the contrary, 78% of temporal bones with positive TG did not involve the VG. Bilateral TG affection is common after experimental infection via relevant natural routes, namely conjunctival sacs and external nares. Except for one cat, all cats harboring FHV-1 DNA were vaccinated. In the unvaccinated cat and the cat vaccinated with non-live-vaccine an intraganglionic presence of field-virus can be postulated, whereas the virus type in the other cats remains unclear. Cats vaccinated with modified-live-vaccine (MLV) could either exhibit FHV-1 positive ganglia due to infection with field-virus, exposition to vaccine-virus or superinfection. Intranasally applied MLV has been shown to establish latency in the TG [10]. MLV applied subcutaneously is less virulent because of the higher core temperature at the injection site, compared to the upper respiratory airways, that interferes with viral replication. The absence of marker FHV-1 vaccines, however, makes any further discrimination between vaccine and field-virus in these cats impossible [7]. Since infected VG were associated with infected TG but not vice versa, a primary involvement of the TG with subsequent infection of the VG seems probable. In humans, HSV-1 is thought to ascend within the lingual nerve from the primary site of infection to the trigeminal and geniculate ganglion.

Viral reactivation is then followed by infection of the VG via faciovestibular anastomosis [8]. This anastomosis has also been described in dogs and cats [2], but it seems to be rather inconsistent (unpublished data). Anterograde spread from the TG to the trigeminal brain stem nuclei with subsequent infection of the vestibular nuclei, via trigeminovestibular projections, and the associated ganglion is the most likely infection route.

Whether the FHV-1 infection of VG neurons is of latent type or lytic is unknown. Detection of latency-associated transcripts (LATs) or the absence of other viral gene transcripts may provide evidence for latency, whereas the absence of LATs and the presence of other viral gene transcripts could indicate a productive infection [9]. All of the FHV-1 positive cats may have been immunosuppressed due to underlying systemic diseases so that reactivation of a latent infection seems plausible. In order to elucidate the impact of FHV-1 infection on the vestibular function, further cats with vestibular signs need to be investigated. In-situ hybridization or RT-PCR for detection of LATs would be helpful to determine the type of infection. Furthermore, quantification of infected and/or damaged neurones in the VG would be necessary to correlate an infection with the degree of possible vestibular impairment.

In conclusion, we detected FHV-1 DNA in the VG of 14% of the cats with a unilateral distribution in six and bilateral affection in one of the cats. There was no detectable FHV-1 DNA in the VL of any cat. Infected VG were associated with infected TG but not vice versa. None of the infected cats had vestibular signs related to the VG infection.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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3.2 Naturally-occuring canine herpesvirus (CHV-1) infection of the vestibular labyrinth and ganglion in dogs

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Naturally-occurring canine herpesvirus (CHV-1) infection of the vestibular labyrinth and ganglion in dogs

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Figures: 1

Tables: 1

Abstract

In humans, the involvement of herpesviruses in vestibular diseases has been subjected to research for many years whereas detailed information in companion animal species is restricted to cats. This study was conducted to assess the prevalence of canine herpesvirus 1 (CHV-1) in the canine vestibular labyrinth (VL) and vestibular ganglion (VG) by PCR. Field-virus was detected in the VL of 17% and in the VG of 19% of 52 dogs.

In two of five dogs with vestibular dysfunction, the possible contribution of the CHV-1 infection could not be excluded. Distribution of CHV-1 infected tissues in dogs differed significantly from FHV-1 infected tissues in cats thereby indicating different infection routes. As in the other investigated species, the causative role of herpesviruses in canine vestibular diseases remains to be further elucidated.

Keywords: Vestibular ganglion, herpesvirus, canine, labyrinth, infection

Introduction

Canine herpesvirus 1 (CHV-1) is a worldwide distributed alpha-herpesvirus, which causes fatal hemorrhagic disease in newborn puppies and mild upper airway and genital tract infections in adult dogs [4]. Like other alpha-herpesviruses, sensory ganglia as the trigeminal and lumbosacral ganglia are considered a site of latency [9].

In humans, various herpesviruses have been detected in the vestibular nuclei, the vestibular labyrinth (VL) and ganglion (VG) and in the endolymphatic sac [1; 5; 15]. Reactivation of latent virus is thought to cause inflammation and vestibular dysfunction in Ramsay Hunt syndrome, benign paroxysmal positional vertigo, vestibular neuritis and Ménière's disease [6; 10]. In a previous study, we identified feline herpesvirus-1 (FHV-1) DNA in the VG of cats. Within the investigated population, the infection could not be related to vestibular signs [12]. In order to shed light on the epidemiology of vestibular CHV-1 infection in dogs and its possible clinical implications, we screened the VG and VL of randomly selected dogs for the presence of virus DNA using polymerase chain reaction (PCR).

Material and methods

Tissue specimens were obtained from 52 dogs (26 males and 26 females; various breeds) that underwent post-mortem examination at the University of Munich in 2007 and 2008. Their age ranged from 3 days to 15 years (mean ± standard deviation [SD]: 5.68 ± 4.53 years).

Altogether, the VL and the VG of 100 temporal bones and 65 trigeminal ganglia (TG) were harvested as described earlier [11]. All samples were collected within 48 h after death, snap frozen in liquid nitrogen, and stored at -80°C until further investigation. DNA extraction was performed using two commercial kits (DNA Micro Kit, Blood and Tissue Kit, Qiagen) followed by spectrophotometric measurement of total DNA content (Nano-Drop 1000, peQLab). The presence of amplifiable DNA was confirmed by PCR of a 229 base pair (bp) segment of the canine GAPDH gene [7]. For PCR-based detection of CHV-1, 0.3 µg of total DNA was used to amplify a 450 bp fragment of the CHV-1 glycoprotein gene B [13]. DNA extracted from CHV-1 infected cultured cells was used as positive control and water as no template control in each PCR run.

All clinical and pathological records were screened for neurological and non-neurological diseases and the pet owners and/or referring veterinarians were questioned about the vaccination status. In accordance with the literature, vestibular signs were classified as peripheral, central or paradox [3].

