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A pilot study contributing to the virome of water buffaloes in Switzerland

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ZUSAMMENFASSUNG

Im Jahr 1996 wurden die ersten 15 Asiatischen Wasserbüffel *(Bubalus bubalis)* in die Schweiz eingeführt. Mittlerweile ist die Büffelpopulation auf über 1900 Tiere angestiegen. Das Virom von Wasserbüffeln in der Schweiz wurde bisher nicht untersucht. Ziel der vorliegenden Pilotstudie war es deshalb, erste Erkenntnisse auf diesem Gebiet zu gewinnen. Dazu wurden auf insgesamt drei Schweizer Betrieben Blutproben von Wasserbüffeln entnommen. Falls auf den drei Betrieben vorhanden, wurden kleine Wiederkäuer ebenfalls beprobt. In einer gesonderten Studie wurden die Büffelproben mittels Next Generation Sequencing (NGS) untersucht, welches auch die Detektion neuartiger Viren ermöglichte. Die vorliegende Arbeit befasst sich mit der Untersuchung der Büffel- und Kleinwiederkäuerproben mittels konventioneller spezifischer diagnostischer Methoden (PCR, ELISA, SNT), mit dem Ziel die NGS Ergebnisse zu bestätigen und zu erweitern.

Mittels NGS wurde lediglich ein Virus nachgewiesen, nämlich ein neuartiges Gemycircularvirus (GyCV). Im konventionellen Ansatz hingegen wurden fünf verschiedene Viren direkt oder indirekt in den Wasserbüffeln nachgewiesen. Die spezifische PCR ergab positive Resultate für das bovine Herpesvirus 6 (BoHV-6) und GyCV. In der Serologie wurden Antikörper gegen das bovine Herpesvirus 2 (BoHV-2), das Pestivirus bovines Virusdiarrhoe Virus (BVDV) und das Arbovirus Blauzungenvirus (BTV) in den Wasserbüffeln nachgewiesen. Das Virom der Wasserbüffel beinhaltete somit sowohl apathogene Viren (BoHV-6) als auch potentiell pathogene Viren, die Krankheiten der Haut (BoHV-2), des Respirations-, Urogenital- und Gastrointestinaltrakts (BVDV), aber auch generalisierte Krankheiten verursachen (BTV). Trotz Hinweisen für eine systemische Verbreitung von GyCV auf einem Bestand bleibt die Bedeutung dieser Beobachtung unklar.
Die negativen Befunde für bovines Herpesvirus 1 (BoHV-1) und bovines Leukämievirus (BLV) waren von besonderer Bedeutung, da diese Infektionen in der Schweiz als ausgerottet gelten. Infektionen mit den pathogenen ovines Herpesvirus 2 (OvHV-2), border disease Virus (BDV) und Schmallenbergvirus (SBV) wurden nur in den Kontaktschafen und im Fall von SBV in den Kontaktziegen gefunden, während die Wasserbüffel negativ getestet wurden. Es ist wohlbekannt dass OvHV-2 von Schafen auf Wasserbüffel übertragen werden kann und in letzteren das bösartige Katarrhalfieber (BKF) verursacht. Die Beobachtung, dass BVDV, nicht aber BDV auf die Wasserbüffel übertragen wurde, erfordert zusätzliche Aufmerksamkeit, vor allem in der Hinsicht dass BVDV in der Schweiz aktiv ausgerottet wird.

Abschliessend lässt sich sagen, dass die vorliegende Pilotstudie unser Wissen über das Virom von Schweizer Wasserbüffeln erweitert hat und diese Spezies als empfänglich für Interspeziesübertragung angesehen werden muss. Weitere Untersuchungen sind erforderlich, um ihre Rolle in der Erhaltung und Übertragung viraler Infektionen zu evaluieren, v.a. bezüglich BoHV-2, BVDV, BTV und GyCV.

Stichworte: Virom, Schweizer Wasserbüffel, Herpesvirus, Pestivirus, Arbovirus, Gemycircularvirus

ABSTRACT

In 1996, the first 15 Asian water buffaloes *(Bubalus bubalis)* were introduced to Switzerland. In the meantime, the Swiss buffalo population increased to over 1900 animals. So far, the water buffaloes in Switzerland have not been investigated for their virome. Therefore, the present pilot study aimed at shedding light into that issue. For this purpose, blood samples were taken from water buffaloes on three Swiss farms and, if present, from the co-housed small ruminants. In a separate study the buffalo samples were investigated by Next Generation Sequencing (NGS) for detection of even as yet unknown viruses. The present study addressed the analysis of the buffalo and the small ruminant samples by conventional specific diagnostic methods (PCR, ELISA and SNT) in order to corroborate and expand the findings of the NGS approach.

While NGS detected just one single virus, i.e. a novel Gemycircularvirus (GyCV), evidence for five different viruses affecting water buffaloes was provided by the classical approach. By specific PCR bovine herpesvirus 6 (BoHV-6) as well as GyCV were detected. By serology, evidence was provided for past infections with bovine herpesvirus 2 (BoHV-2), the pestivirus bovine viral diarrhea virus (BVDV), and the arbovirus bluetongue virus (BTV). Thus, the virome of water buffaloes comprised potentially apathogenic viruses, such as BoHV-6, as well as potentially pathogenic viruses affecting skin (BoHV-2), the respiratory, urogenital, and gastrointestinal tract (BVDV), and viruses causing generalized disease (BTV). Although a systemic spread of GyCV was observed on one farm, the relevance of this infection remains unclear.
There was no evidence for present or past infections of the water buffaloes with bovine herpesvirus 1 (BoHV-1) and bovine leukemia virus (BLV). These were important results, since these infections are considered to be eradicated in Switzerland. However, some limitations of the present pilot study became evident as infections with the pathogenic ovine herpesvirus 2 (OvHV-2), border disease virus (BDV) and Schmallenberg virus (SBV) were detected among co-housed sheep and with SBV in the goats, but not among the water buffaloes. It is well known that OvHV-2 may be transmitted from sheep to water buffaloes, causing malignant catarrhal fever (MCF) in the latter. The observation that of the pestiviruses BVDV, but apparently not BDV was successfully transmitted to the water buffaloes needs further attention, particularly because BVDV is actively being eradicated in Switzerland.

In conclusion, the present pilot study expanded our knowledge about the virome of Swiss water buffaloes, identifying them as susceptible for interspecies transmission. Further research will be required in order to assess their role in maintaining and transmitting virus infections, particularly BoHV-2, BVDV, BTV, and GyCV.

Keywords: virome, Swiss water buffalo, herpesvirus, pestivirus, arbovirus, Gemycircularvirus

VIRUS GLOSSARY

BAstV	Bovine astrovirus
BDV	Border disease virus
BEFV	Bovine ephemeral fever virus
BIV	Bovine immunodeficiency virus
BLHV	Bovine lymphotropic herpesvirus
BLV	Bovine leukemia virus
BoHV-1	Bovine herpesvirus 1
BoHV-2	Bovine herpesvirus 2
BoHV-4	Bovine herpesvirus 4
BoHV-5	Bovine herpesvirus 5
BoHV-6	Bovine herpesvirus 6
BPV-2	Bovine papillomavirus type 2
BPXV	Buffalopox virus
BTV	Bluetongue virus
BuCoV	Bubaline coronavirus
BuHV-1	Bubaline herpesvirus 1
BVDV	Bovine viral diarrhea virus
CpHV-1	Caprine herpesvirus 1
CpHV-2	Caprine herpesvirus 2
CpLHV	Caprine lymphotropic herpesvirus
FMDV	Foot-and-mouth disease virus
GyCV	Gemycircularvirus
LSDV	Lumpy skin disease virus
MCFV	Malignant catarrhal fever viruses
OvHV-2	Ovine herpesvirus 2
PBV	Picobirnavirus
PI-3	Parainfluenza virus type 3
PPRV	Peste-des-petits-ruminants virus
RABV	Rabies virus
RuRV-1	Ruminant rhadinovirus type 1
RuRV-2	Ruminant rhadinovirus type 2
SBV	Schmallenberg virus

1 INTRODUCTION

The Asian water buffalo (*Bubalus bubalis*) has its origin in the hot and humid region of Southeastern and Western Asia, where it was domesticated about 5000 years ago. In the 8th century the water buffalo was introduced to Europe, where since it is kept for milk and meat production. Compared to cattle, water buffalo farming in Switzerland is in its infancy. It has been only 20 years, since the first animals were introduced to Switzerland. Still, this species is gaining increasing importance in Swiss animal farming.

The dissemination of this exotic cloven-hoofed species all over the world generates new infectiological problems. First of all it is well known that a virus may have different effects on different animal species. While in one species the same virus causes only mild symptoms or even subclinical infections, other species may suffer from acute and fatal diseases. Examples for such viruses in ruminants are malignant catarrhal fever viruses (MCFV), bovine herpesvirus 1 (BoHV-1) and bluetongue virus (BTV). Thus, where exotic animals like water buffaloes get in contact with native species such as cattle, sheep and goats, the species-associated difference in the clinical manifestation of viral infections may have severe impact on animal health. Consequently, water buffaloes may represent a subclinically infected reservoir, which is particularly hazardous concerning epizootic viruses. Upon transmission to native farm animals, epizootics may occur that entail considerable economic losses due to animal deaths, control measures of epizootics and trade restrictions. Concerning epizootic viral diseases, Swiss livestock is exemplary and bovine diseases like infectious bovine rhinotracheitis (IBR) and enzootic bovine leukosis (EBL) have successfully been eliminated in cattle.
Another challenge for animal health is coming up with worldwide climatic changes, which go along with the spreading of exotic arthropods, e.g. the Asian Tiger Mosquito *(Aedes albopticus)*. Arthropods frequently act as vectors for viral agents. As a result, exotic viruses (= emerging viruses) may be introduced to Switzerland via arthropods or the spreading of classic agents may be aggravated with potentially severe effects on the animals. As a matter of fact, the two most recent emerging diseases in Swiss farm animals were caused by arboviruses, i.e. BTV and Schmallenberg virus (SBV).

These facts clearly depict that in order to maintain the high animal health standard in Switzerland, it is indispensible to collect data about viruses circulating in Swiss farm animals, including exotic species like the water buffalo.
Not having been investigated so far, the overall aim of the present study was therefore to make a contribution to the establishment of the virome of Swiss water buffaloes. In order to cover a preferably broad range of viruses, the virome was investigated by two fundamentally different approaches. In a first approach, which was the subject of a separate study, blood samples of water buffaloes from three Swiss farms were analyzed by Next Generation Sequencing (NGS), a high-throughput sequencing technology, which in the last few years has gained increasing importance in detection of known and novel viruses. The present manuscript describes a second approach, in which the same buffalo samples as well as

samples from co-housed small ruminants were tested by conventional diagnostic methods in order to complement metagenomic analysis. Several polymerase chain reaction (PCR) techniques were applied to validate and potentially expand the analysis of the samples by NGS. As some viruses may only cause transient or very low level viremia, PCR detection methods may provide negative results despite the circulation of these viruses among the animals tested. In these cases, an actual viral infection may be demonstrated by antibody detection. Therefore, the findings were additionally validated against serological tests, namely enzyme-linked immunosorbent assay (ELISA) and serum neutralization test (SNT), thereby also expanding the range of viruses addressed by the specific methods.
By these means it was intended to specifically target viruses, which are known to circulate among water buffaloes and/or among Swiss ruminants (e.g. ovine herpesvirus 2 (OvHV-2)) as well as viruses expected to be absent (e.g. BoHV-1, BTV). In addition, the NGS results needed corroboration by these conventional methods.

Before stating the aims of the present thesis, the origin of the Asian water buffaloes and their history in Switzerland as well as the viruses that were addressed in this study and their associated diseases shall briefly be reviewed. Herpesviruses, including bovine herpesviruses 1, 2, 5 (BoHV-1, BoHV-2, BoHV-5), bubaline herpesvirus 1 (BuHV-1), OvHV-2, caprine herpesviruses 1 and 2 (CpHV-1, CpHV-2) comprise a big part of the investigations, as they represent a vast family of viruses and constitute major pathogenic agents among mammals. Next to various herpesviruses, the animals were tested for ruminant pestiviruses, namely bovine viral diarrhea virus (BVDV) and border disease virus (BDV), as well as for one retrovirus, the bovine leukemia virus (BLV) and finally the two vector-borne emerging diseases, BTV and SBV. By NGS sequences of a novel Gemycircularvirus (GyCV) were detected. The presence of these sequences was confirmed by conventional means.

1.1 The Asian water buffalo

The Asian water buffalo (*Bubalus bubalis*) belongs to the same family of animals as goats and sheep (*Bovidae)* but to a different subfamily, namely *Bovinae* (Tab. 1). The Asian buffalo must be distinguished from the African buffalo *(Syncerus caffer)*, which is also a member of the tribe *Bovini* but constitutes its own genus *Syncerus*. Next to the water buffaloes, the genus *Bubalus* comprises lowland anoa *(Bubalus depressicornis)* and mountain anoa *(Bubalus quarlesi)*, as well as the tamaraw (*Bubalus mindorensis)*, which are all native to Asia. There are two subspecies of Asian water buffaloes: river buffaloes and swamp buffaloes. River buffaloes have 50 chromosomes, are mainly found in Western Asia and are kept for milk production. The swamp type has 48 chromosomes and is used for draught purposes, especially on the rice fields in south-east Asia.
The common ancestor of river and swamp buffaloes is the wild Asian water buffalo (*Bubalus arnee*), which can still be found in parts of India. Domestication of the water buffalo is thought to have occurred 5000 years ago on the Asian continent. In the 8th century, the river buffalo was introduced to Europe. European water buffaloes belong to the Mediterranean breed and are kept for milk and meat production.

The world's water buffalo population in the meantime counts about 182 million head, 95% of which are found in Asia and 0.25% in Europe. In Europe the largest buffalo populations are found in Italy (Borghese, 2013). Compared to the world's cattle population of approximately one billion (Statista, www.statista.com/statistics/263979/global-cattle-population-since-1990/. Accessed 2017 Jan 12) water buffaloes only take a small part. Still, the water buffalo has an enormous economic impact, since it is the domesticated species most people in the world depend on (FAO, 2000).

Taxonomy	**Water buffalo**	**Sheep**	**Goat**
Family	*Bovidae*	*Bovidae*	*Bovidae*
Subfamily	*Bovinae*	*Caprinae*	*Caprinae*
Tribe	*Bovini*	n.a.*	n.a.*
Genus	*Bubalus*	*Ovis*	*Capra*
Species	*B. bubalis*	*O. aries*	*C. hircus*

Tab. 1. Taxonomic status of water buffalo, sheep, and goat.
*n.a. = not applicable

1.2 Swiss water buffaloes

In 1996 five farmers from Canton Bern imported the first 15 water buffaloes (14 pregnant cows, 1 bull) from Romania to Switzerland (Rüeger et al., 2010). Since two decades, this exotic species has spread to all parts of Switzerland and now counts over 1900 animals, allocated to about 90 farms (H. Schwermer, personal communication, 2016). In order to avoid inbreeding in this comparatively small population, breeding at first was mainly performed with buffalo semen imported from Italy.
The import of a "new" species to Switzerland was motivated by various advantages of water buffalo housing compared to cattle farming. Water buffaloes more efficiently convert poor-quality roughage into milk and meat (Borghese, 2013). While the average milk yield in water buffaloes is four times lower compared to cattle (5 vs. 20 liters per day), the milk price per liter is five times higher (3 vs. 0.60 Swiss Francs). Compared to cow milk, milk from water buffaloes has twice as much fat and a higher protein content (Borghese, 2013). Especially in summer months, buffalo milk is in great demand, as it is the main ingredient for the original Mozzarella cheese. As a final point, the meat from buffaloes has a 50% lower cholesterol and a higher protein content compared to beef (Rüeger et al., 2010).

1.3 Herpesviruses

The order *Herpesvirales* comprises a large number of viruses infecting mammals, birds, reptiles, fish, frogs, and even non-vertebrates (Davison, 2010). It is divided into three families of which the family *Herpesviridae* comprises viruses infecting mammals, birds and reptiles. This family is further subdivided into at least three subfamilies, *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (Davison, 2010). The range of host species is very wide. Herpesviruses

are able to cross the species barrier and cause severe diseases and even death in non-adapted species (Pellett and Roizman, 2007). A characteristic of herpesviruses is the ability to establish lifelong infections by inducing latency in various cell types, such as neurons and/or lymphocytes. During the latent state, no infectious virus can be isolated and no structural viral antigen can be demonstrated, only the viral DNA can be detected. At intervals, the virus may be reactivated from its latent state and re-enter the lytic cycle, resulting in viral shedding (Engels and Ackermann, 1996).