Results

CHV-1 DNA was detected in the VL of nine dogs (17%) with a unilateral affection in three and bilateral involvement in six animals. Ten dogs (19%) harbored CHV-1 DNA in the VG with a unilateral and bilateral affection in any five animals. Four out of thirty-three dogs with available TG (12%) showed bilateral infection of the TG (Table 1). Distribution of CHV-1 infected tissues in dogs with a complete data set (14) is depicted in Fig. 1. CHV-1 positive dogs showed no sex predilection and ranged from 17 days to 12 years of age (mean ± SD: 4.65 ± 3.84). None of the infected dogs had been vaccinated against CHV-1.

A 20 day old pup, which revealed CHV-1 DNA in the VL, VG, and TG, had presented with acute fatal CHV-1 infection. Histology revealed multifocal necrosis in several organs and a variety of other tissues such as lung, liver, and spleen proved positive for CHV-1 (data not shown). All the other dogs suffered from systemic diseases of non-CHV origin.

A vestibular dysfunction was reported in five animals. Two CHV-1 negative dogs exhibited a central unilateral vestibular disease. Another dog showed central vestibular signs caused by a meningioma and associated brain edema. This dog had a contralaterally infected VG. Two other dogs had presented with a paradox vestibular syndrome due to an ependymoma at the cerebellopontine angle and an asymmetric descending (caudal) transtentorial herniation caused by a pituitary macroadenoma, respectively. CHV-1 DNA was detected in both VL in the former and in one VG in the latter dog. None of the examined dogs had presented with peripheral vestibular signs.

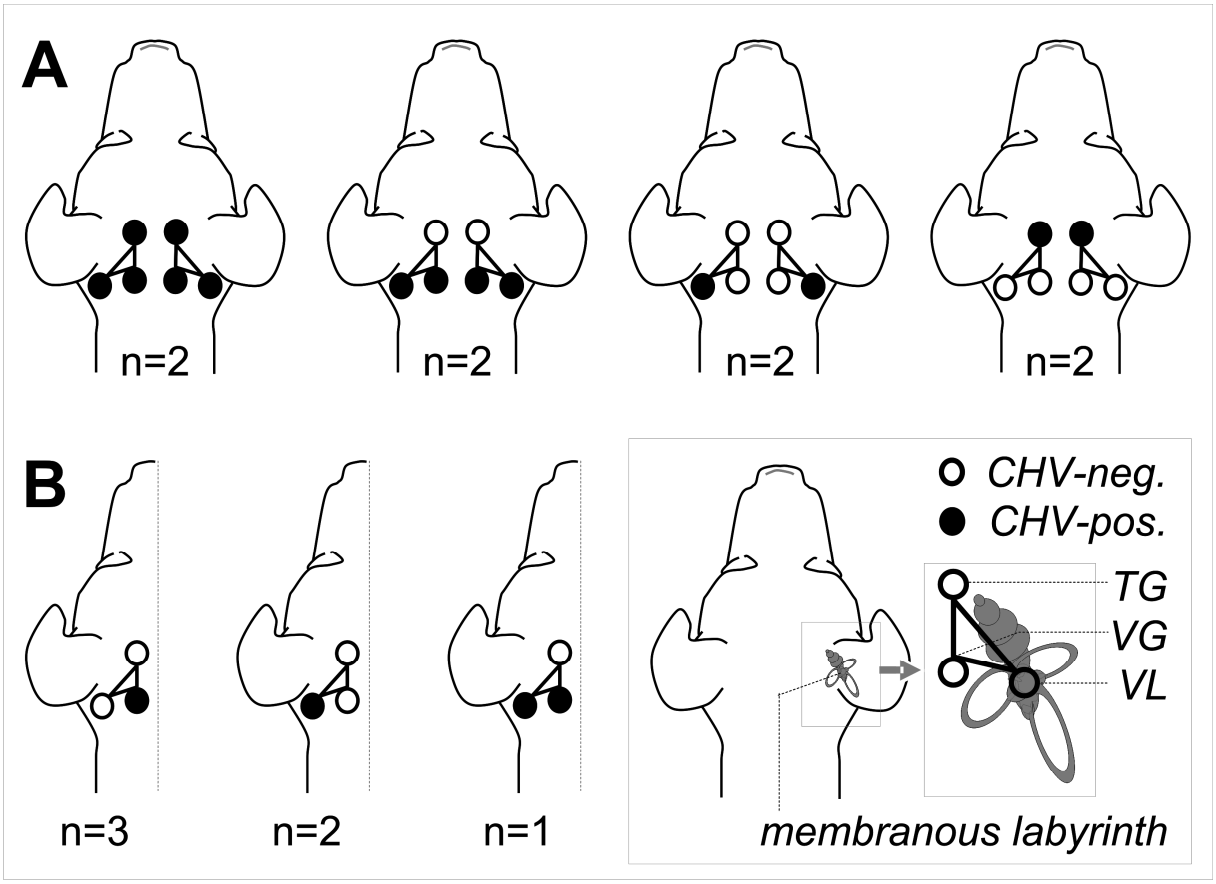

Figure 1: Schematic drawing of the distribution of CHV-1 infected tissues in dogs (n=14) with a complete data set. A, bilateral affection; B, unilateral affection of examined tissues; TG, trigeminal ganglion; VG, vestibular ganglion; VL, vestibular labyrinth.

Legend to Table 1 (next page):

d, days; w, weeks; y, years; n/a, not available; L, left; R, right.

Table 1: Spatial distribution of CHV-1 infected tissues.