1.3.1 Alphaherpesviruses

The subfamily of *Alphaherpesvirinae* consists of four genera, two of which, *Varicellovirus* and *Simplexvirus*, predominantly infect mammals. Viruses of the other two genera, *Iltovirus* and *Mardivirus*, have birds as hosts (Pellett and Roizman, 2007). Of the Varicelloviruses, bovine herpesvirus 1 (BoHV-1), bovine herpesvirus 5 (BoHV-5), bubaline herpesvirus 1 (BuHV-1) and caprine herpesvirus 1 (CpHV-1) were specifically tested for. All of them share close genetic and antigenic properties with BoHV-1, which is considered as a prototype within the genus (Schwyzer and Ackermann, 1996). Among the Simplexvirus it was tested for bovine herpesvirus 2 (BoHV-2), which is closely related to herpes simplex virus 1 and 2 (HSV-1, HSV-2) (Ehlers et al., 1999a).

Bovine herpesvirus 1

BoHV-1 is the causative agent of an epizootic disease called infectious bovine rhinotracheitis (IBR) or infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB). As the names imply, viral entry and shedding either take place in the upper respiratory tract or the genital tract. From these two sites the virus can spread locally, systemically by viremia or invade the peripheral nervous system with establishment of latency in the trigeminal or sacral ganglia (Engels and Ackermann, 1996; Muylkens et al., 2007).

Transmission of BoHV-1 occurs directly by close contact or aerosols, or indirectly via contaminated food, water and personnel, or venereally by semen (Engels and Ackermann, 1996).

Clinical signs of IBR include high fever, apathy, anorexia, milk drop, hyperemia of nasal mucosa ("red nose"), serous to mucopurulent nasal discharge, tracheal stridor, conjunctivitis and ocular discharge, abortion (4^{th} - 8^{th} month of gestation) or embryonic death ($\leq 3^{rd}$ month). In calves it causes a multisystemic infection. The genital form (IPV/IPB) goes along with vesicular exanthema on genital mucosae (Muylkens et al., 2007).

The virus, which causes considerable economic losses, is distributed worldwide with varying prevalences (Ackermann and Engels, 2006). In Switzerland and a few other European countries, BoHV-1 was successfully eradicated by serological testing of blood or milk samples from bovine species and consequent culling of seropositive animals. The disease free status is monitored by annual serological surveys of the Swiss bovine population and serological testing of bovine animals and bovine semen upon importation from non-BoHV-1 free countries (Ackermann et al., 1990). In other countries, disease control is achieved by

regular vaccination with gE deleted marker vaccines and eradication of gE seropositive animals (Ackermann and Engels, 2006).
Infection with BoHV-1 was also shown in sheep, goats and several cervid species, making them potential reservoirs for the virus. However, they do not seem to play a major role in transmission to cattle (Hage et al., 1997; Ackermann and Engels, 2006). In water buffaloes, serological evidence for BoHV-1 infection was reported by Scicluna et al. (2007), showing a seroprevalence of 30.5% in gB/gE blocking ELISA. In 2010 susceptibility of water buffaloes for BoHV-1 was proven by experimental infection of water buffaloes (Scicluna et al., 2010). Strikingly, in both studies none of the animals tested showed any clinical signs, suggesting a possible role of water buffaloes as subclinically infected reservoir (Scicluna et al., 2010). This hypothesis was disproven in 2015, when the first isolation of BoHV-1 in naturally infected water buffaloes was reported (Fusco et al., 2015a). By gB real-time PCR, 14 water buffaloes (11 aborted fetuses, 1 calf with hind leg malformation and 2 dead newborn calves) were shown to be BoHV-1 positive, using a technique based on pyrosequencing, which was able to distinguish between BoHV-1, BoHV-5, BuHV-1 and CpHV-1 (Fusco et al., 2015b).

Bovine herpesvirus 5

BoHV-5 is characterized by its neurotropism and neurovirulence (Perez et al., 2002). In former days it was considered a neurovirulent strain of BoHV-1, called BoHV-1.3 (Belknap et al., 1994; d'Offay et al., 1995). The virus causes severe non-suppurative meningo-encephalitis in young cattle (Belknap et al., 1994; Megid et al., 2015). Disease due to BoHV-5 infection is reported only sporadically. Typical clinical signs of a BoHV-5 infection include apathy, anorexia, depression, bruxism, ptyalism, circling, head pressing, tongue protrusion, blindness, opisthotonus, seizures and recumbency, which usually end in death of affected animals (Ely et al., 1996; Megid et al., 2015). Latency is established in several areas of the central nervous system, tonsils and peripheral blood leukocytes (PBL) (Vogel et al., 2003; Favier et al., 2014). Sheep were shown to be susceptible after experimental infection, developing clinical signs and histological lesions similar to those observed in cattle (Belák et al., 1999). However, so far there is no evidence that they play an epidemiologic role in BoHV-5 transmission. To the author's knowledge, there is no report of BoHV-5 infection in water buffaloes.
The distribution of the virus is mainly limited to the American continent. In South America the virus is highly prevalent with frequent fatal outbreaks (Perez et al., 2002; Megid et al., 2015). In Switzerland BoHV-5 has not been reported yet.

Bubaline herpesvirus 1

Water buffaloes are the primary host of BuHV-1, which was first isolated in 1972 from the prepuce of clinically healthy buffalo bulls (St George and Philpott, 1972). In 2004, the virus could be isolated for the first time in Europe, from nasal swabs of two clinically healthy water buffaloes in Italy, after reactivation with dexamethasone (De Carlo et al., 2004). Phylogenetic analysis of sequences obtained for the gD and gB genes of the isolated viruses indicated that BuHV-1 was more closely related to BoHV-5 than to BoHV-1 (De Carlo et al., 2004).
BuHV-1 has so far been associated with respiratory disease characterized by fever, depression, cough, sneezing, nasal and ocular discharge, diarrhea and death (Petrini et al.,

2012) and abortion (Amoroso et al., 2013), but also with subclinical infection (St George and Philpott, 1972; De Carlo et al., 2004; Scicluna et al., 2007; Petrini et al., 2012; Amoroso et al., 2013; Maidana et al., 2014). In a seroepidemiological study based on the combined use of BoHV-1 gB/gE blocking ELISAs, Scicluna et al. (2007) reported a BuHV-1 seroprevalence of 42% in water buffaloes and apparently, the virus is also able to infect cattle (Scicluna et al., 2007; Petrini et al., 2012). However, due to the close genetic and antigenic relationship of BuHV-1 and BoHV-1, serological assays usually are unable to reliably distinguish between anti-BuHV-1 and anti-BoHV-1 antibodies and thus, the real seroprevalence of BuHV-1 is not known. In Switzerland the virus has not been reported yet.

Caprine herpesvirus 1

Pathogenesis of CpHV-1 is quite similar to BoHV-1. A local respiratory or genital infection is followed by a mononuclear cell-associated viremia, which may lead to systemic infection and abortion (Chenier et al., 2004; Thiry et al., 2006). In kids of one to two weeks of age, CpHV-1 infection causes a febrile systemic disease with high morbidity and mortality, characterized by severe haemorrhagic and ulcerative gastroenteritis. In adult goats, infection mostly takes a subclinical course or expresses itself as vulvovaginitis and balanoposthitis, respectively. Abortion usually occurs in the late gestation period (Mettler et al., 1979; Buonavoglia et al., 1996; Roperto et al., 2000).

CpHV-1 was first isolated from goats in California in 1974 (Saito et al., 1974) and shortly after in kids from Switzerland (Mettler et al., 1979). It is distributed worldwide (Papanastasopoulou et al., 1991), but disease is reported only rarely (Roperto et al., 2000). Sheep and cattle have been shown to be experimentally susceptible, but they do not seem to play an epizootiologic role of CpHV-1 infection in goats, as reactivation in these species has not been achieved so far (Engels et al., 1992; Six et al., 2001).

Bovine herpesvirus 2

BoHV-2 is distributed worldwide. Cattle, small ruminants and several wild ruminant species were shown to be susceptible to infection (Janett et al., 2000; Torres et al., 2009).

Infection with BoHV-2 may be subclinical or result in mild to severe disease, which occurs sporadically or in local outbreaks. Two clinical pictures are differentiated. The local form affects teats and udder and is called bovine herpes mammillitis (BHM). Cows in first lactation are predominantly affected and cases are mostly reported in autumn and winter. Small plaques evolve to vesicles and painful ulcers, leading to resistance during milking. The disease can result in reduced milk production and secondary bacterial mastitis. Calves of affected cows can show identical lesions on the muzzle and in the mouth (Kemp et al., 2008; Torres et al., 2009). The generalized form is called Pseudo-lumpy skin disease (PLSD). PLSD is characterized by fever and sudden appearance of circumscribed hard nodules on the whole body. The nodules undergo necrosis, ulceration and scab formation (Torres et al., 2009).

The ways of transmission of BoHV-2 are not yet clear. Direct and indirect transmission via contaminated material, such as the milking machine, as well as mechanically through insect vectors have been suggested (Janett et al., 2000; Kemp et al., 2008; Torres et al., 2009; Syring et al., 2010). Latency could be demonstrated in nerve ganglia and lymphoid tissues (Torres et al., 2009; Campos et al., 2014).

BoHV-2 is known to circulate in Swiss cattle. However, clinical disease is only reported rarely (Engels et al., 1979; Müller et al., 1984; Janett et al., 2000; Syring et al., 2010), which is probably due to the widespread immunity in the cattle population.
To the author's knowledge, BoHV-2 infection in water buffaloes has not been reported yet.

1.3.2 Gammaherpesviruses

Veterinary medically relevant ruminant Gammaherpesviruses belong to the genus *Rhadinovirus* and *Macavirus* (from malignant catarrhal fever), respectively. Among the Gammaherpesviruses it was specifically tested for ovine and caprine herpesvirus 2 (OvHV-2, CpHV-2), the two most prevalent MCF causing Macaviruses in Switzerland.

Ovine herpesvirus 2 and caprine herpesvirus 2
Malignant catarrhal fever (MCF) is an acute, generalized and mostly fatal systemic disease of many domestic and wild ungulate species, including members of the subfamilies *Bovinae* and *Tragelaphinae*, the family *Cervidae* as well as pigs and giraffes (OIE, 2008). It is characterized by lymphoproliferation and inflammation of mucosal surfaces and blood vessels (Li et al., 2014). Several MCF viruses (MCFV) have been identified. The three most important MCFV are OvHV-2, CpHV-2 and alcelaphine herpesvirus 1 (AlHV-1) (Li et al., 2001). AlHV-1 is found in wildebeest in Africa and zoological gardens, while the latter two are endemic in sheep (OvHV-2) and goats (CpHV-2) worldwide (Li et al., 2005a). These species represent the well-adapted reservoir hosts, in which the infection remains subclinical. They shed virus mainly via nasal secretions and transmit it through direct contact, aerosols or indirectly via contaminated food or water. In indicator hosts like cattle, bison, water buffaloes, pigs, giraffes and various cervid species, infection with MCFV usually leads to clinical disease, but subclinical infections may also occur (Powers et al., 2005; Stahel et al., 2013). Indicator hosts do not excrete virus and therefore are no source of infection (Li et al., 2014).
Clinical MCF can express itself in various forms, the most common affecting head and eyes. It goes along with fever, mucopurulent nasal and ocular discharge, bilateral corneal opacity due to keratitis, erosions on the muzzle and on buccal mucosae, salivation and lacrimation. More rarely reported are peracute (animals found dead without typical clinical signs), neurologic (hyperaesthesia, incoordination, nystagmus and head pressing), alimentary (diarrhea) or cutaneous forms (folliculitis and alopecia) (Crawford et al., 2002; Li et al., 2014).
In Switzerland, MCF occurs sporadically in cattle. There are two reports of MCF in Swiss water buffaloes. In 2011, Switzerland reported the first possible case of CpHV-2 associated MCF in a water buffalo (Dettwiler et al., 2011). Until this time, CpHV-2 associated MCF had only been reported in cervids (Keel et al., 2003). Following these findings, the prevalence of OvHV-2 and CpHV-2 was investigated on three Swiss farms housing water buffaloes. Both OvHV-2 and CpHV-2 were shown to be prevalent in Swiss water buffaloes. For the two viruses found, susceptibility apparently was higher for CpHV-2 than OvHV-2, but the penetration rate was higher with OvHV-2 than with CpHV-2. Compared to cattle, water

buffaloes seemed to be more susceptible to infection, but at the same time had a higher rate of subclinical infections. It is well known, that there are marked differences in the susceptibility towards OvHV-2 in different species. Domestic cattle and zebus seem to be relatively resistant, while water buffaloes and bison seem to represent an intermediate type. Père David's deer and Bali cattle are highly susceptible to MCF (Li et al., 2014).

1.4 Ruminant pestiviruses

Pestiviruses are positive-sense single stranded RNA viruses and belong to the family *Flaviviridae* (Collett, 1992). Bovine viral diarrhea virus (BVDV) with its natural reservoir in cattle and border disease virus (BDV) with sheep as its natural host represent the most important ruminant pestiviruses (Bachofen et al., 2008; Danuser et al., 2009).

Bovine viral diarrhea virus and border disease virus

BVDV and BDV infect several species of the order Artiodactyla worldwide, with BVDV being mostly reported in cattle and BDV in sheep (Bachofen et al., 2013). Despite a high genetic variability, serological cross-reactivity among pestiviruses is high (Vilček et al., 2001; Danuser et al., 2009). However, cross-neutralization assays can be used to attribute particular infections to particular virus subtypes (Bachofen et al., 2008; Kaiser, V., Doctoral Thesis, Berne, 2016).

According to their effect on cell cultures, cytopathogenic (cp) and non-cytopathogenic (ncp) biotypes of pestiviruses are distinguished (Becher et al., 1997).

Transmission occurs directly or indirectly via the oropharyngeal route. Pestivirus infection may take two different courses: acute infection ("hit and run") with transient viremia (BVDV one to two weeks) resulting in long-lasting immunity and clearing of infection, or persistent infection ("infect and persist") with highly strain specific immunotolerance, absence of seroconversion and lifelong virus shedding. Acute infections with BVDV are frequently subclinical or accompanied by mild clinical signs like fever, diarrhea and enhancing of respiratory infections due to immunosuppression. Persistent infections are established in fetuses which are infected with a ncp strain via the placental route between the 2^{nd} and 4^{th} month of gestation. After birth, these animals remain persistently infected (PI). They shed high amounts of virus all their lives and are therefore the main source of infection. Most frequently, PI animals may show no or only unspecific clinical signs, such as reduced performance. However, they are prone to mucosal disease (MD). MD may be caused by mutation of the resident ncp strain to a cp strain or, less frequently, by superinfection with an antigenically closely related cp strain. Clinical signs of MD result from erosions and ulcerations throughout the gastrointestinal tract and include high fever, anorexia, profuse diarrhea and dehydration with 100% lethality. Intrauterine infection at another time may lead to resorption (1^{st} month), abortion, stillbirth, malformations (5^{th} month) or birth of seroconverted healthy or malformed animals (6^{th} - 9^{th} month) (Schweizer and Peterhans, 2014). Transient infection of sheep with BDV is mostly inapparent. PI animals, also called "hairy shakers", are generated when intrauterine infection with a pestivirus occurs between day 20 and 80 of gestation. In goats, transient infection with BVDV or BDV is usually

subclinical and transplancental transmission to the fetus often leads to abortion. Persistently infected goats are thus rather rare (Bachofen et al., 2013).
Following the example of different Scandinavian countries, Switzerland initiated a national eradication program for BVDV in 2008 (Schwermer et al., 2013). Until 2012 all bovine animals were tested for BVDV antigen in order to detect PI animals. Virus-positive animals were culled. Since 2013, herds are periodically monitored for BVDV antibodies, either in blood or in bulk milk. An increased antibody titer of the herd is an indication for re-infection with a pestivirus and in these cases animals are specifically tested for persistent viremia with BVDV. From the beginning of the program, BVDV vaccination was striktly banned. Thanks to this program the PI prevalence among newborn calves could be diminished from approximately 1.4% in 2008 to 0.02% in 2012 (Presi et al., 2011). The eradication of the main source of infection, the PI animals, was accompanied by a continuous increase of a naive, seronegative population. Before the eradication program started, the seroprevalence of BVDV in Swiss cattle amounted to 60% (Rüfenacht et al., 2000). However, BVDV and BDV are not strictly species specific and BVDV may still circulate in small ruminants. In Swiss small ruminants, antibodies to BVDV were found in 2.1% of sheep and 2.6% of goats (Danuser et al., 2009).
There is no systematic program for BDV eradication and BDV seroprevalences of approximately 9% in sheep and 6% in goats were recently reported in Switzerland. The prevalence of BDV PI animals is unknown (Danuser et al., 2009).
Various serological and molecular studies have shown that, as in cattle, pestivirus infections in water buffaloes are widely distributed and that they are a possible cause of abortion in this species (Becher et al., 1997; Sudharshana et al., 1999; Bhatia et al., 2008; Craig et al., 2008; Martucciello et al., 2009; Mingala et al., 2009; Craig et al., 2015).

1.5 Retroviruses

Among the retroviruses, it was tested for bovine leukemia virus (BLV), a virus that is distributed worldwide with low prevalences.

Bovine leukemia virus
BLV is a member of the family *Retroviridae* and causes an epizootic disease mainly in cattle called enzootic bovine leukosis (EBL). Its genome consists of two segments of positive-sense single stranded RNA. The virus predominantly infects B-lymphocytes. Transmission of BLV occurs via direct or indirect contact with blood or milk, transplacentally, venereally or via arthropods. In the host, BLV establishes a lifelong infection with chronic course. A big part of affected animals remain subclinically infected, while, after an incubation time of months to years, 30% to 70% develop persistent lymphocytosis and 0.1 - 10% suffer from malignant lymphoma.
Clinical signs are the manifestation of the lymphatic leukemia and/or tumorous infiltration of various organs and therefore vary depending on the organs affected. Symptoms include inappetence, weight loss, reduced performance, general enlargement of lymph nodes with possible impairment of the affected organ's function (OIE, 2008).

Thanks to an eradication program, Switzerland is officially BLV-free since 1994, while there are still cases reported from other European countries. Together with BoHV-1, the Swiss cattle population is yearly sampled and monitored for BLV antibodies in order to maintain the disease-free status.
Small ruminants were shown to be susceptible after experimental infection, but they do not seem to play a role in virus spreading (OIE, 2008). Water buffaloes are susceptible to natural infection (Molnár et al., 1999; OIE, 2008).

1.6 Emerging viruses

Among the emerging viruses it was specifically tested for bluetongue virus (BTV) and Schmallenberg virus (SBV). Both viruses are mainly transmitted through biting midges and only recently caused outbreaks in Swiss farm animals.
After having found DNA sequences of a novel gemycircularvirus in several water buffalo samples, the presence of this virus was additionally investigated by conventional PCR. Although detailed information on gemycircularviruses is lacking, the novel agent is listed within the emerging virus group, as it has been described here for the first time.

Bluetongue virus
BTV is the prototype of the *Orbivirus* genus in the family *Reoviridae* (Mertens et al., 2004). The genome consists of 10 segments of double-stranded RNA. Twenty-seven serotypes of BTV have been defined so far, showing only low or no cross-reactivity (Hofmann et al., 2008; Schulz et al., 2016). All ruminant species are susceptible to infection with BTV. However, clinical manifestation is mostly seen in sheep, while cattle usually represent a subclinically infected virus reservoir (Hofmann et al., 2008; Casaubon et al., 2013). Virus transmission mainly occurs through insect vectors of the genus *Culicoides* spp., mainly *C. imicola*. Circulation of BTV among ruminants coincides with the occurence of susceptible vectors, which are mainly found in tropical, subtropical and temperate regions but keep spreading northwards due to climatic changes (Wilson et al., 2009). Clinical signs are associated with vascular injury including fever, depression, erosive and ulcerative lesions on the muzzle and oral mucosae, salivation, nasal discharge, facial edema, zyanotic tongue (blue tongue), conjunctivitis, lameness due to coronitis and laminitis, and abortion in pregnant animals (Hofmann et al., 2008; Maclachlan, 2011).
In Europe, bluetongue disease has occured for decades. Climatic changes have probably contributed to the continuous spreading of different BTV serotypes in Europe since the end of the 20th century. Since 1998, at least eight different BTV serotypes have been detected (Zientara et al., 2013). The outbreak with the highest economic losses in northern Europe occured from 2006 to 2009 and was caused by the unexpected emergence of BTV serotype 8, which until this time point had never been detected on the continent (Hofmann et al., 2008; Zientara et al., 2013). In 2007, this serotype also emerged in the Swiss ruminant population and caused several local outbreaks (Hofmann et al., 2008; Casaubon et al., 2013). The virus strain had the atypical feature that it was highly virulent not only in sheep but also in cattle and South American camelids (Zientara et al., 2013). Furthermore, BTV-8 had the potential to

cause transplacental infection in fetuses (Maclachlan, 2011). Surprisingly, the virus was successfully eradicated from Switzerland following a compulsory vaccination campaign in Swiss livestock, which took place from 2008 to 2010. This success suggested that BTV-8 had not been able to establish a permanent reservoir in the local insect populations.
Water buffaloes are susceptible to BTV infection as shown by seroepidemiological studies (Lage et al., 1996; Gür et al., 2011).

Schmallenberg virus

The name of the Schmallenberg virus (SBV) derives from the German city, where it was first detected. In 2011, dairy cattle in Germany and the Netherlands showed clinical signs of an unidentified disease, including fever, milk drop and diarrhea. The symptoms disappeared after a few days. However, in winter 2011 numbers of abortions, stillbirths and malformed fetuses among cattle and sheep rised. Thanks to metagenomic analysis (NGS), a novel orthobunyavirus in the family *Bunyaviridae* could be identified as the causative agent (Hoffmann et al., 2012; Schorer et al., 2012). The virus was classified in the Simbu serogroup, which had previously not been detected in Europe (Hoffmann et al., 2012). The genome consists of three segments of negative-sense single stranded RNA (Doceul et al., 2013). Similar to other members of the *Orthobunyavirus* genus and as for BTV, transmission of SBV occurs through biting midges (*Culicoides* spp.) or transplacentally (Schorer et al., 2012). Susceptible species include a wide range of domestic and wild ruminants (Wernike et al., 2014). Clinical signs are most apparent in cattle and include fever, inappetence, drop in milk production and diarrhea in adult animals. Viremic state lasts for one to six days. Transplacental transmission may result in abortion, stillbirth and/or congenital defects affecting brain, spinal cord and skeletal muscles (Hoffmann et al., 2012; Lievaart-Peterson et al., 2012; Schorer et al., 2012; Doceul et al., 2013).
In contrast to BTV, whose nationwide spread could be prevented by rigorous vaccination, SBV has rapidly spread through Europe. In 2012, Belgium and the Netherlands reported seroprevalences in cattle of 91% and 72.5%, respectively (Elbers et al., 2012; Garigliany et al., 2012). By september 2013, SBV had been reported from 27 European countries (Wernike et al., 2014). In Switzerland, the first cases were diagnosed mid-July 2012. By December 2012, between-herd seroprevalence in cattle herds was 99.5% (Balmer et al., 2014).
Water buffaloes were shown to be susceptible to SBV infection (Azkur et al., 2013).

Gemycircularvirus

The genus *Gemycircularvirus* belongs to the newly created family of *Genomoviridae* (Krupovic et al., 2016). The only recognized and isolated species in this genus is Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), a virus infecting the plant pathogenic fungus *Sclerotinia sclerotiorum* (Yu et al., 2010). It's a small, non-enveloped virus with a genome of circular single-stranded DNA of about 2.17kb. The genome encodes a replication protein and a capsid protein, which are separated by two intergenic regions (large and small intergenic region). Thanks to metagenomic analyses, in the past few years genomes of over 100 SsHADV-1-like putative viruses have been reported, deriving from a wide range of sample materials, including plants (Dayaram et al., 2012; Male et al., 2015), insects (Rosario et al., 2012), feces of various animal species and humans (Sikorski et al., 2013; Steel

et al., 2016), sewage (Kraberger et al., 2015; Phan et al., 2015), rat blood (Li et al., 2015), bovine and human serum/plasma (Lamberto et al., 2014; Uch et al., 2015), human brain (Lamberto et al., 2014) and cerebrospinal fluid (Phan et al., 2015; Zhou et al., 2015). With the exception of SsHADV-1, not much is known about these novel viruses, neither about the natural hosts nor about their clinical impact. However, they seem to be widespread in the environment (Krupovic et al., 2016).

1.7 Aims

The overall aim of this project was to analyze the "virome" of Swiss water buffaloes. The NGS approach to the problem had been assigned to a different sub-project. The present study addressed the following aims:

1. To test the samples for physical evidence of herpesviruses, ruminant pestiviruses, and/or GyCV by PCR methods.
2. To test the samples by serological methods (ELISA) for indirect evidence of specific herpesviruses, ruminant pestiviruses, BLV, BTV and/or SBV.
   If serological evidence for ruminant pestiviruses was detected by ELISA, to discriminate between infection with BVDV and BDV by means of SNT.
3. To compare the results with those obtained from the NGS approach.
4. To integrate the results as a building block for the determination of the water buffalo "virome".
5. To assess the risk of interspecies transmission between water buffaloes and native ruminants.

1.8 Hypotheses

Based on the existing knowledge of viruses occurring in water buffaloes and cattle, and taking account of the current situation of viral infections in Swiss ruminants, hypotheses were stated for presence or absence of targeted viruses in the animals tested.

1. Herpesviruses are widely distributed among mammals and they cause lifelong infections with potential latency in PBL. Also, with the tests applied (panherpes nested PCR and specific real-time PCRs), a wide range of herpesviruses was covered by the applied tests. Therefore, detection of various ruminant herpesviruses in the three species was expected.
2. Among the pestiviruses it was quite probable to still find seropositive animals on the three farms, either for BVDV or BDV. The samples were taken in 2013, shortly after the stage of antigen-testing and elimination of PI animals. At this time, seroprevalences for pestiviruses, particularly BVDV, were still high.
3. After the results of NGS suggested GyCV related sequences with a high prevalence in the water buffaloes, confirmation by means of specific real-time PCR was assumed.

4. After the quick and widespread wave of SBV infections in 2012/13, presence of seropositive ruminants on the three farms was hypothesized.
5. Detection of persistent or transient BVDV viremia by PCR methods was considered rather unlikely due to the success of the eradication campaign. However, chances existed that viremia due to BDV might be detected in some sheep. In contrast, pestivirus viremia in the water buffaloes was not expected, unless these viruses would be able to establish an independent cycle among these animals.
6. Although small ruminants and water buffaloes may serve as reservoir for epizootic viruses infecting cattle, it was to be expected that BoHV-1 (and related), BLV and BTV were absent after having been eradicated in Switzerland. Still, the corroboration of this hypothesis was of great importance.
7. Possible interspecies transmission could be expected for herpesviruses as well as pestiviruses, as they are known to cross the species barrier.

2 MATERIAL AND METHODS

2.1 Farms and animals

Three Swiss farms housing water buffaloes were investigated in this study.
Farm 1 was located in the Canton of Schwyz. The animal stock consisted of 21 water buffaloes, including 17 cows, 1 adult bull and 3 calves, as well as 30 cattle. The two species were housed separately under the same roof, having direct contact only in the courtyard. The age of all the water buffaloes was around two years. The animals were kept for milk production and spent the summer on an alp.
Farm 2 was also situated in the Canton of Schwyz and housed 33 water buffaloes and 20 sheep. As far as known, the age of the water buffaloes ranged from two to seven years. The water buffaloes were kept in two barns, each with access to a courtyard. The sheep were separated from the buffaloes only through a wooden wall. The two species did not share the pasture. The water buffaloes spent the summer on an alp.
Farm 3 was located in the Canton of Solothurn. It housed 5 water buffaloes (2 cows and 3 calves), 65 cattle and 7 goats (adults and kids). The adult buffaloes were kept together with the cattle in one barn, while the buffalo calves were kept in another premise, separated from the goats only through a wooden wall. The water buffaloes were mainly used for meat production and therefore did not reach a high age. Pasturing of buffaloes and goats was always strictly separated.
None of the animals had been reported to be diseased at the time of sampling.

2.2 Sample collection

In may and june 2013, EDTA blood samples were taken from water buffaloes and from co-housed small ruminants by the private veterinarian. Animals tested included 17 water buffaloes on farm 1, 26 water buffaloes and 19 sheep on farm 2, 5 water buffaloes and 7 goats on farm 3. No samples were taken from cattle.

2.3 Sample preparation

The plasma fraction of the blood was separated from the cell fraction by centrifuging the samples at room temperature with 868x g for ten minutes.
For buffy coat production one part of blood was mixed with four parts of lysisbuffer (0.15M NH_4Cl, 10mM $CHKO_3$, 0.1mM EDTA disodium salt [pH 7.2]) and centrifuged at 4°C with 868x g for ten minutes. After disposal of the supernatant, this step was repeated. The leukocyte pellet was subsequently resuspended in 40ml of phosphate-buffered saline (PBS). After another centrifugation step the supernatant was discarded with a little PBS left in the tube, in which the leukocytes were resuspended and centrifuged at 16'060x g for one minute.

The PBS supernatant was removed with a pipette and the buffy coat was stored at -20°C until further processing.
Nucleic acid extraction from all the buffy coats was carried out using the QIAamp® DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. This kit is able to copurify RNA and DNA. Nucleic acids were eluted in 100µl elution buffer. In every extraction series an eluate control was included in order to detect potential contamination during nucleic acid processing. After extraction, a 1:10 dilution with diethylpyrocarbonate-(DEPC) treated water was prepared for every sample. The extracted nucleic acid was stored at -20°C until further use.

2.4 Method overview

PCR was used for detection of viral nucleic acids extracted from buffy coat. As some viruses only cause transient or very low level viremia, an actual infection with these viruses was demonstrated by antibody detection using ELISA and in some cases SNT. Table 2 gives an overview of the viruses investigated and the conventional tests used for their detection.

Classification	Virus	PCR	ELISA	SNT
Herpesviridae	Herpesviridae	Panherpes nested PCR		
Herpesviridae: Alphaherpesvirinae	Bovine herpesvirus 1 Bovine herpesvirus 5 Bubaline herpesvirus 1 Caprine herpesvirus 1 Bovine herpesvirus 2	 Real-time PCR Real-time PCR	Competitive ELISA Indirect ELISA Indirect ELISA Indirect ELISA	 BoHV-2 SNT
Herpesviridae: Gammaherpesvirinae	Ovine herpesvirus 2 Caprine herpesvirus 2	Real-time PCR Real-time PCR		
Flaviviridae	Pestiviruses	Panpesti RT-PCR	Indirect ELISA	BVDV/BDV cross-SNT
Emerging Viruses: Reo-/Bunyaviridae	Bluetongue virus Schmallenberg virus		Competitive ELISA Indirect ELISA	
Retroviridae	Bovine leukemia virus		Indirect ELISA	
Genomoviridae	Gemini-like myco-infecting circular virus	Real-time PCR		

Tab. 2. Viruses targeted by PCR and serological methods.

2.5 Herpesviruses

2.5.1 Panherpes nested PCR

In order to search for a broad range of a priori unspecified herpesviruses, a panherpes nested PCR was conducted according to VanDevanter et al. (1996) and Ehlers et al. (1999b). The PCR targets a highly conserved region of the herpesviral DNA polymerase gene. By using degenerate consensus primers, this PCR is able not only to detect known but also novel

herpesviruses without prior information on the DNA sequence. The degenerate primers are used in a nested format with two forward (DFA and ILK) and one reverse primer (KG1) in the first PCR and one forward (TGV) and one reverse primer (IYG) in the second PCR (Fig. 1) (VanDevanter et al., 1996). Primers for the second PCR bind within the amplified sequence of the first PCR, thereby reducing the amount of unspecific products. Sequences of each primer are shown in Table 3. The product after panherpes nested PCR has an expected size of 215 - 235bp (Ehlers et al., 1999b).