Dog breed	Age	Sex	CHV-1 associated disease	Vestibular disease	Side	VL	VG	TG
Yorkshire Terrier	9 y	♀	—	—	L	+	+	—
					R	—	—	—
Mongrel	20 d	♂	yes	—	L	+	+	+
					R	+	+	+
Labrador cross	6 y	♂	—	—	L	+	+	+
					R	+	+	+
Munsterlander	2 y	♀	—	—	L	+	+	—
					R	+	+	—
Maltese cross	6.5 y	♀	—	—	L	+	—	—
					R	+	—	—
Samoyede	11 w	♂	—	—	L	+	—	—
					R	—	—	—
Puli	4 y	♂	—	yes	L	—	+	—
					R	—	—	—
Mongrel	5 y	♂	—	—	L	+	+	—
					R	+	+	—
Miniature Pinscher	1 y	♀	—	—	L	—	—	—
					R	—	+	—
Bernese Mountain Dog	2 y	♀	—	—	L	—	—	—
					R	+	—	—
Labrador Retriever	9 y	♀	—	yes	L	+	—	—
					R	+	—	—
Poodle	9 y	♂	—	yes	L	—	+	—
					R	—	—	—
Pekinese cross	12 y	♀	—	—	L	—	—	+
					R	—	—	+
Dobermann Pinscher	7 y	♂	—	—	L	—	—	+
					R	—	—	+
Norfolk Terrier	17 d	♀	—	—	L	—	—	n/a
					R	—	+	n/a
Beagle	1.5 y	♀	—	—	L	—	+	n/a
					R	—	+	n/a

Discussion

Clinically silent inner ear infections by CHV-1 affected nearly 20% of the randomly selected dogs investigated in this study. In humans, the involvement of herpesviruses in vestibular diseases has been subjected to research for many years. In veterinary medicine detailed information regarding herpesvirus infection of the vestibular system in companion animal species is restricted to cats [12].

This study was initated in order to elaborate base data on the prevalence of CHV-1 in canine vestibular compartments as a pilot to future investigations regarding the possible association with vestibular dysfunction in this species. An infection of the canine VG was identified in 19% of tested individuals which ranges slightly higher than what we have observed in cats (14%) [12]. In contrast to the latter, herpesvirus DNA was also found in the vestibular labyrinth of 17% of the dogs.

The involvement of this receptor zone for sensation of equilibrium could possibly interfere with mechanoelectrical transduction and signal transmission if the epithelial cells, the calyx or bouton like nerve endings resemble the target structures, as previously shown in hamsters experimentally infected with Herpes simplex virus type-1 (HSV-1) [2].

Concerning the clinical relevance of the vestibular CHV-1 infection, 11 of 14 positive dogs did not show vestibular dysfunction and vestibular signs in one of the remaining dogs was not related to the infection but an intracranial neoplasia. In another two dogs suffering from intracranial neoplasia the contribution of VL or VG infection to vestibular deficits is unlikely but cannot reliably be ruled out. A similarly low evidence of clinical significance was previously observed in cats [12].

Apart from the viremic puppy, the clinical data suggest either latent or infectious virus with subliminal impairment of functional structures. In humans, latent infection of the VG and other cranial nerve ganglia by members of the Herpesviridae have been demonstrated [14]. Distress can stimulate virus reactivation and replication at any time with subsequent host cell damage and associated deficits. Reactivated virus may spread from other cranial nerve ganglia to the VG or it is reactivated within the VG causing neuronal and axonal degeneration in loco. From there, virus travels via vestibular nerve branches and possibly via perilymph to and within the membranous labyrinth [2; 6; 10].

Omission of CHV-1 vaccination identifies the virus detected in our dogs as field-virus. In contrast to the results of human studies, CHV-1 DNA was found in the VL in 4 out of 14 dogs without concurrent VG infection, which could either reflect separate primary infection of the VL or a virus translocation from the VG-based cell body to the axon terminal.

Regarding the route of infection, it is noteworthy that in cats VG infection was always accompanied by an infected TG [12], whereas a simultaneous infection of the TG in dogs does not appear to be mandatory. In humans, an ascending infection of the TG and geniculate ganglion via the lingual nerve, with successive spread to the VG and VL via facio-vestibular anastomosis has been discussed [14]. Similarly in dogs, the oronasal mucosa resembles the most important entry site, followed by local replication and infection of the trigeminal nerve branches [9]. Although our results do not necessarily support a preceding infection of the TG, it needs to be considered that the VG, in contrast to other cranial nerve ganglia such as the geniculate ganglion or the TG has no direct connection with sensory nerve endings of the body surfaces [5]. Thus, spread from the TG to trigeminal brain stem nuclei with subsequent infection of the vestibular nuclei, via trigemino-vestibular projections, followed by centrifugal virus dissemination to the VG and/or VL would be a rather likely route. Moreover, an infection of the VG and/or VL via facio-vestibular anastomosis needs to be considered.

Apart from the neurogenic route, the VL can also be infected from the meninges via endolymphatic duct and cochlear aquaeduct, which resemble the drainage pathways of endolymph and perilymph into the intracranial venous sinuses and the subarachnoid space, respectively [8]. The latter has been demonstrated in HSV-1 infected hamsters. In accordance with these experiments, hematogenic infection of the VL appears unlikely, while, the TG can also be infected secondary to viremia [2].

Conclusions

The results of this study provide the essential basis for future investigation regarding the potential role of herpesvirus infection in vestibular diseases in dogs. Since most of the dogs with VL and/or VG infection lacked related vestibular deficits, the contribution of CHV-1 infection to vestibular disease remains momentarily unclear. Future studies using in-situ techniques will determine the exact cellular targets of CHV-1 and clarify if neurophysiologically relevant structures are affected and/or damaged. Besides, dogs with clinically unambiguously diagnosed peripheral vestibular disease should be examined to further elucidate the significance of the presence of CHV-1 in vestibular compartments. In addition, clinical trials with antiviral treatment in dogs suffering from vestibular disease may help to resolve open questions since application of antiviral drugs achieved control of vertigo in 90% of human patients with vestibular neuritis and Ménière's disease and in almost 70% of patients with benign paroxysmal vertigo [6].

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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4. Discussion

4.1 General aspects of the study

In humans, members of the *Herpesviridae* have been detected in different compartments of the vestibular system [10; 61; 230] and are associated with various vestibular diseases [65]. In cats and dogs, however, herpesvirus infections of vestibular inner ear structures have not been a subject of investigation so far, although vestibular diseases are common [49; 144] and herpesvirus infections show a high prevalence in these species [50; 75].