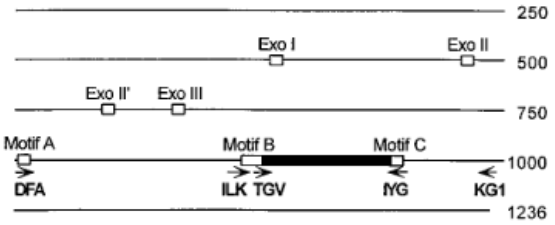

Fig. 1. Priming sites for DFA, ILK, KG1, TGV and IYG primers within the human herpesvirus 1 DNA polymerase gene (VanDevanter et al., 1996).

PCR	Primer	Primer Name	Sequence
1.	Forward	DFA	5'- GAY TTY GCN AGY YTN TAY CC-3'
	Forward	ILK	5'- TCC TGG ACA AGC AGC ARN YSG CNM TNA A-3'
	Reverse	KG1	5'- GTC TTG CTC ACC AGN TCN ACN CCY TT-3'
2.	Forward	TGV	5'- TGT AAC TCG GTG TAY GGN TTY ACN GGN GT-3'
	Reverse	IYG	5'- CAC AGA GTC CGT RTC NCC RTA DAT-3'

Tab. 3. Primer sequences for panherpes nested PCR according to VanDevanter et al. (1996).

The reaction mixtures for first and second round PCR described by Ehlers et al. (1999b) were slightly modified. For first round PCR, 5µl of extracted sample DNA were used as template, for second round PCR 1µl of the product from first round PCR was used. For both PCRs the final mixture had a volume of 25µl and contained 2.5µl PCR Buffer (10x) (Qiagen, Hombrechtikon, Switzerland), 200µM of each deoxynucleotide triphosphate, 1µM of each primer and 2 units HotStarTaq DNA Polymerase (5U/µl) (Qiagen, Hombrechtikon, Switzerland), topped up with DEPC treated water. Each sample was tested undiluted and in 1:10 dilution. The eluate control and DEPC treated water served as negative controls, extracted OvHV-2 DNA from a case of malignant catarrhal fever in a cow served as positive control. For thermal cycling a Peltier Thermal Cycler-200 (MJ Research) was used. Cycling conditions were the same as described by Ehlers et al. (1999b), with the exception of 12 minutes of initial denaturation at 95°C. The products of the second PCR were stained with 5µl of Orange Loading Dye (6x) (Thermo Fisher Scientific, Waltham MA, USA) and loaded on a 2% agarose-gel, which contained Gel Red (1000x) (Biotium, Hayward CA, USA). The 50bp DNA Ladder (New England Biolabs) served as DNA marker. Electrophoresis was carried out at 100V for approximately two hours. Bands of the expected size (215 - 235bp) were visualized under UV light and excised from the gel using a scalpel blade.
Gel-extraction of DNA was conducted with the QIAquick® Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland). The DNA was eluted

in 30µl of elution buffer. Gel-extracted DNA was subsequently amplified in a third sequencing PCR using non-degenerate primers (Tab. 4).

Primer	Primer Name	Sequence
Forward	TGVseq	5'-CAT CTG ATG TAA CTC GGT GTA-3'
Reverse	IYGseq	5'-GAC AAA CAC AGA GTC CGT-3'

Tab. 4. Primer sequences for sequencing PCR according to VanDevanter et al. (1996).

The reaction mixture for sequencing PCR and cycling conditions corresponded to those described for second round PCR with the exception of 200µM of each sequencing primer and 1 unit of HotStarTaq DNA Polymerase (5U/µl). The products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland), eluting with 30µl of elution buffer.
The amount of amplified DNA was measured in the Nanodrop (ND-1000 Spectrophotometer, Scientific AG, Kloten, Switzerland). Of each positive sample, 45ng of DNA were mixed with 8µM of sequencing primer forward or reverse (100µM) and supplemented with DEPC treated water to a final volume of 10µl. Sequencing was performed by Microsynth (Balgach, Switzerland) and sequences were analyzed with NCBI BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Samples were considered herpesvirus positive, if a band of the expected size was visible in the agarose-gel and if sequences showed high identity to herpesviral sequences deposited in GenBank.

Since specific real-time PCRs for particular herpesviruses are known to be more sensitive than conventional panherpes PCR, the samples were additionally subjected to individual real-time PCRs for BoHV-1, BoHV-5, OvHV-2, and CpHV-2, respectively.

2.5.2 Alphaherpesviruses

BoHV-1 real-time PCR
For detection of BoHV-1 DNA, a real-time PCR according to Abril et al. (2004) was conducted, which targets a 97bp sequence of the highly conserved gB gene. The final mixture consisted of 10µl sample DNA, 12.5µl TaqMan® Universal PCR Master Mix (Life Technologies), 240nM of each primer (Microsynth, Balgach, Switzerland), 160nM probe and a top-up of DEPC treated water to a final volume of 25µl (Tab. 5).

Primer/Probe	Sequence
Forward (10µM)	5'-TGT GGA CCT AAA CCT CAC GGT-3'
Reverse (10µM)	5'-GTA GTC GAG CAG ACC CGT GTC-3'
Probe (10µM)	5'-FAM-AGG ACC GCG AGT TCT TGC CGC-TAMRA-3'

Tab. 5. Primer and probe sequences for BoHV-1 real-time PCR according to Abril et al. (2004).

Besides BoHV-1 DNA, this reaction mixture also amplifies BoHV-5 DNA, less efficiently though. Only the water buffalo samples were tested, undiluted and in 1:10 dilution. A plasmid

containing the gB gene of BoHV-1 served as positive control. DEPC treated water and eluate controls served as negative controls.
The PCR was run on a 7900HT Fast Real-Time PCR System (Life Technologies) with 9600 emulation ramping. Cycling conditions corresponded to those described by Abril et al. (2004). Each run was analyzed using the Sequence Detector Software (version 2.4). The baseline for background fluorescence was set from cycles 3 to 15. The threshold value was set manually after each run, above the background noise in the phase of exponential amplification, and ranged around 0.2. Criteria, whereby a sample was considered BoHV-1 positive were a Ct-value of ≤ 40 and a furcation of reporter and quencher fluorescence. If only one or none of these criteria applied, samples were considered negative.

BoHV-1 gB competitive ELISA

For antibodies against BoHV-1, a gB competitive ELISA was conducted according to the manufacturer's instructions (IDEXX IBR gB X3, IDEXX Diavet AG, Bäch, Switzerland). The optical density (OD value) of each well was analyzed in the photometer at 450nm wavelength (Sunrise Tecan) and the percentage of sample blocking was calculated. Samples were considered seronegative, if blocking of the sample was < 45% and seropositive, if blocking was ≥ 55%. Samples with a blocking value between 45% and 55% were considered questionable.

BoHV-5 real-time PCR

Real-time PCR for BoHV-5 targets a 103bp sequence of the gB gene (Abril et al., 2004). PCR setup and analysis were the same as described for BoHV-1 real-time PCR, with the exception of 240nM primer forward, 60nM primer reverse and 80nM probe (Tab. 6).

Primer/Probe	Sequence
Forward (10µM)	5'-ACA TCA TCT ACA TGT CGC CCT TC -3'
Reverse (10µM)	5'-TTG TAG TAG CCC TCG ATT TGC T -3'
Probe (10µM)	5'-FAM-ACC GCG AGC ACA CCA GCT ACT -TAMRA-3'

Tab. 6. Primer and probe sequences for BoHV-5 real-time PCR according to Abril et al. (2004).

In contrast to BoHV-1 real-time PCR, this reaction mixture specifically amplifies BoHV-5 DNA, allowing discrimination of the two viruses in positive samples. Only the water buffalo samples were tested, undiluted and in 1:10 dilution. A plasmid containing the gB gene of BoHV-5 served as positive control. DEPC treated water and eluate controls served as negative controls.

BuHV-1 and CpHV-1 indirect ELISA

For detection of antibodies against BuHV-1 and CpHV-1 two indirect ELISAs were conducted, which are both based on the recombinant gE antigen of the respective virus (Bertolotti et al., 2013; Nogarol et al., 2014). Test principles for both assays were identical and carried out according to the manufacturer's instructions (IN3 diagnostic, Turin, Italy). The optical densities (OD) were read at 450nm wavelength (Sunrise Tecan). Percentage of

reactivity of each sample compared to the positive control (S/P value) was calculated. S/P values of ≤ 30% were considered seronegative, S/P values of ≥ 40% were considered seropositive. Samples with reactivities between 30% and 40% were considered doubtful.

BoHV-2 indirect ELISA

As BoHV-2 only causes low level viremia, this virus was targeted via serological assays. An indirect whole virus ELISA (ID Screen® BHV-2 Indirect, IDvet, Grabels, France) was performed according to the manufacturer's instructions. The optical densities (OD) were read at 450nm wavelength (Sunrise Tecan). Percentage of reactivity of each sample compared to the positive control (S/P value) was calculated. S/P values amounting ≤ 90% were considered seronegative. Sample reactivities ≥ 110% were considered seropositive. Percentages in between were questionable.

BoHV-2 SNT

Samples displaying S/P values of ≥ 90% in the BoHV-2 ELISA were consecutively subjected to a serum neutralization test. Plasma samples were complement inactivated at 56°C for 30 minutes and diluted with DMEM/F-12 Glutamax medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; Thermo Fisher Scientific, Waltham MA, USA) supplemented with 2% fetal calf serum and 1% ABAM (containing 10'000U/ml penicillin, 10'000µg/ml streptomycin and 25µg/ml Fungizone) in two-fold dilution steps. Due to the limited amount of sample plasma left, dilutions ranged from 1:4 and 1:2.44, respectively to 1:256 and 1:156.16. BoHV-2 strain V766 virus stock was diluted in DMEM resulting in a working suspension with a concentration of 2000 $TCID_{50}$/ml. After mixing 220µl of the virus suspension with the same amount of each antibody dilution and incubated for one hour at 37°C and 5% CO_2. Then, 100µl of each plasma-virus mixture were distributed on four wells, coated with Madin-Darby bovine kidney (MDBK) cells, yielding approximately 100$TCID_{50}$ of BoHV-2 per well. Medium and cell controls as well as viral back titration were included. The plate was incubated for three days at 37°C and 5% CO_2. Wells displaying cytopathic effects (CPE) typical for BoHV-2 were identified under the light microscope. Antibody titers were calculated according to Reed and Muench and expressed as the reciprocal of the highest dilution of plasma exhibiting 50% inhibition of CPE. The SNT was valid, if the titration of the working suspension yielded a virus concentration of 600 - 6000 TCID50/ml to result in 30 - 300 TCID50 per well.

2.5.3 Gammaherpesviruses

OvHV-2 and 12S real-time PCR

A TaqMan real-time PCR targeting the ORF 63 tegument protein gene was used in order to specifically detect OvHV-2. Primers and probes, based on the sequences described by Taus et al. (2007; GenBank accession no. DQ198083.1) and Hart et al., (2007; GenBank accession no. AY839756.1), were designed in-house using the Perkin-Elmer Primer Express software (version 1.0, Perkin-Elmer, Foster City, California) (Stahel et al., 2013). The reaction mixture for OvHV-2 had a final volume of 25µl and was previously described by Hüssy et al. (2001).

The final mix contained 12.5µl TaqMan® Universal PCR Master Mix (Life Technologies), 1.25µl DEPC water, 240nM primer forward (Life Technologies Europe BV, Zug, Switzerland), 600nM primer reverse (Life Technologies) and 80nM probe (Microsynth, Balgach, Switzerland) to which 10µl of 1:2 (due to the limited amount of DNA available) and 1:10 diluted template DNA were added (Hüssy et al., 2001).
In addition to OvHV-2, amplification of the 12S rRNA house-keeping gene served as internal control, in order to confirm successful DNA extraction and sensitivity to the PCR reaction. Primers and probes were designed in-house (Perkin-Elmer Primer Express software version 1.0, Perkin-Elmer, Foster City, California) and were based on a consensus sequence between previously published 12S rDNA sequences of various bovid taxa (Gatesy et al., 1997; Stahel et al., 2013). The 25µl reaction mixture for 12S contained 12.5µl TaqMan® Universal PCR Master Mix (Life Technologies), 1.8µl DEPC water, 600nM of each primer (Microsynth, Balgach, Switzerland) and 160nM probe (Life Technologies), to which 10µl of 1:10 diluted template DNA were added. Sequences for primers and probes for both PCRs are shown in Table 7. PCR was run on a 7900HT Fast Real-Time PCR System (Life Technologies) with 9600 emulation ramping. Each run was analyzed using the Sequence Detector Software (version 2.4). The baseline for background fluorescence was set from cycles 3 to 15. The threshold value was set manually after each run, above the background noise in the phase of exponential amplification and ranged around 0.02. Criteria, whereby a sample was considered OvHV-2 positive were a Ct-value of ≤ 40 for OvHV-2 as well as 12S and a furcation of reporter and quencher fluorescence.

Primer/Probe	**Sequence OvHV-2**
Forward (20µM)	5'-GAG AAC AAG CGC TCC CTA CTG A-3'
Reverse (20µM)	5'-CGT CAA GCA TCT TCA TCT CCA G-3'
Probe (10µM)	5'-FAM-AGT GAC TCA GAC GAT ACA GCA CGC GAC A-TAMRA- 3'

Primer/Probe	**Sequence 12S**
Forward (100µM)	5'-GCG GTG CTT TAT AYC CTT CTA GAG-3' (100µM)
Reverse (100µM)	5'-TTA GCA AGR ATT GGT GAG GTT TAT C-3'
Probe (10µM)	5'-VIC-AGC CTG TTC TAT AAY CGA T-MGBNFQ-3'

Tab. 7. Primer and probe sequences for OvHV-2 and 12S real-time PCR according to Stahel et al. (2013).

CpHV-2 real-time PCR

For detection of CpHV-2 a real-time PCR with primers and probe according to Cunha et al. (2009) was performed. The PCR targets an 80bp fragment of the CpHV-2 DNA polymerase gene. In contrast to Cunha et al. (2009), the upstream primer used here contained a guanine instead of an adenine base in position nine, raising specificity for CpHV-2. Further, the probe was labelled with TET™ instead of HEX™ at the 5' end (Tab. 8).
The reaction mixture had a final volume of 25µl and was composed of 12.5µl TaqMan® Universal PCR Master Mix (Life Technologies), 6.8µl DEPC water, 200nM of each primer (Microsynth, Balgach, Switzerland) and 80nM of the probe (Microsynth, Balgach, Switzerland), to which 5µl of undiluted or 1:10 diluted template DNA were added. The cycle protocol corresponded to the one for OvHV-2 (Hüssy et al., 2001; Cunha et al., 2009).

Ramping of the cycler was set to the standard rate. As for OvHV-2, baseline values were set from cycles 3 to 15, and the threshold was set manually after each run, ranging around 0.2. A sample was considered CpHV-2 positive if the threshold cycle was ≤ 40, with a simultaneous furcation of reporter and quencher fluorescence.

Primer/Probe	Name	Sequence
Forward (20µM)	dpol771-F	5'-CAC ACC CA**G** CTG GAG TAT GAC-3'
Reverse (20µM)	dpol831-R	5'-ATG TTG TAG TGG GGC CAG TC-3'
Probe (10µM)	CpHV-2 probe	5'-**TET**-AGT TCC ATT CTG AGC GGG T-BHQ1-3'

Tab. 8. Primer and probe sequences for CpHV-2 real-time PCR according to Cunha et al. (2009).

2.6 Ruminant pestiviruses

Panpesti RT-PCR

A panpestivirus reverse transcription PCR was conducted for detection of pestiviral nucleic acids, namely of bovine viral diarrhea virus (BVDV) and border disease virus (BDV). This PCR is able to detect both viruses, as primers bind to a conserved region of the 5' untranslated region (UTR) of the viral genome (Tab. 9) (Vilček, 1994). As the genome of pestiviruses consists of single stranded RNA with positive polarity, a reverse transcription step preceded amplification of cDNA. PCR was done with the Qiagen® OneStep RT-PCR Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The reaction mixture had a final volume of 20µl and consisted of 9.9µl DEPC water, 4µl of buffer (5x), 400µM dNTP, 0.6µM of each primer (Microsynth, Balgach, Switzerland), 4 units RNasin (RNase inhibitor, Promega) and 0.8µl of enzyme mix (reverse transcriptase and DNA polymerase) to which 2µl of sample RNA were added. Each sample was tested undiluted and in 1:10 dilution. The eluate control and DEPC treated water served as negative controls, extracted RNA from a BVDV persistently infected bull served as positive control. The amplicons had an expected size of 244 - 247bp. Products were stained with Orange DNA Loading Dye (6x) (Thermo Fisher Scientific, Waltham MA, USA) and loaded on a 1.5% agarose-gel containing Gel Red (1000x) (Biotium, Hayward CA, USA). The 50bp DNA Ladder (New England Biolabs) served as DNA marker. Electrophoresis was carried out at 100V for about 90 minutes.