To date, studies of canine and feline vestibular inner ear structures have concentrated on morphological [36; 39; 62; 143] and immunohistochemical [41] investigations. Reports on molecular analyses of the canine and feline vestibular labyrinth (VL) and the vestibular ganglion (VG), however, do not exist in the literature. Conventional temporal bone processing, which includes long-lasting decalcification following fixation procedures [36], is very time-consuming and has negative effects on nucleic acid preservation [15; 54; 154]. The first aim of the present study, therefore, was to establish a suitable and fast method allowing for both morphological and molecular evaluation of the VL and VG harvested from post-mortem samples in cats and dogs.

4.2 New method for feline and canine temporal bone processing

This study, which included 44 temporal bones of 14 cats and eleven dogs presented for post-mortem examination, successfully demonstrated that the described anatomical preparation after Fast Green FCF tracing is a fast method applicable for both DNA analysis and morphological evaluation of vestibular inner ear structures. However, distinct practical skills and the ability of three-dimensional imagination are absolutely necessary for applying this preparation technique. Even though, the basic approach may be achieved within some weeks, preparation remains a great challenge and affords maximal concentration and fingertip feel due to the anatomical variations amongst and between different species.

The foramen vestibuli and the foramen cochleae can easily be located within the temporal bone. These structures serve as landmarks for identification of the three vestibular ampullae. The lateral and anterior ampulla, which are right next to each other, were located caudo-medial to the foramen vestibuli and varied in position within a radius of approximately 1-2 mm.

The posterior ampulla was situated caudal to the foramen cochlea with similar position variations. In particular, the position of the ampullae in relation to the fenestra vestibuli and cochleae, and their location within the petrous temporal bone was found to be individually variable. Ampulles in cats and small dogs were more superficially localized than in middle- and large-sized dogs and did not vary as much in position. In general, the anterior and lateral ampullae are easier to locate, prepare and to harvest than the posterior ampulla. Preparation of feline vestibular inner ear structures does not raise any special difficulties, whereas preparation of canine temporal bones is far more challenging due to the great variety of ampulla location and an increased fragility of the temporal bone if compared to the compact texture in cats. As mentioned above, standard temporal bone processing includes long-lasting fixation and decalcification which further delays vestibular inner ear investigations for several months [36; 37]. Using a dissection microscope and the dye Fast Green FCF, removal of the VL and VG from the left and right inner ear was achieved within 2 h in cats and within 3 h in dogs. In addition, stepwise preparation also allows for thorough gross examination [140] which would be impossible if the entire temporal bone was fixed, decalcified and embedded in accordance to previous protocols.

Peri-/endolymphatic injection of Fast Green FCF resulted in an intense staining of the membranous labyrinth and hence facilitated identification and preparation of these vestibular inner ear structures.

Fast Green FCF is a sea green triarylmethane food dye, that is also used in scientific research [141; 215]. In the past, it has already been applied for visualization of the semicircular ducts and ampulles of the vestibular labyrinth [2; 45]. In addition, Fast Green FCF is an acidophilic dye used in histology [20; 141] and electrophoresis for protein staining [131; 141].

The results obtained in this study demonstrate that the application of Fast Green FCF does not impair tissue morphology on light and electron-microscopic level. Furthermore, there was no evidence of a potential interaction of Fast Green FCF with genomic or viral DNA in our study, possibly impeding downstream DNA-analyses.

Chemicals necessary for conventional vestibular inner ear processing include fixatives and decalcifiers that might exert negative effects on the integrity of DNA. Common fixatives used on vestibulocochlear inner ear specimens are aldehydes (e.g. form- [23; 39; 42], para- [37; 38; 40] and glutaraldehyde [39; 143; 187]) and to a lesser extent alcohols [39].

Aldehydes have been reported to cause cross-linking and degradation of DNA and therefore negatively influence DNA integrity in a time-dependant manner [15; 54]. Alcohol-based fixatives on the other hand act as a good preserving agent of nucleic acids and are, therefore, much more suitable for subsequent DNA-analyses [15; 54; 102]. However, alcohols are rarely used as fixatives for vestibular inner ear structures [39].

Strong decalcifiers such as formic, nitric or hydrochloric-based acids allow rapid decalcification, however, they impede further DNA analyses due to degradation of DNA [4; 133; 177; 224]. Ethylene diamine tetraacetic acid (EDTA) has been reported to be superior to strong decalcifiers in terms of molecular investigations, but relatively slow-acting [4; 177; 224]. Using EDTA, decalcification of osseous samples on the scale of feline or canine temporal bones takes a long time.

In this investigation, spectrophotometric measurements confirmed sufficient amounts and purity of isolated genomic DNA from our vestibular inner ear samples. The preserved integrity of isolated DNA was demonstrated by PCR of a fragment of the housekeeping gene GAPDH (229 bp) and the target genes [FHV-1 (383 bp) and CHV-1 (450 bp)].

Even though autolysis in the membranous labyrinth starts early and may raise difficulties in cadaver studies, the 48-h time frame still allowed for DNA-analysis, gross, microscopic, and ultrastructural evaluation.

Taken together, this novel method for investigation of vestibular inner ear samples is advantageous compared to anatomical standard techniques, since the performance of DNA-analysis has been achieved for the first time in addition to previously reported morphological examinations.

4.3 Experimental design of the study

The present study was designed to address the question if the vestibular labyrinth and ganglion of cats and dogs is a possible target for canine or feline herpesvirus infections.

Herpesvirus infections in general can be more or less confidently diagnosed using a broad range of diagnostic tools, including enzyme-linked immunosorbent assay [176; 183; 185; 228] or serum neutralization test [109; 176; 228] for the detection of antibodies or virus isolation [91; 157; 158], IHC [24; 136], ISH [111; 112], PCR [52; 185; 223] and ELMI [125; 188; 202] for direct virus evidence. These methods differ with respect to their specificity, sensitivity and, therefore, their diagnostic value concerning a potential virus infection.

Seroprevalences in cats and dogs are usually very high due to the widespread use of vaccines especially within the cat populations and the great distribution of FHV-1 and CHV-1 in general [50; 75]. Moreover, seroprevalences are not synonymous with tissue infection and, therefore, detection of anti-herpesvirus-antibodies does not provide sufficient evidence for a possible herpesvirus infection of a distinct tissue type. IHC demonstrates virus antigen and thus proves tissue infection. However, IHC can only detect infectious virus while latent virus is missed due to the lack of antigen during latency [21]. Additionally, in chronic conditions, viral antigens may be bound by secreted antibodies which interferes with binding of the primary IHC antibody and, therefore, may lead to false negative results [134].