Products were visualized under UV light and if present, bands of the expected size were excised using a single-edged razor. DNA was extracted from the gel using the QIAquick® Gel Extraction Kit, following the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland). DNA was eluted in 30µl of elution buffer. The amount of amplified DNA was measured using the Nanodrop (ND-1000 Spectrophotometer, Scientific AG, Kloten, Switzerland). Fourty-five nanograms of extracted DNA were mixed with 8µM of primer forward 324 (100µM) and supplemented with DEPC treated water to a final volume of 10µl. Sequencing was performed by Microsynth (Balgach, Switzerland) and sequences were analyzed with NCBI BLAST®. Samples were considered pestivirus positive, if gel

electrophoresis revealed a band of the expected size (244 - 247bp), and sequences matched pestiviral sequences deposited in GenBank.

Primer	Name	Sequence
Forward	324	5'- ATG CCC WTA GTA GGA CTA GCA-3'
Reverse	326	5'-TCA ACT CCA TGT GCC ATG TAC-3'

Tab. 9. Primer sequences for Panpesti RT-PCR according toVilček (1994).

BVDV/BDV biphasic indirect ELISA

For serological analysis of pestiviruses a biphasic indirect ELISA according to Canal et al. (1998), targeting the conserved NS3 protein of pestiviruses was conducted. The wells on the plate were coated alternately with antigen derived from BVDV infected (positive wells) and non-infected cells (negative wells) to control for unspecific binding. The protocol was slightly modified (Institute of Virology and Immunology, Bern), incubation periods being 60 instead of 90 minutes. For water buffalo samples goat anti-bovine IgG was used as conjugate, while peroxidase-labelled protein G was used for small ruminant samples. With protein G, the plate was incubated only for 30 minutes. Optical densities were analyzed at 405nm (Sunrise Tecan). OD values of each sample were corrected by subtracting the OD value of the negatively coated well from the positively coated well. Percentage of reactivity of each sample compared to the positive control (S/P value) was then calculated. Samples showing reactivities of < 20% were considered seronegative, samples with reactivities of ≥ 30% were considered seropositive. Percentages in between were considered doubtful.

BVDV/BDV cross-SNT

Due to the marked cross-reactions among pestiviruses, the pestivirus ELISA is not able to distinguish between anti-BVDV and anti-BDV antibodies. In order to identify the specificity of the antibodies, positive and doubtful ELISA samples were checked by cross-neutralization test. Each sample was tested against the three pestiviral strains BVDV-1a R1935/72 (Oregon C24V, cp), BVDV-1h CH-04-01b (ncp) and BDV Swiss a R9336/11 (ncp). SNT was carried out as described by Kaiser (Kaiser, V., Doctoral Thesis, Berne, 2016). The antibody titers for BVDV-1a, the cytopathogenic strain, were evaluated on the basis of cytopathic effects visible under the light microscope. For the ncp viruses a polyclonal immunoperoxidase staining was performed, which colors viral antigen in the cytoplasm of the infected cells. Every well was checked under the light microscope. Successful staining expressed itself as red brown coloring in the cytoplasm of infected cells. Wells displaying a red brown staining were considered positive (Kaiser, V., Doctoral Thesis, Berne, 2016). The antibody titers were calculated according to Spearman and Kaerber. Sample titers > 8 were considered as seropositive for pestivirus, while titers of ≤ 8 were considered seronegative. In order to relate the antibodies to either BVDV or BDV, the titer ratios (Q value) of BVDV-1a and BDV as well as BVDV-1h and BDV were calculated for each sample. Q values of ≥ 4 were considered as significant for antibodies against BVDV (1a or 1h), Q values of ≤ 0.25 as significant for BDV antibodies. Intermediate ratios (0.25 < Q < 4) pointed towards an unresolved source of infection (Danuser et al., 2009; OIE, 2008).

2.7 Retroviruses

BLV indirect ELISA

Detection of antibodies against bovine leukemia virus (BLV), the causative agent of enzootic bovine leukosis (EBL), was carried out with IDEXX Leukosis Serum X2 Test Kit (IDEXX Diavet AG, Bäch, Switzerland), which is based on whole virus as antigen. The optical density (OD) of each well was read in the photometer at a wavelength of 450nm (Sunrise Tecan). The percentage of OD compared to the positive control (S/P values) was calculated. S/P values amounting < 30% were considered seronegative. Sample reactivities ≥ 40% were considered seropositive. Percentages between 30% and 40% were questionable.

2.8 Emerging viruses

BTV competitive ELISA

A blocking ELISA was performed in order to detect antibodies against bluetongue virus (BTV) (VMRD, Pullman, Washington, USA). The assay targets a conserved epitope of the VP7 protein and is able to detect antibodies of 24 BTV serotypes.
Plasma samples were inactivated in a thermomixer for 30 minutes at 56°C. Further steps were carried out according to the manufacturer's instruction. OD values were determined by reading the plate at 650nm in the photometer (Sunrise Tecan). Percentage of sample reactivity compared to the negative controls (S/N values) was calculated. S/N values of < 50% were considered seropositive. S/N values ≥ 60% were considered seronegative. OD values between 50% and 60% were considered doubtful.

SBV biphasic indirect ELISA

Evidence for past infection with SBV was tested by serological means. A biphasic indirect ELISA was done, according to the manufacturer's instructions (SVANOVIR® SBV-Ab, Svanova, Boehringer Ingelheim, Uppsala, Sweden). Plates were coated alternately with SBV whole virus antigen and control antigen (uninfected cells). Optical densities were measured in the microplate photometer at 450nm wavelength (Tecan Sunrise). The corrected OD values (OD_{Corr}) of each sample were calculated by subtracting the OD values of the wells with control antigen from the OD values of the corresponding wells containing the SBV antigen. Percentage of reactivity of each sample compared to the positive control (S/P value) was then calculated. According to the manufacturer, S/P values of ≥ 10% are considered seropositive, values of < 10% are considered seronegative.

GyCV real-time PCR

Next Generation Sequencing revealed 14 water buffalo samples with different amounts of reads for a novel virus of the genus *Gemycircularvirus*. In order to specifically detect this virus and to investigate the prevalence of GyCV in water buffaloes and small ruminants, a real-time TaqMan® PCR was designed, targeting a 60bp sequence located in the small intergenic region of Rep and Cap gene. The reaction mixture had a final volume of 20µl and consisted of 10µl TaqMan® Universal PCR Master Mix (Life Technologies), 5µl DEPC

treated water, 0.5µM of each primer (10µM) and 250nM probe (5µM) to which 2µl of DNA were added (Tab. 10). If no DNA was available, plasma was used, which had been complement inactivated by heating it to 95°C for three minutes and then cooling it in the ice bath during five minutes.

Primer/Probe	**Sequence**
Forward (10µM)	5'-GAT CGT TCG CTT CTT TCG GTA T-3'
Reverse (10µM)	5'-TGG CTA GGC GCA CAA AAA C-3'
Probe (5µM)	5'-FAM TGT CCG TGA TGA CAA AT-MGB-3'

Tab. 10. Primer and probe sequences for GyCV real-time PCR.

PCR was carried out in a QuantStudio™ 7 Flex Real-Time PCR System. Cycling was carried out according to the manufacturer's instructions (TaqMan® Universal PCR Master Mix, Life Technologies). Results were evaluated using the QuantStudio™ 7 Flex Real-Time PCR System Software. Baseline values were set automatically, the threshold was set manually after each PCR run above the background noise in the exponential phase of amplification. Amplified DNA of one water buffalo sample showing a high amount of GyCV reads after NGS served as positive control. Samples were considered GyCV positive, if they had a Ct value of ≤ 45 and a furcation of reporter and quencher fluorescence at the same time.

3 RESULTS

Forty-eight water buffaloes, 19 sheep and 7 goats from three Swiss farms were tested for the presence of a range of viral nucleic acids and antibodies against viruses that might be circulating in these species.
Overall, five different viruses were detected by the PCR methods: bovine herpesvirus 6 (BoHV-6) and a novel gemycircularvirus (GyCV) in water buffaloes, OvHV-2, type 2 ruminant rhadinovirus of domestic sheep (RuRV-2) as well as GyCV in sheep, and caprine lymphotropic herpesvirus (CpLHV) in a goat.
Serology yielded evidence for another five different viruses circulating in water buffaloes and small ruminants: seropositivity was demonstrated against BoHV-2, BVDV and BTV in water buffaloes, BDV, BTV as well as SBV in sheep, and finally BVDV and SBV in goats.
The results, sorted for individual viruses, are described below.

3.1 Herpesviruses

3.1.1 Panherpes nested PCR

For detection of a broad range of herpesviruses a conventional panherpesvirus nested PCR was used, targeting a conserved segment of the herpesviral polymerase gene. In nine of the 74 samples – one water buffalo, seven sheep and one goat sample – gel electrophoresis of the amplified DNA revealed discernible bands with expected sizes of 215 - 235bp. The resulting amplicons were sequenced and the sequencing results were subjected to BLAST analysis and sequence alignments (Appendix). A phylogenetic tree (Fig. 2) derived from these alignments suggested that all the viruses identified by panherpes nested PCR clustered with different members of the *Gammaherpesvirinae* subfamily, rather than with the outlier BoHV-1, which is a member of the *Alphaherpesvirinae*. However, these viruses were not all equal, but could be divided into at least three groups. (1) Four sheep viruses (sheep #9, #11, #14, #16) clustered with OvHV-2, which represents the former type 1 of ruminant rhadinoviruses, but is now classified in the genus *Macavirus*. (2) Three viruses (sheep #4, #15, #18) clustered more closely with a type 2 ruminant rhadinovirus that had previously been isolated from sheep, tentatively designated type 2 ruminant rhadinovirus of domestic sheep and closely related to bighorn lymphotropic herpesvirus (LHV). (3) Finally, the viruses identified from the goat and the water buffalo seemed to be highly related to a type 2 ruminant rhadinovirus isolated from bison and, according to BLAST analysis, could be assigned to caprine lymphotropic herpesvirus (CpLHV) and bovine herpesvirus 6 (BoHV-6), in former days called bovine lymphotropic herpesvirus (BLHV) (Li et al., 2005b).
The samples of the remaining 65 animals did not provide a discernible product after panherpes PCR and were therefore considered negative.
Thus, at least according to this test, various herpesviruses circulated to various degrees on the individual farms but did not seem to be shared easily among the different animal species.

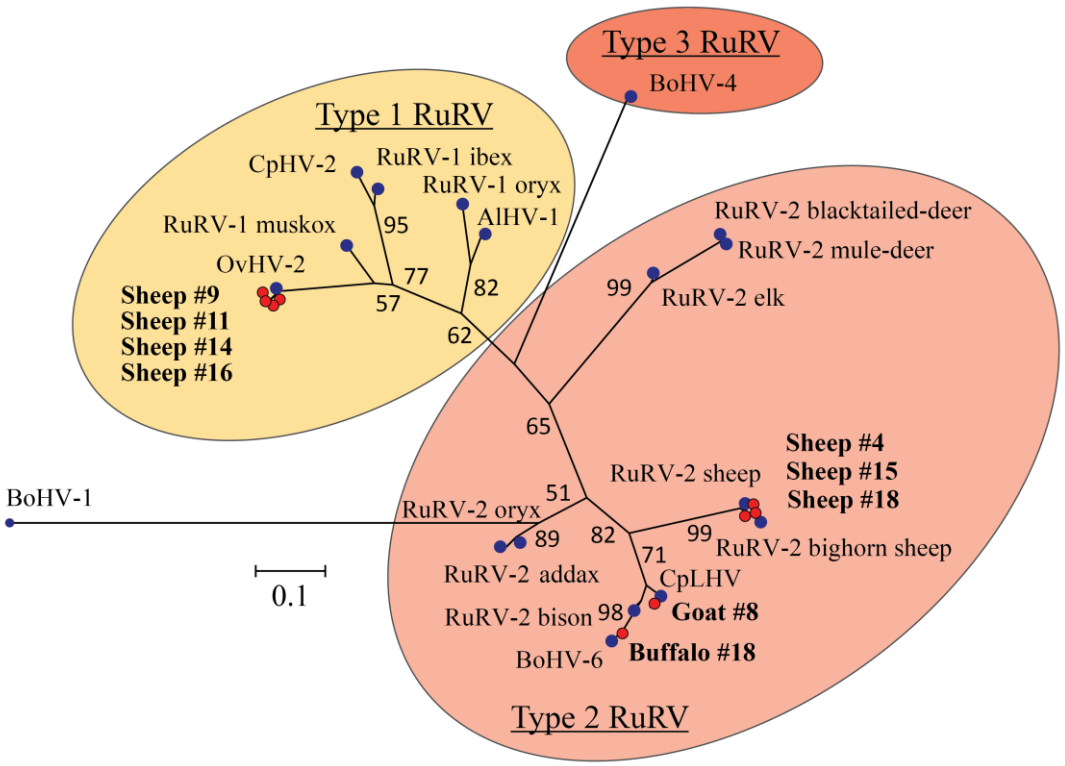

Fig. 2. Clustering of sequences detected by panherpes PCR (bold) in a phylogenetic tree of ruminant herpesviruses.

3.1.2 Alphaherpesviruses

BoHV-1 real-time PCR and gB competitive ELISA

Since the 1990s Switzerland is considered officially free of BoHV-1, a status, which is verified at intervals by serological screening of Swiss bovine animals. However, the virus can also infect non-bovine species, such as sheep and goats, which are exempt from the screening procedure. Therefore, it was of interest to test the animals for virus and antibodies against BoHV-1.

All the 48 water buffaloes were tested negative by both real-time PCR and blocking ELISA. The small ruminant samples were only tested by ELISA. They all showed blocking activities below 45% and were therefore considered negative.

Thus, there was no evidence for this notifiable virus among the animals on either farm.

BoHV-5 real-time PCR

BoHV-5 is closely related to BoHV-1 and known to cause encephalitis in bovines.

As BoHV-1, all the 48 samples from water buffaloes were tested negative for BoHV-5. Small ruminant samples were not tested.

BuHV-1 indirect ELISA

BuHV-1 infections of water buffaloes have lately been reported from Italy and South America. All the water buffaloes (except one from farm 1 of which not enough plasma was available) were subjected to an indirect gE ELISA for BuHV-1 antibodies. The small

ruminants were not tested with this ELISA . Since all tested samples had S/P values below the negative cut-off value of 30%, all the water buffaloes were regarded as seronegative, indicating that BuHV-1 did not circulate among the buffaloes on the three farms. As this test is also able to detect antibodies directed against gE of BoHV-1, though with less sensitivity (Nogarol et al., 2014), the results also argue against infection with BoHV-1 and thus reinforce findings of the BoHV-1 gB competitive ELISA.

CpHV-1 indirect ELISA

CpHV-1 is genetically and antigenically related to BoHV-1 and, in addition, is known to circulate among Swiss goats. It was therefore of interest to screen the plasma of water buffaloes, sheep and goats for antibodies against gE of CpHV-1 in order to learn, whether or not the agent was present on any of the three farms. However, as for BuHV-1, all the samples (except one water buffalo of farm 1 of which not enough plasma was available) showed reactions below the negative cut-off value of 30% and were, thus, considered negative.

BoHV-2 indirect ELISA and SNT

Cattle are the most common host of BoHV-2. On farms 1 and 3 of the study, the water buffaloes were kept in close contact to cattle, for which reason it was of interest to analyze the samples for antibodies against BoHV-2. Unfortunately, the cattle present on farm 1 and 3 were not available for sampling. Plasma samples from all the 48 water buffaloes and the 26 small ruminants were tested by ELISA. Indeed, by ELISA, one water buffalo from farm 1 had an S/P value of 111.95%, which was above the positive cut-off value (110%), whereas one water buffalo from farm 2 showed an S/P value of 109.36%, which was just below the positive cut-off, but above the negative cut-off value of 90%. Therefore, this sample was considered doubtful. The remaining 72 animals had S/P values of less than 90% and were, thus, regarded as seronegative for BoHV-2.

The two buffalo samples which were positive/questionable in the ELISA were subjected to SNT. Both showed neutralizing activity against BoHV-2 at titers of 80 and 62, respectively.

This is the first evidence that BoHV-2 may infect Swiss water buffaloes to a degree that leads to the production of neutralizing antibodies.

3.1.3 Gammaherpesviruses

OvHV-2 real-time PCR

OvHV-2 is known to endemically circulate in the Swiss sheep population and able to cause MCF in water buffaloes. By OvHV-2 real-time PCR, all the samples were tested in 1:2 and 1:10 dilution. OvHV-2 DNA was detected in 15 of 19 sheep samples. The Ct values ranged from 29.3 to 39.8 (Tab. 11). The four positive results for OvHV-2 detection by panherpes PCR were confirmed in the real-time PCR, all showing Ct values below 35. However, two sheep displaying Ct values in the same range (Ct 33.3 and 34.5, respectively) and seven sheep with Ct values of above 35 in the OvHV-2 PCR had not been detected by panherpes PCR. Interestingly, two of the three sheep that had been positive for sheep-derived RuRV-2 in panherpes PCR, showed positive signals in the OvHV-2 PCR.

Thus, presence of OvHV-2 was confirmed among the sheep on farm 2, even at higher prevalence than according to the panherpesvirus test. However, there was no evidence for transmission of the virus from the infected sheep to the water buffaloes on the same farm, neither was OvHV-2 detected in the water buffaloes or in the goats on farms 1 and 3.

Sample	Ct value 1:2	Ct value 1:10	Panherpes PCR
Sheep #11	29.3	31.9	OvHV-2
Sheep #16	31.8	36.2	OvHV-2
Sheep #6	33.3	35.7	-
Sheep #4	33.7	35	RuRV-2
Sheep #9	33.7	36	OvHV-2
Sheep #3	34.5	> 40	-
Sheep #14	34.8	37	OvHV-2
Sheep #15	34.9	39.8	RuRV-2
Sheep #5	35.2	38.9	-
Sheep #10	35.4	35.6	-
Sheep #2	36.3	> 40	-
Sheep #7	36.5	39.5	-
Sheep #17	36.7	> 40	-
Sheep #1	37.3	35.8	-
Sheep #13	38.9	38.3	-
Sheep #12	> 40	> 40	-
Sheep #18	> 40	> 40	RuRV-2
Sheep #19	> 40	> 40	-
Sheep #8	> 40	> 40	-

Tab. 11. Detection of OvHV-2 and RuRV-2 DNA in buffy coat cells from sheep (sorted according to OvHV-2-related Ct values).

CpHV-2 real-time PCR

CpHV-2 is the second gammaherpesvirus known to cause MCF in water buffaloes.

In contrast to OvHV-2, which was detected with high prevalence in the sheep on farm 2, CpHV-2 was not detected in any of the 74 samples.

3.2 Ruminant pestiviruses

Panpesti RT-PCR

Water buffaloes, sheep and goats are known to be susceptible to both BVDV and BDV, respectively. For this reason, it was of interest to test the three species by conventional panpestivirus RT-PCR, in order to detect viremic or persistently infected animals.

Neither any of the 26 small ruminants nor 47 of 48 water buffaloes showed a band by gel electrophoresis, which after sequencing and BLAST analysis could be attributed to a pestivirus. However, one water buffalo sample from farm 1 showed a well discernible band of the expected length, which according to BLAST analysis displayed 99% sequence identity to the 5' UTR of BVDV-1 isolate Carlito (GenBank Accession: KP313732.1). This isolate was also used as positive control. However, upon re-testing of the sample twice, the result of gel

electrophoresis could not be reproduced and the sample did not show any band. Accordingly, the band appearing in the first round of testing was suggestive for contamination.
Thus, all the 74 samples were considered negative for pestiviral nucleic acids, suggesting that none of the animals had been viremic with a pestivirus at the time of sampling, neither with BVDV nor with BDV. Importantly, this also rules against the possibility that any of the animals tested had been persistently infected with a pestivirus.

BVDV/BDV biphasic indirect ELISA and cross-SNT

A high percentage of animals experience a transient infection with pestiviruses, which leads to a long-lasting seroconversion. To investigate, whether the water buffaloes, the sheep or the goats in the present study had seroconverted, the 74 plasma samples were tested for antibodies directed against the highly conserved pestiviral NS3 protein by ELISA. Indeed, the ELISA revealed seven samples, which exceeded the negative cut-off value of 20% (Tab. 12): four water buffaloes, one goat and one sheep exceeded the positive cut-off value of 30%, whereas one water buffalo was classified as questionable with a reaction of 21% compared to the positive reference serum. With reactions of 20% or less, the remaining 67 samples did not provide evidence for pestivirus antibodies. These animals were, therefore, considered seronegative.
In order to assign the antibodies to either BVDV or BDV, the seven samples revealing ELISA reactions greater than the negative cut-off value (> 20%) were subsequently subjected to cross-neutralization tests, where each sample was tested against three different pestivirus strains: BVDV-1a (cp), BVDV-1h (ncp) and BDV Swiss a (ncp). All the seven samples showed titers > 8 against at least one virus strain and were therefore confirmed seropositive for pestivirus. In terms of assignment of the antibodies to BVDV or BDV, the SNTs allowed an unambiguous result in six out of the seven samples. Four water buffaloes as well as the goat had significantly higher titers against BVDV than against BDV (Q value $\geq$ 4). In contrast, the sheep had significantly more neutralizing antibodies against BDV than against any of the two BVDV strains tested (Q value $\leq$ 0.25). One water buffalo from farm 2 showed rather high neutralizing antibody titers against both BVDV and BDV, thus providing an insignificant difference between antibody titers against the two viruses ($0.25 < Q < 4$) (Tab. 12).
According to these results, at least two different pestiviruses had been circulating among the ruminants on the three farms.

Farm	Species	ELISA	SNT Titer BVDV - 1a	BVDV - 1h	BDV Swiss a	Q value BVDV/BDV	Interpretation
1	Buffalo	41%	14	80	8	10	BVDV
2	Buffalo	42%	67	190	95	2	Ambiguous
	Buffalo	74%	95	538	80	6.37	BVDV
	Buffalo	57%	190	1076	160	6.37	BVDV
	Sheep	56%	8	10	190	0.05	BDV
3	Buffalo	21%	40	48	≤ 7	≥ 6.86	BVDV
	Goat	50%	57	320	28	11.43	BVDV

Tab. 12. Results of the seven samples exceeding the negative cut-off value by ELISA and correspondent SNT titers against the three pestiviral strains BVDV-1a, BVDV-1h and BDV Swiss a.

3.3 Retroviruses

BLV indirect ELISA

Small ruminants as well as water buffaloes are known to be susceptible to infection with BLV. However, as small ruminants are excluded from the sample testing, these species may act as reservoirs for the retrovirus.

The plasma samples of 72 animals were tested (no plasma available from two water buffaloes from farm 1). As all the samples exhibited S/P values below the negative cut-off (< 30%), they were regarded as BLV seronegative.

Thus, there was no evidence for circulation of the notifiable BLV among water buffaloes and small ruminants on any of the three farms.

3.4 Emerging viruses

BTV competitive ELISA

As water buffaloes and small ruminants are known to be susceptible to infection with BTV, which has only recently caused infections in Swiss ruminants, it was of interest to test for antibodies against BTV in the plasma samples. Four out of 74 tested samples exceeded the positive cut-off value showing S/N values of less than 50% against VP7 of BTV, indicating a distinct blocking activity. Accordingly, three water buffaloes on farm 3 (25.5%, 37.2%, 17.2%) and one sheep on farm 2 (29.9%) were considered BTV seropositive. All the other samples reacted in the negative range, with S/N values of more than 60%. These 70 animals were therefore considered as BTV seronegative (Fig. 3).

Thus, it could be shown that three years after the official elimination of BTV serotype 8 in Switzerland, sheep as well as water buffaloes may still carry anti-BTV antibodies.

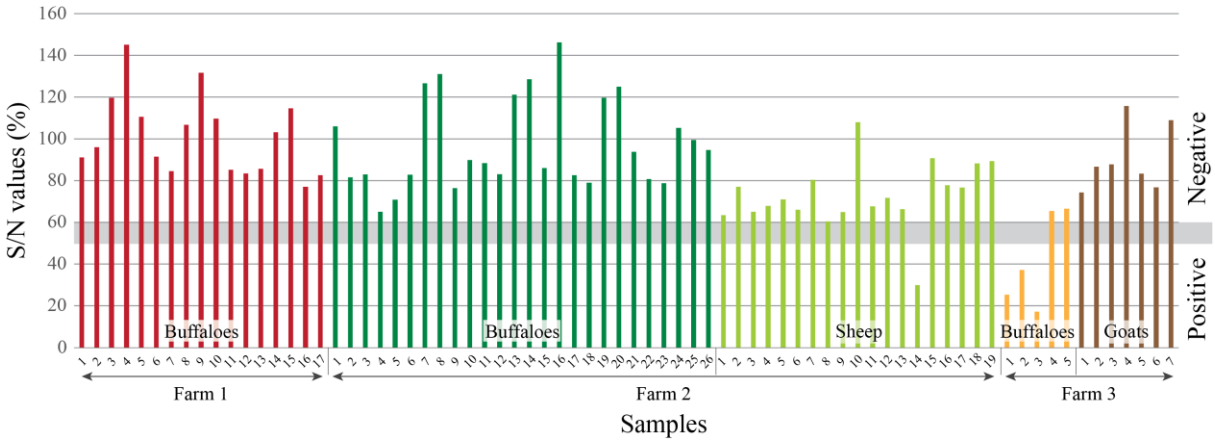

Fig. 3. S/N values of the 74 plasma samples tested in BTV competitive ELISA.

SBV biphasic indirect ELISA

In 2012, SBV spread rapidly across the Swiss ruminant population, leaving behind a high percentage of seroconverted animals. In order to investigate, whether the water buffaloes and the small ruminants in the present study had been affected by SBV infection, an indirect biphasic ELISA was conducted. As the 72 water buffaloes tested (no plasma left from two buffaloes on farm 1) showed S/P values below the negative cut-off of 10%, they were considered seronegative against Schmallenberg virus. Among the small ruminants, eight sheep on farm 2 and two goats on farm 3 showed S/P values of more than 10%, indicating seropositivity against SBV (Fig. 4).

These findings suggest past infection of small ruminants with SBV, while the water buffaloes apparently were not affected.

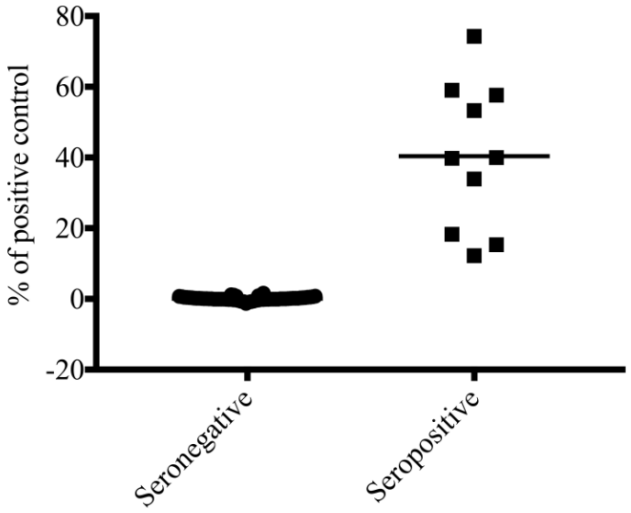

Fig. 4. S/P values of the 72 plasma samples tested in SBV indirect ELISA.

GyCV real-time PCR

Previous results of Next Generation Sequencing revealed various amounts of GyCV sequences in the blood samples (buffy coat and plasma fraction) of 14 water buffaloes. In order to specifically detect GyCV DNA in the samples, PCR primers were designed and buffy coat extracted DNA from all the 48 water buffaloes as well as the 26 small ruminants were

tested for GyCV DNA by real-time PCR. Interestingly, 22 of 48 water buffaloes gave a detectable signal with Ct values ranging from 25.9 to 40.4 (Fig. 5). The 14 samples that had already been detected GyCV positive by NGS were confirmed positive by real-time PCR. The other eight water buffaloes were newly detected as carriers of GyCV DNA by PCR. With an R^2 value of 0.5, the samples with low Ct values had a tendency to also provide a relative high number of reads by NGS (Fig. 5). As a rule of thumb, the samples could be divided into three categories: (1) PCR-negative samples that did not yield any reads in NGS; (2) PCR-positive samples that were not confirmed by NGS; (3) PCR-positive samples that were confirmed by NGS reads in a tentatively dose-dependent manner. In addition to the 22 water buffaloes, 11 sheep showed positive signals by PCR with Ct values ranging from 35.0 to 39.8. Compared to the sheep, a part of the GyCV positive buffaloes had distinctly lower Ct values, suggesting a higher amount of viral DNA in the samples and possibly indicating ongoing virus replication in these animals (Fig. 6).
The remaining animals did not give a Ct value and were therefore considered negative.
According to these findings, for the first time evidence is provided for the presence of GyCV DNA in blood samples of water buffaloes and sheep.

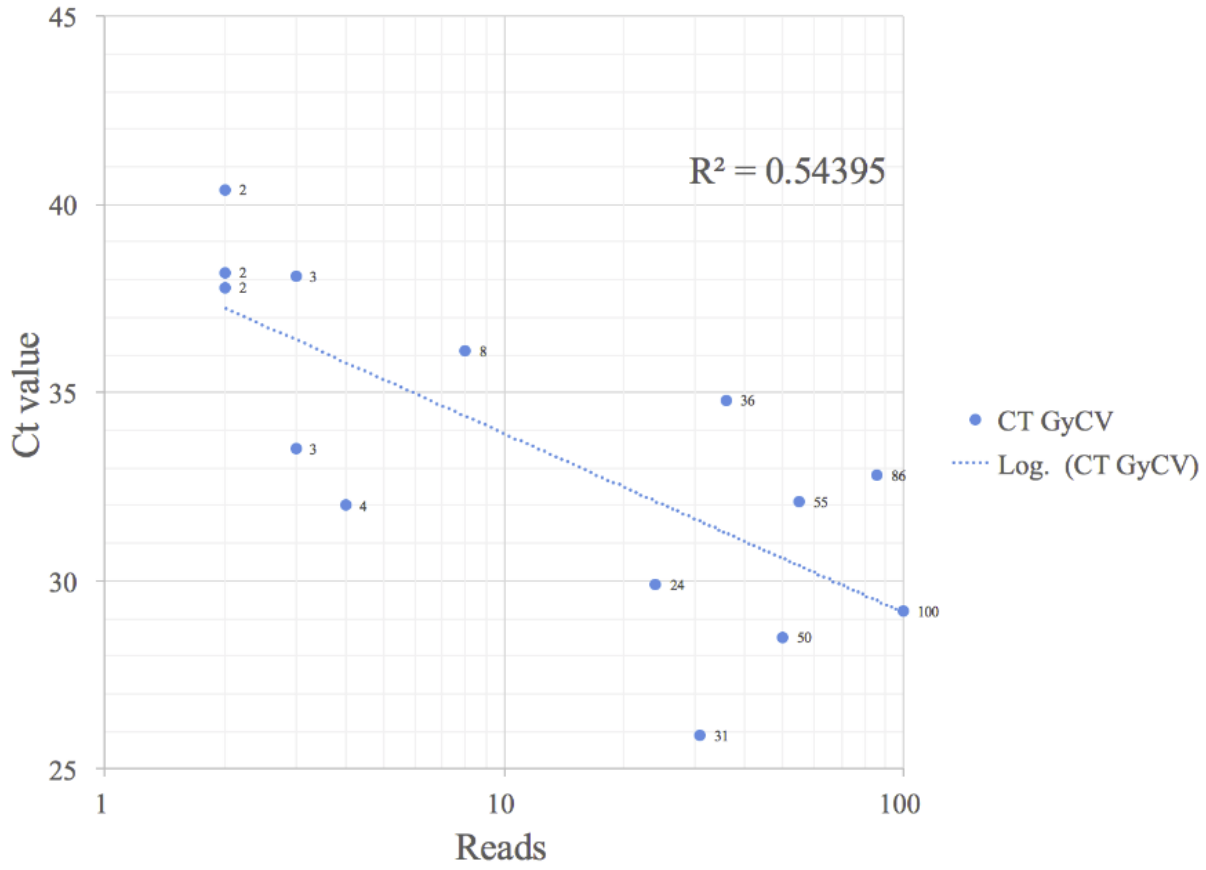

Fig. 5. Comparison of Ct values and NGS reads of water buffaloes on farm 2 with regard to GyCV detection. Numbers adjacent to the graphed points indicate the numbers of GyCV reads found by NGS.