Virus isolation procedures are often used in the diagnosis of herpesvirus infections and serve as an excellent tool for the confirmation of infectious virus [21]. However, detection of latent virus, immature virus, which is not fully infective, or detection of virus with damaged envelope due to improper sample handling, cannot be achieved applying these methods [24]. Diagnostic tools such as electron microscopy or ISH can exactly localise virus particles, or viral DNA, respectively, within different cells and provide additional information about possible morphological changes induced by virus infection. However, ELMI is not capable of detecting a latent herpesvirus infection and ISH is known to have a comparably low sensitivity for demonstrating viral DNA in tissue sections [21]. In addition, in a study designed as a large scale screen of vestibular inner ear samples for the presence of herpesvirus infections, proper application of these methods would afford unreasonably high technical efforts.

Since a qualitative detection of herpesvirus-DNA is representative for both infectious and latent herpesvirus [21], the possible presence of FHV-1 and CHV-1 DNA in vestibular inner ear structures, analysed in the present study, was performed by PCR. Compared to other standard techniques commonly used in herpesvirus diagnostics, PCR has previously been shown to be more sensitive and specific [24; 136; 211]. Arbusow et al. [10] were the first to demonstrate HSV-1 DNA in the VL and VG of human temporal bones via PCR. Since PCR allows for a rapid and repeatable screening of large sample numbers, it was, therefore, designated as the method of choice for the present experiments.

Former studies in humans have estimated that only 1%–10% of ganglion cells may be infected with HSV-1 and that infected neurones are grouped in clusters [21; 68; 71; 72]. For the spatial distribution and exact cellular assignment of herpesvirus infected cells within the human vestibular labyrinth, no information exists. This knowledge, however, is essential for the understanding of the underlying pathobiology and, therefore, it is absolutely necessary for estimating the impact of vestibular labyrinth infection on vestibular function.

Assuming the possibility of a low share of herpesvirus infected cells and their potential uneven distribution within the tissues to be investigated, each the complete VL and VG were subjected to further DNA-analysis in the present study, in order to exclude false negative results due to analysis of non-representative sample sizes. Since PCR performed in our study exclusively provided qualitative information on the presence of genomic or viral DNA sequences in a given sample, it did not determine the viral load within the investigated samples or the transcriptional activity of the virus genome. The answer of these further questions will remain subject of future research. In order to address the question if the vestibular labyrinth and ganglion is a possible target for canine and feline herpesvirus infections, PCR is the method of choice.

4.4 Field versus vaccine virus

While omission of CHV-1 vaccination identifies the virus detected in the dogs as field-virus, the situation in the investigated cats is not as clear. Except for two animals, all cats harboring FHV-1 DNA had been vaccinated with a modified live virus vaccine (MLV). One cat had been vaccinated with a none-live virus vaccine and one cat had not been vaccinated at all. In these two cases, infection with field-virus can be assumed. Due to the lack of commercially available marker vaccines, however, differentiation between field-virus and MLV is impossible, using standard PCR protocols for detection of FHV-1 [75; 135; 203; 211]. Weigler et al. [221] showed that frequency and distribution of FHV-1 infected tissues for vaccine-only and vaccine-challenge groups were identical thus indicating that MLV applied intranasally has the capability of establishing latency. MLV applied subcutaneously, however, is less virulent because of the higher core temperature at the injection site, compared to the upper respiratory airways that interferes with viral replication [75; 119]. While MLV applied intranasally has the ability to become latent in the vaccinated cat, the biologic properties of subcutaneously applied MLV concerning the possibility of latency are still unclear. Thus, cats subcutaneously vaccinated with MLV could either exhibit FHV-1 positive VG due to infection with field-virus, exposition to vaccine virus or superinfection.

In the future, introduction of commercial marker vaccines and establishment of PCR protocols for unambiguous identification of vaccine-virus will allow to distinguish between field and vaccine-virus. The implementation of such marker vaccines is nowadays mainly applied in productive livestock [59; 104; 216], e.g. for classical swine fever vaccination [128]. For cats, different promising FHV-1 marker vaccines have been developed in the past [178; 231]. Their application, however, has been restricted to experimental research so far.

As soon as the genomes of various field-virus strains, and importantly, also of distinct vaccine viruses are fully sequenced, and these sequences are made available in public data bases, sequence analyses of eligible genes will probably enable a distinction between different field-virus strains and known vaccine viruses. Performance of sequence analyses on virus isolates from investigated sample materials could then also be applied as a diagnostic tool.

4.5 Type of virus infection

In general, detection of viral DNA in the investigated tissues could either represent infectious (primary, low-level persistent infection or reactivation of latent virus) or latent virus [21]. Depending on the age and immune status of the animal, primary infection with FHV-1 or CHV-1 can either be subclinical or cause mild or severe clinical signs [50; 75]. Latent infections and low level persistent infections differ in terms of transcriptional activity of the viral genome [21]. During latency, only latency-associated transcripts (LATs) are abundantly produced, and the transcription of other viral genes is largely suppressed, whereas low-level persistent infection is accompanied by a typical lytic virus cycle with expression of the entire set of viral proteins [21; 107]. Reactivation of latent virus may be induced by different types of distress and causes a change from the latent to the productive/lytic virus cycle with subsequent damage of infected cells [21; 65; 107]. After reactivation, infectious virus may travel from its site of latency (e.g. the neuron) to the periphery where further virus replication particularly within mucosal sites takes place and virus is shed and possibly transmitted to susceptible hosts [21; 75; 182]. In humans, it has been proposed that different strains of HSV-1 have the ability to either travel retrograde or anterograde after reactivation from the VG thus determining to a large degree the clinical expression of viral ganglionitis [68; 70; 72]. While the McIntyre B strain of HSV-1 flows towards the periphery after reactivation from the VG and may cause sensorineural hearing loss together with episodic vertigo (Ménière's disease), the H 129 strain is preferentially carried towards the brain and, therefore, may also cause central signs [68; 70; 72]. Virus reactivation in general may be but is not necessarily accompanied by overt clinical signs [21]. Whether the FHV-1 infection of the VG is a latent or lytic type is unkown. Qualitative detection of LATs or the absence of other viral gene transcripts may provide evidence for latency, whereas the absence of LATs and the presence of other viral gene transcripts could indicate a productive/lytic infection [213].