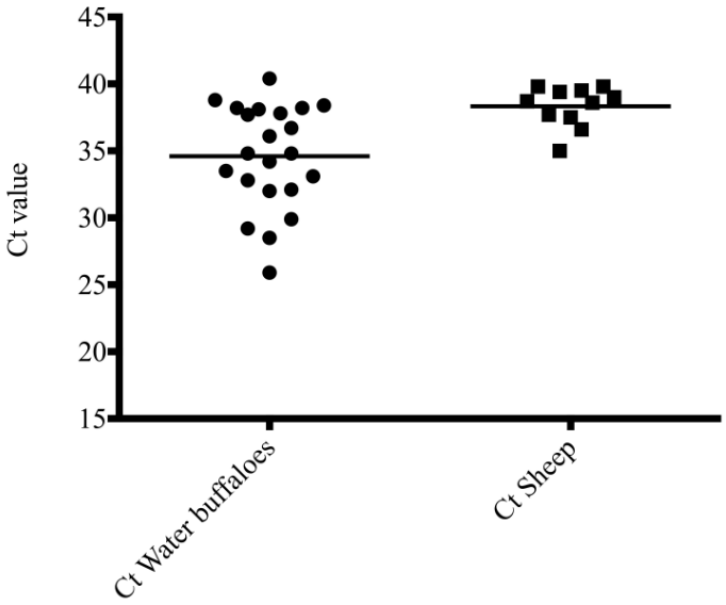

Fig. 6. Ct values of 22 water buffaloes and 11 sheep according to GyCV real-time PCR.

4 DISCUSSION

The salient features of the present study are the detection of BoHV-6 and a novel GyCV in the water buffaloes, OvHV-2, RuRV-2 of domestic sheep and GyCV in sheep and CpLHV in a goat by means of PCR.
By serological methods water buffaloes were found seropositive for BoHV-2, BVDV and BTV, sheep seropositive for BD, BTV and SBV and goats seropositive for BVDV and SBV.
Neither by NGS nor by conventional methods evidence was found for the physical and/or serological presence of BoHV-1, BoHV-5, BuHV-1, CpHV-1, CpHV-2, BVDV or BDV in the study-species.

In the following, the results shall be discussed referring to the aims and the hypotheses stated earlier and in the context on the current situation of viral diseases in ruminants.

4.1 Aims 1 and 2

The first two aims concerned the detection of various herpesviruses (herpesviruses in general, BoHV-1, BoHV-2, BoHV-5, BuHV-1, OvHV-2, CpHV-1, CpHV-2), pestiviruses (BVDV and BDV), BLV, BTV, SBV and GyCV either directly by PCR or indirectly by ELISA. In the cases of BoHV-2 and pestiviruses, ELISA positive samples were additionally tested by SNT.

(1) According to hypothesis 1, detection of various ruminant herpesviruses was expected in the animals tested. While no alphaherpesviruses were detected by this approach, hypothesis 1 was still confirmed, since - by means of panherpes PCR followed by sequencing - the presence of several gammaherpesviruses could be demonstrated in the samples, namely BoHV-6 in a water buffalo, RuRV-2 of domestic sheep and OvHV-2 in the sheep, as well as CpLHV in a goat.
The present case is the first report of BoHV-6 infection in Swiss water buffaloes. Previously, BoHV-6 had been detected in a buffalo herd in Brazil in the context of a lymphoproliferative disease (De Oliveira et al., 2015). However, the same authors detected BoHV-6 also in buffalo herds with no history of lymphatic disease. At the time, prevalence of BoHV-6 was estimated to be 2.23% among water buffaloes and 66.66% among cattle, which confirmed that BoHV-6 rather infects cattle than buffaloes. With one BoHV-6 positive buffalo among 48 tested in the present study, these prevalence data among buffaloes were pretty much confirmed. Indeed, BoHV-6 has been first detected in PBL and B-lymphoma cells of cattle (Rovnak et al., 1998). Later on, the virus was found in the most diverse tissue samples of diseased cattle, including bovine PBL (Collins et al., 2000; Kubis et al., 2013), vaginal exsudate and vaginal swabs from cows suffering from non-responsive post-partum metritis (Cobb et al., 2006; Banks et al., 2008; Garigliany et al., 2013), in a bovine aborted fetus (Gagnon et al., 2010) and in brain samples of Swiss cows suffering from neurologic disease (Lechmann, J., Master's Thesis, Zurich, 2014). Although BoHV-6 has been detected in some

diseased cattle and water buffaloes, a causative association to a disease has never been firmly established and neither was it confirmed here.
The detection of the other gammaherpesviruses in sheep and goats (OvHV-2, RuRV-2 of sheep, CpLHV) also supported the first hypothesis but was no surprise, since these viruses are known to subclinically circulate among these species. Particularly the high OvHV-2 infection rate in sheep was to be expected, as the virus is widely spread in the Swiss sheep population.
OvHV-2 was initially detected in four sheep by panherpes nested PCR (see also Fig. 2). By real-time PCR presence of the virus in the sheep was confirmed, even at higher prevalence (79%) than according to panherpes PCR (21%), which may be explained by a higher sensitivity of real-time PCR compared to panherpes nested PCR. Two sheep were positive for RuRV-2 of domestic sheep by panherpes PCR and OvHV-2 positive by real-time PCR, suggesting a co-infection with two different gammaherpesviruses in these animals. In case of herpesviral co-infections, panherpes nested PCR only detects the most abundant virus, in this case RuRV-2.

(2) Many alphaherpesviruses can cause transient viremia, which is important for generalized clinical manifestations as well as incidences of abortion. The duration of such viremia is usually too short to be detected by random single time collection of blood samples. However, just like all herpesviruses, alphaherpesviruses establish lifelong latency, which can be reactivated at intervals. In the absence of vaccines, detection of corresponding antibodies therefore is a safe indicator of infection. Since BoHV-1 has been successfully eradicated in Switzerland, whereas infections with the serologically related viruses BoHV-5 and BuHV-1 have thus far never been reported, according to hypothesis 6, detection of any serological evidence for these viruses among the water buffaloes or small ruminants was not expected. Indeed, this hypothesis was confirmed by the present data. The only uncertainty regarding these serologies were due to CpHV-1, which is known to circulate among Swiss goats (Mettler et al., 1979; Plebani et al., 1983). However, since CpHV-1 seems to be a regional problem among goats (Plebani et al. 1983) and since the goats in the present study reacted negatively, it was no surprise to see that also the buffaloes remained seronegative.
In contrast, serological evidence was gained for BoHV-2 infection in two water buffaloes. The results, initially detected by ELISA, were confirmed by serum neutralization. Although the virus apparently has a wide host range, including cattle, sheep, goats and many wild ruminants (Janett et al., 2000; Torres et al., 2009), this outcome was unexpected, yet, confirmed in part hypothesis 7, according to which interspecies transmission of herpesviruses was expected. To the author's knowledge, the two present cases are the first water buffaloes with evidence for BoHV-2 infection. In Swiss cattle the virus is known to circulate with estimated seroprevalences of between 7% and 25% (Engels et al., 1979; C. Bachofen, personal communication, 2016). However, there is no eradication program against BoHV-2, likely due to the fact that infections most often take a subclinical course and bovine herpes mammillitis is reported only rarely (Engels et al., 1979; Müller et al., 1984; Janett et al., 2000; Syring et al., 2010). Indeed, no clinical disease attributable to BoHV-2 infection was reported from the two farms, where the affected water buffaloes were housed. It will be interesting to look for mammilitis among water buffaloes in the future.

(3) In contrast to herpesviruses, the PCR-based detection of ruminant pestiviruses in the samples was, according to hypothesis 5, less likely. Indeed, this hypothesis was confirmed inasmuch as there was no physical evidence for an ongoing acute or persistent pestivirus infection in any of the animals tested. BVDV has successfully been brought to the brink of extinction by the effective application of a national eradication program for cattle (Schwermer et al., 2013). Precise knowledge does not exist but considering the present number of persistently infected cattle at below of 0.02%, it was unlikely that the number of persistently infected animals among the foreign hosts (buffaloes, sheep, goats) would exceed the percentage of persistently infected animals in the natural host of this virus (cattle). It was therefore comforting to see that no novel BVDV reservoir among the Swiss water buffaloes was detected.

(4) On the other hand, considering the unavailability of anti-pestivirus vaccines in Switzerland, the serological evidence of the present study clearly indicated that pestiviruses, i.e. BVDV, were able to infect water buffaloes causing seroconversion, although not to a high prevalence. These findings confirmed hypothesis 2 as well as hypothesis 7. Interestingly, with just one sheep seropositive for BDV and one goat seropositive for BVDV, small ruminants seropositive for pestivirus antibodies were even less frequent than buffaloes, which suggested that, due to the absence of persistently infected animals, the infection pressure was not very high.

(5) BLV in Switzerland has been eradicated for a long time. Therefore, it was no surprise that no evidence of BLV seropositive animals was found in any of the samples. Thus, hypothesis 6 was also confirmed in this respect.

(6) After compulsory vaccination against BTV-8, which is followed by a long-lasting antibody response of up to six years (Eschbaumer et al., 2012), Switzerland is again officially free of BTV since 2012. Mandatory vaccination included cattle, sheep and goats but not buffaloes. Therefore, it was hypothesized that at least the water buffaloes would be tested seronegative, whereas goats and sheep may or may not retain antibodies dating back to the vaccination campaign. Indeed, one sheep from farm 2 was tested seropositive, whereas all the other sheep and the goats on farm 3 were tested negative. Surprisingly and contrary to hypothesis 6, three out of five water buffaloes on farm 3 were tested seropositive to BTV. Using a competitive ELISA with an antigen comprising a fragment of VP7 that is conserved among 24 serotypes of BTV, it was not possible to determine the exact BTV type that caused this reaction. Unfortunately, not enough serum was left for further serotyping. Accordingly, three scenarios may explain this seroreaction in the water buffaloes: (A) although *vaccination* had not been mandatory for the water buffaloes, on farms 1 and 2 vaccination was carried out in 2008 and 2009. The buffaloes on farm 3 were delivered from three different farms, that had also vaccinated their animals in 2008 and 2009. In cattle, vaccination-induced antibodies have been shown to persist at high levels in serum and milk for at least three years (Oura et al., 2012). Consequently, there may still have been animals with detectable antibody titers at the time of sampling in 2013. The fact that the three seropositive water buffaloes on farm 3 had no vaccination history clearly argues against vaccination-derived antibodies. (B) However,

the three seropositive buffaloes originated from vaccinated dams and might be considered as individuals carrying *maternal antibodies*. Maternally derived BTV antibodies in cattle calves and lambs were shown to persist for more than three months (Vitour et al., 2011; Leemans et al., 2013). The seropositive twins on farm 3 were 3.7 months old when sampled, which could explain colostral derivation of the antibodies in these animals. With blocking activities of 17.2% and 25.5% this would suggest, that even at the age of four months maternal antibodies may persist at high titers in the water buffaloes. However, at an age of ten months, the third buffalo was probably too old to still have colostral antibodies. (C) The third and most disquieting possibility is that the animals actually had experienced a *BTV infection*. It is known from previous reports that water buffaloes are susceptible to BTV infection (Lage et al., 1996; Gür et al., 2011). To address this possibility for the present case, it would be extremely useful to know the causing serotype. Thus far, only BTV-8 has been recorded in Switzerland. However, the Swiss water buffaloes originate from Romania, where numerous outbreaks of BTV serotype 4 have been reported (Niedbalski, 2015). Moreover, on a regular basis semen for artificial insemination is being imported from Italy, where several types of BTV are known to circulate (Kyriakis et al., 2015). Although option (C) cannot be substantiated, with the knowledge of their susceptibility it is advisable to include water buffaloes into a future monitoring of BTV.

(7) Taking into account the rapid and countrywide spread of Schmallenberg virus in 2012/2013 as well as the fact that others have found antibodies against SBV in Anatolian water buffaloes (Azkur et al., 2013), seropositive animals were expected in the present study. As a matter of fact, eight sheep on farm 2 and two goats on farm 3 were found seropositive by indirect ELISA. However, none of the water buffaloes showed positive results. Thus, hypothesis 4 was confirmed for the small ruminants, but not for the water buffaloes.
In the Anatolian water buffaloes, SBV antibodies were detected at a prevalence of 1.5%, while seroprevalences in cattle, sheep and goats were 39.8%, 1.6% and 2.8%, respectively (Azkur et al., 2013). Considering that 40% of sheep on farm 2 reacted seropositively against SBV, this seroprevalence exceeded that of sheep in the study from Turkey. It was therefore a particular surprise to observe all the water buffaloes on the same farm to be seronegative.

(8) After detection of a novel member of the *Gemycircularvirus* genus by NGS in 14 water buffaloes on farm 2, detection of GyCV sequences in these animals by a newly established real-time PCR was expected to be confirmed. Indeed, all the plasma samples positive by NGS were confirmed, while even eight more water buffaloes were found positive by the conventional PCR approach. Moreover, 11 sheep, which had not been tested by NGS, also reacted positively by GyCV PCR. Thus, hypothesis 3 was confirmed.
Hitherto, among ruminants GyCV had only been detected in serum of healthy cattle (Lamberto et al., 2014). The present study provides the first evidence of a novel GyCV in plasma samples of water buffaloes and sheep.
It is not yet clear, whether GyCV other than SsHADV-1 do establish real infections in the "hosts" in which they were detected. As gemycircularviruses apparently are widely spread in the environment (Krupovic et al., 2016), the possibility of GyCV being a sample contaminant needs to be considered. The fact that the novel GyCV was found with a high prevalence only

on farm 2, in water buffaloes as well as in sheep, would make an infectious event plausible. However, a serological confirmation of infection would be more conclusive.

4.2 Aim 3

An important aim of this study concerned the comparison of the two approaches applied for virus detection, namely established conventional diagnostic tests (PCR, ELISA, SNT) on the one hand and the relatively novel NGS technology on the other hand.

By the conventional approach evidence was gained for infection of the water buffaloes with BoHV-6, GyCV, BoHV-2, BVDV and BTV, while by the metagenomic approach GyCV was the only virus detected in the buffalo samples. By overall comparison, this would clearly argue in favor of conventional methods, which in contrast to NGS have the advantage that viral infections can be demonstrated directly by detection of viral genome but also indirectly via antibody detection. This circumstance is of particular importance for viruses that establish no or only transient viremia. Comparing only the viruses that were physically detected, conventional PCR still was superior to NGS. While GyCV was detected in both approaches, BoHV-6 was only detected by conventional PCR but not by NGS, indicating a higher sensitivity of the conventional method. This was underlined by the fact that by specific real-time PCR eight more water buffaloes were detected GyCV positive than by NGS. One reason, why metagenomic techiques lag behind in terms of sensitivity of virus detection lies in the relatively small size of the viral genome compared to the contaminating host genome in a sample (Barzon et al., 2013). Taking conventional PCR as standard, specificity of NGS appeared very satisfactory, as there were no viral sequences detected that were not found by PCR. However, the novel gemycircularvirus could only be detected by PCR after priorily having been discovered by NGS, whereas it would have been missed by applying conventional tests solely. As a matter of fact, in the past few years NGS technology has become a valuable tool for identification of novel viral agents (Barzon et al., 2013; Van Borm et al., 2015), as it has the big advantage of being a culture- and sequence-independent technique (Barzon et al., 2013; Granberg et al., 2015; Van Borm et al., 2015). Indeed, only recently a novel Orthobunyavirus causing disease in ruminants, now known as Schmallenberg virus, could be identified thanks to NGS (Hoffmann et al., 2012). If sequences of novel viruses are obtained by NGS, presence of the identified viruses is usually confirmed by using targeted and more sensitive conventional tests (Van Borm et al., 2015).
Another advantage of NGS is its ability to generate a wealth on sequence data of potentially all genomes present in a sample. However, in order to reduce the amount of data and to optimize detection of the desired genomes, sample preparation steps should precede the actual sequencing (Granberg et al., 2015). For the NGS sub-project of the present study, pre-sequencing steps were carried out with the objective of reducing the amount of host DNA and of enriching viral nucleic acids in the blood samples. After a homogenization and a filtration step, samples were treated with nucleases, leading to the degradation of unprotected nucleic acids. In consequence, only actively replicating viruses, protected by the viral capsid, were detected by NGS (Granberg et al., 2015). This also explains why in this study detection of

BoHV-6 was missed by NGS: latent episomal herpesvirus DNA was degraded in the course of nuclease treatment. Thus, detection of gammaherpesviruses, which occur in the blood stream during the latent state, cannot be expected by NGS. In contrast, alphaherpesviruses like BoHV-1, which usually occur in the blood during active infection with systemic spreading or after reactivation from the latent state, should readily be detected by NGS. In the present study only one alphaherpesvirus was detected by conventional means, namely BoHV-2. The reason why this virus was not found in the NGS approach may be explained by the fact that BoHV-2, if at all, only establishes low level viremia. This clearly points out a limitation of virus detection by NGS: the procedure of sample preparation for concentration of viral DNA/RNA and removal of host-DNA and other big sized particles inevitably leads to a certain degree of selection or loss of viruses. As demonstrated in the present study, these viruses are reliably detected by conventional methods.