The degree and progress of post-mortem RNA degradation, and in particular, degradation of distinct transcripts [12; 31] is variable, and, basically, depends on the presence and activity of RNA degrading enzymes [56; 98] (RNAses), the temperature [121], the pH-value [31] and humidity [160], the post-mortem time-interval [31] and the type of tissue [12; 56] and its pathological alterations [31].

Therefore, common procedures for RNA-detection generally require the use of fresh sample materials [53; 56]. The feasibility of detection and quantification of distinct RNAs also depends on the applied RNA extraction method, as well as on specific properties of the investigated transcripts themselves (transcript length, abundance, sequence, and degree of degradation), and additionally differs with respect to the applied analytical methods (as e.g. RT-PCR, real-time PCR, gene array, etc.) [12; 31; 56]. The acceptable timeframe for generation of sample materials for analyses of distinct transcripts by a particular analytical method, has therefore in each case to be estimated in preliminary experiments under defined conditions [12].

Since the described preparation technique permits recovery of vestibular inner ear samples from both ears within 2 h in cats and 3 h in dogs, retrieval of these structures immediately after death may allow for performance of RNA-based analyses. Thus, the qualitative detection of viral transcripts could allow for distinction of latent and lytic herpesvirus infections.

Since all of the FHV-1 positive cats in this study may have been immunosuppressed due to underlying systemic diseases, reactivation of a latent infection seems possible. The puppy suffering from fatal hemorrhagic disease displayed CHV-1 DNA not only within vestibular inner ear structures, but also within the liver, spleen and lung. Besides, multifocal necrosis was present in a variety of organs, thus indicating viremia and the presence of infectious virus [30; 166; 226]. Comparable to the cats, reactivation of latent virus or low-level persistent infection in the remaining dogs seems plausible, since they also suffered from systemic diseases.

4.6 Distribution of FHV-1/CHV-1 infection

FHV-1 DNA was detected in the VG of 14% of the cats with a unilateral distribution in six and a bilateral affection in one of the cats. Thirty-two percent of examined cats harboured FHV-1 DNA in the TG while no virus DNA could be found in the VL of any cat. Notably, in all cats for which the complete data set was obtainable, infection of the VG was accompanied by involvement of the TG. On the contrary, 78% of temporal bones with positive TG did not involve the VG. Analogous to studies performed in human cadavers, the percentage of infected TG in cats is higher than of infected VG [60; 210]. Since infected VG were always associated with infected TG (but not vice versa), a primary involvement of the TG with subsequent infection of the VG seems probable.

CHV-1 DNA was detected in the VL of nine dogs (17%) with a unilateral affection in three and bilateral involvement in six animals. Ten dogs (19%) harbored CHV-1 DNA in the VG with a unilateral and bilateral affection in any five animals. Four out of thirty-three dogs with available TG (12%) showed a bilateral CHV-1 infection. Here, peripheral vestibular system infection was not always accompanied by an infected TG. Thus, only 22% of VL and/or VG infected temporal bones revealed a simultaneous TG infection. The dogs in contrast to the cats also showed infection of the VL. The involvement of this receptor zone for sensation of equilibrium could possibly interfere with mechanoelectrical transduction and signal transmission if the epithelial cells, the calyx or bouton like nerve endings resemble the target structures. While VL infection was always accompanied by VG infection in a study of 21 human temporal bones, simultaneous infection of the VG was not always observed in our dogs [10]. Besides, infectious rates of the TG in the investigated dogs were lower than in the cats and even below the ones of VL or VG infection. Furthermore and opposed to the cats, TG infection was not mandatory for peripheral vestibular system infection. Hence, a CHV-1 infection of vestibular inner ear structures was only accompanied by CHV-1 infection of the TG in 22%.

4.7 Potential routes of virus infection

On the basis of FHV-1 distribution pattern within the investigated tissues it can be assumed that TG infection precedes VG infection in cats. Anterograde spread from the TG to the trigeminal brain stem nuclei with subsequent infection of the vestibular nuclei via trigemino-vestibular projections, and the associated ganglion seems to be the most likely infection route in the cat. In the dog, distribution of CHV-1 infected tissues was somewhat different compared to both cats and humans, and therefore permits several routes of infection. In humans, an ascending infection to the TG and geniculate ganglion via the lingual nerve with successive spread to the VG and VL via facio-vestibular anastomosis has been discussed [9; 156; 189; 210]. Similarly in dogs, the oronasal mucosa resembles the most important entry site, followed by local replication and infection of the trigeminal nerve branches [50; 142]. Although our results do not necessarily support a preceding infection of the TG, it should be considered that the VG, in contrast to other cranial nerve ganglia such as the geniculate ganglion or the TG, has no direct connection with sensory nerve endings of the body surfaces [61].

Thus, spread from the TG to trigeminal brain stem nuclei with subsequent infection of the vestibular nuclei, via trigemino-vestibular projections, followed by centrifugal virus dissemination to the VG and/or VL would be a rather likely route. Moreover, an infection of the VG and/or VL via facio-vestibular anastomosis needs to be considered.

Apart from the neurogenic route the VL can also be infected from the meninges via endolymphatic duct and cochlear aquaeduct, which resemble the drainage pathways of endolymph and perilymph into the intracranial venous sinuses and the subarachnoid space, respectively [124]. The latter has been demonstrated in HSV-1 infected hamsters [46]. In accordance to these experiments, hematogenic infection of the VL appears unlikely, while the TG can also be infected in the course of viremia [46].