In conclusion, for diagnosis of viral infections, particularly of emerging viruses, conventional diagnostic tests and NGS should be used in a complementary manner, by taking advantage of two synergistic features of both approaches: the high-throughput and sequence-independent nature of NGS technology as well as the higher sensitivity of targeted diagnostic tests (Barzon et al., 2013; Van Borm et al., 2015). With the further development, metagenomic techniques have more and more found their way to diagnostic laboratories. However, the implementation of NGS only precedes slowly, as in contrast to conventional methods, NGS data analysis requires cost-intensive expertise in bioinformatics and computing resources (Barzon et al., 2013).

4.3 Aim 4

A main intention of the present study was to contribute to the determination of the water buffalo virome. The viruses that so far constitute the buffalo virome are numerous and include viruses causing generalized, respiratory, gastrointestinal, urogenital and neurologic disease (Tab. 13).

Disease	Virus	References
Systemic	OvHV-2, CpHV-2, BVDV, BTV, BLV, BIV, BPXV, FMDV, PPRV, LSDV, BEFV, Flaviviruses	Stahel et al., 2013; Dettwiler et al., 2011; Martuciello et al., 2009; Hofmann et al., 2008; Molnar et al., 1999; Albernaz et al., 2015; Singh et al., 2006; Klein et al., 2008; Govindarajan et al., 1997; Sharawi and Abd El-Rahim, 2011; Patel et al., 1993; Casseb et al., 2014
Respiratory	BoHV-1, (BuHV-1), PI-3	Fusco et al., 2015a; Petrini et al., 2012; Maidana et al., 2012
Gastro-intestinal	BVDV, BuCoV, Rotavirus, PBV, BAstV	Martuciello et al., 2009; Decaro et al., 2008; Manuja et al., 2010; Mondal et al., 2013; Alfred et al., 2015
Urogenital	BoHV-1, BPV-2	Fusco et al., 2015a; Roperto et al., 2013
Neurologic	BoHV-5, SBV, RABV	Megid et al., 2015; Hoffmann et al., 2012; Zhang et al., 2011
Asymptomatic	BoHV-6, (BuHV-1)	De Oliveira et al., 2015; Petrini et al., 2012

Tab. 13. Viral infections and associated diseases hitherto reported in water buffaloes

These viruses have mostly been reported in Asia or South America, while reports of viral infections from European buffaloes are rather scarce. This fact can be contributed to the smaller buffalo population in Europe and to the superior animal health status compared to other continents. For example, in Switzerland water buffaloes were subjected to a mandatory test for antibodies against BoHV-1 before the permit for importation was issued. Most likely, this precluded not only importation of animals carrying latent BoHV-1 but also BuHV-1, BoHV-5, and probably CpHV-1.
Due to financial restrictions, the present study was confined to only one sample material for virome analysis. EDTA blood represented the material of choice for the following reasons: (I) it allowed testing for both viral nucleic acids in the leukocytes and antibodies in the plasma, (II) emerging viruses, which are often transmitted by arthropods, cause prolonged viremia with a relatively high amount of virus in the blood stream/blood cells, (III) a wide range of non-arboviruses, such as herpesviruses or pestiviruses cause viremia, (IV) blood offered the possibility for detection of novel viral agents, (V) professional blood withdrawal favored low probability of sample contamination and finally, (VI) blood collected in EDTA tubes is known for high consistency regarding quality and DNA/RNA content.
The present report therefore did not claim to complete, but rather intended to contribute to the establishment of the buffalo virome. Indeed, with the results gained from the two methodical approaches, two viruses - BoHV-2 and GyCV - were found that can newly be recorded as part of the buffalo virome. Further it could be confirmed that water buffaloes are susceptible to infection with BoHV-6, BVDV and BTV.
In order to expand the spectrum of viruses infecting water buffaloes, other tissue sites, e.g. the respiratory tract and the digestive tract, should be included in future investigations. Also, the probability of virus detection may be improved by increasing the sample size on herd level and on individual level.

4.4 Aim 5

The present study was motivated by the increasing number of water buffaloes in Swiss livestock. In the background of the infectiological hazards accompanied by this trend, one of the important questions of this report was to assess the risk of interspecies transmission between water buffaloes and native ruminants.

An important evidence was the absence of infections with BoHV-1 and BLV in the three study-species. Both viruses have been eradicated in Swiss cattle but not in water buffaloes and small ruminants, which may act as virus reservoirs.
Five different herpesviruses were shown to have infected the animals in this study. Three of them - BoHV-6, RuRV-2 of domestic sheep and CpLHV - are considered apathogenic and unlikely to pose a risk to the ruminants. Anyway, with the exception of BoHV-6, interspecies transmission of these gammaherpesviruses has not been reported to date. They are supposed to have co-evolved with their hosts and to be highly adapted to them (McGeoch et al., 2005).
It is interesting to note that the BoHV-6-positive buffalo was housed on farm 2, where no cattle were present. Since the virus was not found among the samples of sheep on the same

farm, it is possible that this particular animal had either been in contact to neighbouring cattle or else that BoHV-6 may also infect small ruminants.
The other two herpesviruses - OvHV-2 and BoHV-2 - are known to be potentially pathogenic in several ruminant species. In contrast to the herpesviruses mentioned above, OvHV-2 and other MCFV are known to cross the species barrier with often severe consequences in non-adapted species, including water buffaloes (Stahel et al., 2013). Thus, having found OvHV-2 with a prevalence of nearly 80% in the sheep flock on farm 2, there is a high risk for virus transmission from the sheep to the co-housed water buffaloes, causing clinical MCF in the latter. Strikingly, none of the co-housed water buffaloes was tested OvHV-2 positive, which was corroborated by the preliminary report of the farmers, according to which no clinical MCF cases had occurred on their farms. The absence of clinical MCF may be ascribed to the strict separation of the water buffaloes and the sheep on this farm, which is known to be an efficacious method for preventing transmission of MCFV (Li et al., 2014). These results also confirmed that the virus did not circulate subclinically in the water buffaloes, which has been reported before (Stahel et al., 2013). Although not detected among water buffaloes in this study, CpHV-2 was previously shown to infect water buffaloes, resulting in subclinical infection or clinical MCF (Dettwiler et al., 2011; Stahel et al., 2013). Consequently, both domestic sheep and goats clearly pose a risk for native cattle and water buffaloes. The other way round, water buffaloes and cattle are considered as dead end hosts for both viruses and therefore are not believed to play a role in virus transmission to other ruminants. In order to prevent clinical disease in indicator hosts, which would be particularly indicated for the buffaloes on farm 2, separation from small ruminant reservoir hosts should be strictly maintained. Another possibility is the establishment of virus-free sheep or goat flocks, by serparating lambs/kids from their dams at an appropriate time (Li et al., 1999; Hüssy et al., 2001; Li et al., 2005a).
Co-housed cattle would seem the most probable source of BoHV-2 infection for the two buffaloes on farms 1 and 2. Interestingly, there were no cows present on farm 2, suggesting another source of infection, for example insects (Janett et al., 2000; Kemp et al., 2008), or contact to neighbouring cattle or small ruminants.

It is known from the literature that interspecies transmission of BVDV and BDV from the primary hosts (cattle and sheep, respectively) to less adapted species occurs (Cranwell et al., 2007; Bachofen et al., 2008; Krametter-Froetscher et al., 2008; Danuser et al., 2009; Braun et al., 2015; Kaiser, V., Doctoral Thesis, Berne, 2016). Recently, Braun et al. (2015) described the transmission of BDV from a PI calf to seronegative heifers, resulting in persistently infected fetuses. Bachofen et al. (2008) reported transmission of BVDV from a PI goat to seronegative pregnant goats, which also resulted in a second generation of PI offspring. In the present study it could be confirmed that BVDV successfully crossed the species barrier and caused transient infections in buffaloes on all the three study farms as well as in one goat on farm 3. In contrast, past BDV infection was only detected in a sheep, the main host, while interspecies transmission to water buffaloes apparently did not occur. Indeed, to the author's knowledge BDV infection in water buffaloes has so far not been reported. In the light of the ongoing BVDV eradication program in Switzerland, two critical points arising from these results must be emphasized. Firstly, small ruminants but also water buffaloes must be

regarded as potential BVDV virus reservoirs which may hamper BVDV eradication in Swiss cattle (Danuser et al., 2009; Bachofen et al., 2013). Secondly, after eradication of BVDV in cattle, BDV, which is currently mainly circulating in sheep, may spill over to susceptible cattle herds. This may cause considerable economic losses due to expensive follow-up examinations in order to clearly distinguish between BVDV and BDV infections. As already implied earlier, BDV should be taken into account in BVDV eradication (Krametter-Froetscher et al., 2008; Bachofen et al., 2013; Braun et al., 2015; Kaiser, V., Doctoral Thesis, Berne, 2016).
Although seropositive results for both BTV and SBV were found in the present study, interspecies transmission was not expected for these viruses, as (with exception of BTV-26) direct horizontal transmission could not be demonstrated to date (Batten et al., 2014). However, it is known from previous reports that native ruminants as well as water buffaloes are susceptible to infection with these viruses and, although not in a direct manner, that these species contribute to the spreading of viral infections among susceptible species.
The example of BTV-26 demonstrates that, in terms of transmission and clinical outcome, emerging serotypes may behave differently from known serotypes. Also, epidemiologic data of the recent BTV and SBV outbreaks in Europe raise the question whether there is another mode of transmission than only by vectors and via the placental route, for example horizontally with a reservoir in wild species (Doceul et al., 2013), or venereally via infected semen. In fact, BTV and SBV genome were detected in semen from viremic bulls. However, venereal transmission could not be proven until today (Osburn, 1994; Vanbinst et al., 2010; Hoffmann et al., 2013). In any case, if future eradication of (re-)emerging BTV or SBV is intended, water buffaloes should be included.

Two of the most surprising findings were firstly the detection of a novel GyCV by NGS, and secondly the confirmation of its high prevalence in water buffaloes and sheep on farm 2. Many aspects of the virus are yet to be investigated, including the host range and its clinical impact. Assuming a real infection event in water buffaloes and sheep, the apparently systemic spreading in both species on farm 2 would give a first hint for interspecies transmission of the virus.

In conclusion, previous knowledge on the buffalo virome and the present results clearly indicate that water buffaloes share a great viral spectrum with our domestic cattle. In Switzerland, where co-housing of different ruminant species is very common, interspecies transmission is very likely to occur. As demonstrated in the present study, interspecies transmission not only occurs between native species like cattle, sheep and goats, but also affects the more exotic water buffaloes. For some viruses, economic losses resulting from clinical disease after interspecies transmission are limited. Other viral infections, however, particularly epizootic viruses and MCFV may cause considerable damage. The significance of the observations of possible interspecies transmission is in the notion that water buffaloes have to be taken into account if any of those viruses should emerge in the Swiss cattle population and if their eradication would be addressed. At present, this may be the case particularly with regard to BVDV and BTV.

A. APPENDIX

Sequence alignments panherpes nested PCR

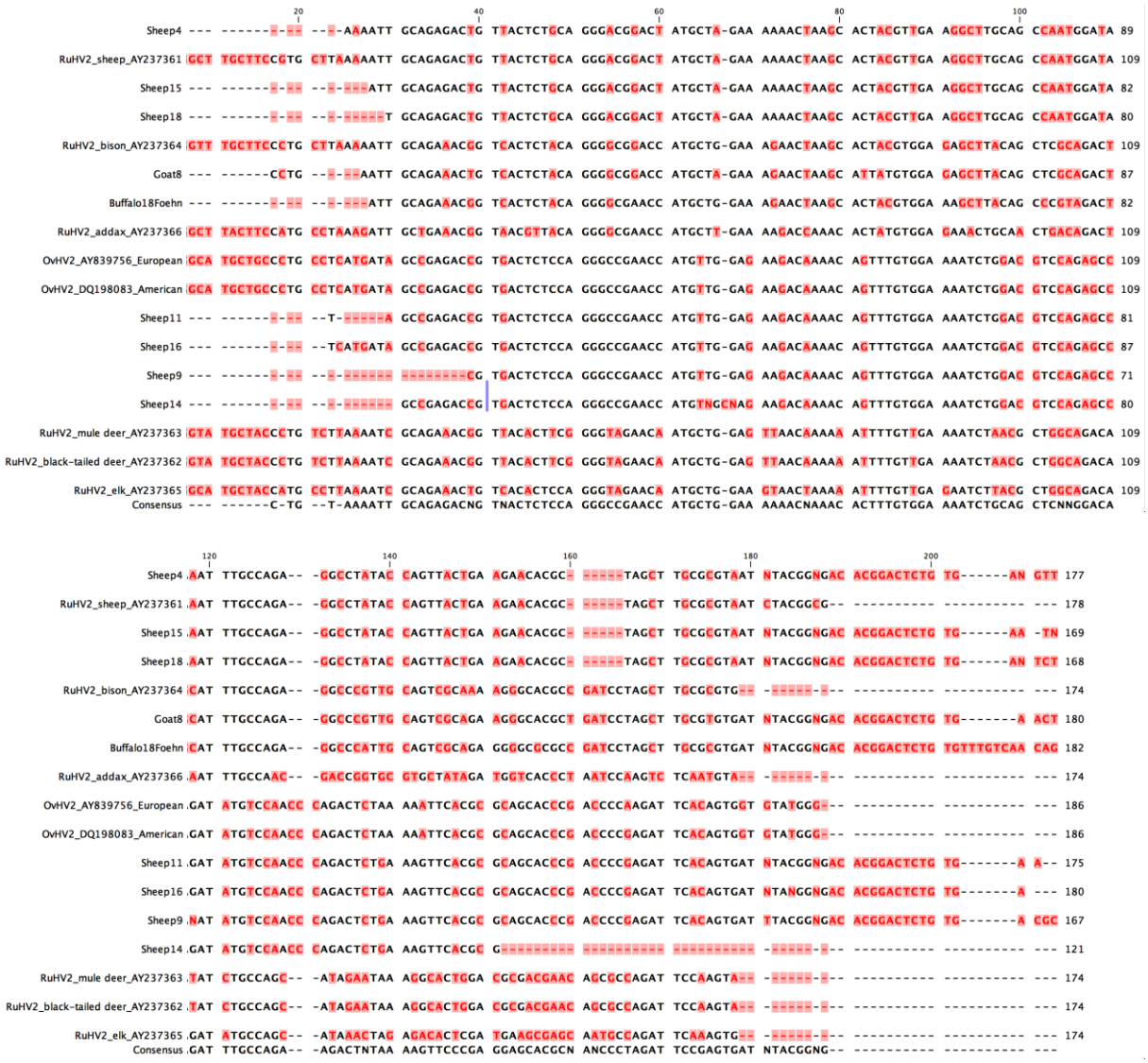

Fig. A1. Sequence alignment of the PCR products, targeting a highly conserved region of the herpesviral DNA polymerase gene. The alignment includes selected ruminant herpesviruses, with representatives of the former types 1 and 2 ruminant rhadinoviruses (GenBank numbers included in their identification) as well as amplicons from the present study. Identical nucleotides are in black, differences are highlighted in red. This alignment was used as the basis for the phylogenetic tree shown in Fig. 2.

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