4.8 Clinical relevance of vestibular herpesvirus infection

Concerning the clinical relevance of peripheral vestibular system infection with FHV-1 /CHV-1 in cats and dogs, most of the animals with an infected VG and/or VL did not show vestibular deficits. Vestibular disease in the remaining animals with VG and/or VL infection was attributed to other causes than herpesvirus infection. Thus, only two of seven cats with infected VG revealed vestibular signs which resulted from otitis media/interna in the one and a mass effect due to vasogenic brain edema in the other cat. Analogous to the cats, 11 of 14 dogs with VL and/or VG infection did not show vestibular dysfunction and vestibular signs in three dogs were related to an intracranial neoplasia. Despite intensive research, it is still controversial if herpesviruses in men are the causative agent for different vestibulopathies including vestibular neuritis, Ménière's disease, benign paroxysmal positional vertigo, etc. since they have been detected in vestibular compartments of both healthy and diseased humans [66; 74; 159; 173; 205; 222; 230]. In summary, the present study demonstrated the presence of FHV-1 and CHV-1 DNA in vestibular inner ear samples of cats and dogs for the first time. Since infection of the VG and/or VL was not associated with vestibular disease in the animals investigated in the present study, a possible contribution of vestibular herpesvirus infection to vestibular dysfunction remains to be further clarified. For investigation of potential subclinical alterations following herpesvirus infections of vestibular inner ear structures, future studies could employ methods as e.g. ISH or ELMI for identification of the infected cell types, and the degree of potential morphological injury.

The identification of such cellular patterns of herpesvirus infections and subsequent alterations in the vestibular system could then elucidate its possible functional consequences. For example, vestibular hair cells of adult mammals only retain a limited capacity of proliferation and regeneration [153; 162]. Infection and possible injury of these receptors might, therefore, directly result in hair cell loss and secondary vestibular ganglion cell degeneration. Infection and damage of supporting cells, which surround the hair cells [113], however, may not immediately cause subsequent hair cell loss. Thus, infection and impairment of hair cells as compared to supporting cells, could faster lead to vestibular dysfunction.

4.9 Conclusions and future prospects

We were the first to establish and publish a fast and successful method for retrieval of vestibular inner ear samples in cats and dogs suitable for molecular analyses in epidemiological studies. In the following, we were able to show for the first time that FHV-1 and CHV-1, very much like human herpesviruses [10], infect the VL and/or VG in cats and dogs. Thus, FHV-1 DNA could be demonstrated in the VG of 14% of 50 randomly selected cats while CHV-1 DNA has been detected in the VL of 17% and in the VG of 19% of 52 examined dogs.

Our studies provide the essential basis for future investigations regarding the potential role of herpesvirus infection in vestibular diseases in cats and dogs. In order to estimate the relevance of herpesvirus infections of the vestibular system in the pathogenesis of peripheral vestibular disorders, animals with clinically unambiguously diagnosed peripheral vestibular disease should be examined. Here, investigation of the peripheral vestibular system of cats and dogs with, and during episodes of benign idiopathic vestibular disease would be of particular interest. This approach will answer the question if the incidence of herpesvirus infections of the vestibular system correlates with idiopathic peripheral vestibular disease. A major limitating factor of these investigations, however, seems to be the retrieval of inner ear samples from these animals, since vestibular signs usually resolve within two weeks without therapy [144; 191; 212], and, therefore, it is rather unlikely that these animals will be euthanized and donated for post-mortem examination.

Appliance of the methodological approaches described in the present study would allow for resolving of important, yet unanswered questions. As mentioned above, these future queries would e.g. concern the identification of cell types in the vestibular system infected by herpesviruses, possible morphological alterations and their quantification in order to estimate their impact on vestibular function. Furthermore, studies based on the analyses of viral RNA- (LATs) and protein-expression in fresh samples of infected vestibular tissue by means of ISH [155], RT-PCR [213] and ICH [24; 142] would provide additional information on the respective type of infection (latent or lytic) and their morphologic pendants.

5. Summary

PCR-based investigation of the presence of herpesvirus in the peripheral vestibular system in cats and dogs

Peripheral vestibular diseases are a common cause of disequilibrium in both man and domestic mammals. In humans, herpesvirus infections of the peripheral vestibular system have been discussed as potential causes for various vestibular disorders. Vestibular inner ear structures are difficult to access and their examination is very time-consuming when using standard techniques. Up to now, only few morphological and immunohistochemical studies have been performed in cats and dogs, while molecular analyses have not been reported.

The aim of the present study was to evaluate if vestibular inner ear structures of dogs and cats are infected by herpesvirus. Consequently, a fast and reliable method for isolation of the vestibular labyrinth and ganglion had to be established. To ensure the preservation of tissue morphology and to avoid DNA degradation by autolytic processes, the inner ear preparation was performed within a 48-h post-mortem timeframe. After gross preparation of the temporal bone, a suitable dye was injected into the endo- and/or perilymphatic space for better visualization of the membranous labyrinth, followed by gradual removal of cortical bone under a dissection microscope. Retrieval of vestibular samples from both temporal bones per animal was achieved within 2 h in cats and 3 h in dogs. Light- and transmission electronmicroscopic investigations confirmed well-preserved vestibular cell morphology. Genomic DNA was isolated from the remaining samples of the vestibular labyrinth and from the vestibular ganglion, using standard protocols. Spectrophotometric measurement of DNA content and polymerase chain reaction (PCR) for amplification of housekeeping gene sequences (Gapdh) proved for sufficient amounts and integrity of generated DNA samples. Using specific PCR's, Feline herpesvirus-1 (FHV-1) and Canine herpesvirus-1 (CHV-1) - DNA sequences were detected in vestibular inner ear structures of cats and dogs for the first time. Clinical and pathological records were screened for underlying diseases especially with regard to vestibular dysfunction. Pet owners and veterinarians were questioned about additional anamnestic data and the vaccination status of the respective animals.

FHV-1 DNA was detected in the vestibular ganglion of 14% of the cats, whereas 17% and 19% of the dogs showed CHV-1 DNA in the vestibular labyrinth and ganglion, respectively. Besides, 32% and 12% of the cats and dogs displayed a bilateral infection of the trigeminal ganglion. In cats, FHV-1 infection of the vestibular ganglion was always accompanied by simultaneous involvement of the trigeminal ganglion, indicating a primary infection of the trigeminal ganglion. In dogs, however, a CHV-1 infection of vestibular inner ear structures was only accompanied by CHV-1 infection of the trigeminal ganglion in 22%. In 5 of 7 cats and in 11 of 14 dogs, vestibular herpesvirus infection did not cause vestibular dysfunction, and vestibular signs in the remaining animals were attributed to other causes than herpesvirus infections.

The results of these studies have been published in the Journal of Neuroscience Methods [163] and in The Veterinary Journal [164]. A manuscript entitled “Naturally-occurring canine herpesvirus (CHV-1) infection of the vestibular labyrinth and ganglion in dogs” is currently under revision at The Veterinary Journal.

The present studies, therefore, provide the essential basis for future examinations regarding the potential role and relevance of herpesvirus infections for vestibular diseases in cats and dogs. These investigations will identify the distinct cell types within the vestibular system infected by infectious or latent herpesvirus and quantitatively characterize the morphological alterations following infection.

6. Zusammenfassung

PCR-basierte Untersuchungen zum Vorkommen von Herpesvirusinfektionen des peripheren vestibulären Systems von Hunden und Katzen

Krankheitsbedingte Alterationen des peripheren vestibulären Systems treten als häufige Ursache von Gleichgewichtsstörungen bei Mensch und Tier in Erscheinung. In der Humanmedizin werden Herpesvirusinfektionen als mögliche Ursache unterschiedlicher vestibulärer Erkrankungsentitäten diskutiert. Die Entnahme einzelner vestibulärer Innenohrstrukturen ist äußerst anspruchsvoll und zeitaufwendig. Bei Hunden und Katzen wurden daher an diesem Sinnesorgan bislang nur wenige morphologische und immunhistochemische Studien durchgeführt während molekularbiologische Untersuchungen gänzlich fehlen.

Ziel der vorliegenden Arbeit war es, das mögliche Vorkommen von Herpesvirusinfektionen peripherer vestibulärer Strukturen bei Hunden und Katzen zu untersuchen. Hierzu wurde eine schnelle und sichere Präparationstechnik entwickelt. Um einen möglichst hohen morphologischen Erhaltungsgrad als auch die Integrität der DNA zu gewährleisten, wurden die Proben innerhalb eines Zeitrahmens von 48 Stunden post-mortem gewonnen. Zur besseren Darstellung des häutigen Labyrinths im Felsenbein wurde ein geeigneter Farbstoff in den endo- und/oder periplymphatischen Raum des Innenohres injiziert und der kompakte Knochen unter einer Stereolupe abgetragen. Vestibuläre Strukturen beider Innenohren konnten so bei Katzen binnen zwei und bei Hunden binnen drei Stunden gewonnen werden. Der gute Erhaltungszustand der Proben wurde durch licht- und elektronenmikroskopische Untersuchungen bestätigt. Genomische DNA wurde aus dem verbleibenden vestibulären Probenmaterial mittels Standardverfahren isoliert. Spektrophotometrische Messungen des DNA-Gehaltes sowie die Amplifikation eines Fragments des GAPDH Gens mittels Polymerase Kettenreaktion (PCR) belegten eine für weitere molekularbiologische Untersuchungen ausreichende Menge und Integrität der extrahierten genomischen DNA.

Die Anwendung der beschriebenen Präparationstechnik gestattete es im Folgenden erstmalig, das Vorkommen und die Prävalenz von Caninen (CHV-1) und Felinen Herpesvirus-1 (FHV-1) Infektionen in vestibulären Strukturen nachzuweisen.

Die klinischen Untersuchungsbefunde und Sektionsberichte der jeweiligen Tiere wurden mit besonderem Augenmerk auf Anzeichen für vestibuläre Alterationen studiert. Weitere anamnestische Daten sowie der Impfstatus der Tiere wurden bei den entsprechenden Tierbesitzern und behandelnden Haustierärzten erfragt.

Bei 14% der Katzen und 19% der Hunde wurde FHV-1 bzw. CHV-1 DNA in wenigstens einem Vestibularganglion nachgewiesen. Im vestibulären Labyrinth wurde CHV-1 DNA bei 17% der Hunde detektiert. Bei 12% der Hunde und 32% der Katzen konnte eine bilaterale Herpesvirusinfektion beider Trigeminalganglien nachgewiesen werden. Hinweisend auf einen möglichen Ausbreitungsweg der Herpesvirusinfektion zeigten sämtliche Katzen, bei denen FHV-1 DNA im Vestibularganglion detektiert wurde, ebenfalls eine Infektion des entsprechenden Trigeminusganglions. Ein derartiges Verteilungsmuster war hingegen lediglich bei 22% der Hunde festzustellen. Bei 5 von 7 Katzen und 11 von 14 Hunden war die nachgewiesene Herpesvirusinfektion peripherer vestibulärer Strukturen nicht mit einer klinisch feststellbaren vestibulären Dysfunktion vergesellschaftet. Die vestibulären Symptome der übrigen Tiere wurden auf andere Ursachen als eine Herpesvirusinfektion des peripheren vestibulären Systems zurückgeführt.

Die Ergebnisse der vorliegenden Arbeit wurden in den Fachzeitschriften des Journal of Neuroscience Methods [163] und The Veterinary Journal [164] veröffentlicht. Ein weiteres Manuskript mit dem Titel „Naturally-occurring canine herpesvirus (CHV-1) infection of the vestibular labyrinth and ganglion in dogs“ befindet sich derzeit beim The Veterinary Journal in Revision. Die methodischen und inhaltlichen Ergebnisse dieser Arbeit stellen somit die entscheidende Grundlage für weiterführende Untersuchungen bezüglich der Pathogenese und Relevanz von Herpesvirusinfektionen vestibulärer Innenohrstrukturen bei Hunden und Katzen dar. Gegenstand der unmittelbar nachfolgenden Studien wird beispielsweise die Identifikation der von infektiösen oder latenten Herpesvirusinfektionen betroffenen Zelltypen des peripheren vestibulären Systems sowie die quantitative Charakterisierung ihrer morphologischen Alterationen sein.

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