Final report on the research project 1.13.g - The virome of Swiss water buffaloes

Introduction

The Asian water buffalo (Bubalus bubalis) is gaining increasing importance in Swiss animal farming. Over the last two decades, the animal population has risen from 14 to over 1200. The introduction of this exotic species to Switzerland created new infection risks for water buffaloes as well as the native livestock. It is well known that viruses may have different effects on different species. 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. Particularly with regard to epizootic viral agents this circumstance poses a big hazard. Water buffaloes may represent a subclinically infected reservoir for epizootic viruses that may cause disease outbreaks upon transmission to native farm animals. Further, new challenges for animal health are arising with worldwide climatic changes, which go along with the spread of arthropods and the viruses they carry.

Since water buffaloes in Switzerland and their pathogens have not been investigated so far, the present study aims at gaining more information about the viral spectrum present in this species. In order to investigate potential inter-species transmissions, co-housed small ruminants were also included in the study.

Material and methods

Three Swiss farms were chosen for the study. Two were located in the Canton of Schwyz, one in the Canton of Solothurn. In May and June 2013, EDTA blood samples were taken from the water buffaloes and if present, from the co-housed small ruminants by the private veterinarian of the farmers (Tab. 1). 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. Cattle were not sampled.

Tab. 1: Overview of the total number of and sampled animals on the three study farms

	Farm 1 (SZ)		**Farm 2 (SZ)**		**Farm 3 (SO)**	
	Total	Sampled	Total	Sampled	Total	Sampled
Water buffaloes	21	17	33	26	5	5
Sheep	-	-	20	19	-	-
Goats	-	-	-	-	7	7
Cattle	30	-	-	-	65	-

The virome was investigated by two approaches: firstly, by Next Generation Sequencing (NGS), that allows unspecific detection of viruses and therefore also enables detection of unexpected and novel viruses, and secondly, by conventional specific diagnostic methods, including PCR, ELISA and SNT, that are not as broad as NGS but more sensitive (Tab. 2). Sequencing results of NGS suggestive of infection with a novel virus were confirmed by generation of conventional primers and overlapping sequencing.

Tab. 2: Overview of viruses targeted by conventional diagnostic methods

Classification	Virus	PCR	Target	ELISA	Target	SNT
	Herpesviridae	Panherpes nested PCR	DNA polymerase gene			
Alphaherpes-virinae	Bovine Herpesvirus 1	qPCR	gB gene	Competitive ELISA	gB	
	Bovine Herpesvirus 5	qPCR	gB gene			
	Bubaline Herpesvirus 1			Indirect ELISA	gE	
	Caprine Herpesvirus 1			Indirect ELISA	gE	
	Bovine Herpesvirus 2			Indirect ELISA	Whole virus	BoHV-2 SNT
Gammaherpes-virinae	Ovine Herpesvirus 2	qPCR	ORF 63 tegument protein			
	Caprine Herpesvirus 2	qPCR	DNA polymerase gene			
Flaviviridae	Pestiviruses	Panpesti RT-PCR	5'UTR	Indirect ELISA	NS3	BVDV, BDV SNT
Emerging Viruses Reo/Orthobunya-viridae	Bluetongue Virus			Competitive ELISA	VP7	
	Schmallenberg Virus			Indirect ELISA	Whole virus	
Retroviridae	Bovine Leukaemia Virus			Indirect ELISA	Whole virus	
Viruses found by NGS: Mycodnaviridae	Gemini-like myco-infecting circular virus	qPCR	Small intergenic region			

Results and Discussion

Tab. 3: Results of specific tests for detection of viral nucleic acids and *antibodies (in italic)* in water buffaloes and small ruminants

Virus targeted	Farm 1	Farm 2		Farm 3	
	Water buffaloes	Water buffaloes	Sheep	Water buffaloes	Goats
Herpesviridae	-	1 BLHV	4 OvHV-2 3 RuRhV type 2	-	1 CpLHV
OvHV-2	-	-	15	-	-
CpHV-2	-	-	-	-	-
BoHV-1 PCR	-	-	n.d.	-	n.d.
BoHV-1 ELISA	-	-	-	-	-
BoHV-5	-	-	n.d.	-	n.d.
BuHV-1	-	-	*n.d.*	-	*n.d.*
CpHV-1	-	-	-	-	-
BoHV-2 ELISA *BoHV-2 SNT*	*1 positive* *1 (Titer 80)*	*1 questionable* *1 (Titer 62)*	- -	- -	- -
Pestivirus PCR	-	-	-	-	-
Pestivirus ELISA *Pestivirus SNT*	*1* *1 BVDV*	*3* *2 BVDV, 1 ambig.*	*1* *1 BDV*	*1* *1 BVDV*	*1* *1 BVDV*
BTV ELISA	-	-	*1*	*3*	-
SBV ELISA	-	-	*8*	-	*2*
BLV ELISA	-	-	-	-	-
GyCV NGS	-	14	n.d.	-	n.d.
GyCV PCR	-	22	11	-	-

By Panherpes PCR we found nine positive animals, one water buffalo positive for Bovine Lymphotropic Herpesvirus (BLHV), three sheep positive for Ruminant Rhadinovirus type 2 (RuRhV 2) of domestic sheep and one goat positive for Caprine Lymphotropic Herpesvirus (CpLHV). These three

viruses are apathogenic and were only found in the species which they are adapted to. Regarding BLHV, it is the first report of this cattle virus in peripheral blood leukocytes of a Swiss water buffalo. It is not known, if this infection originated from interspecies transmission from cattle or if BLHV may circulate within the water buffalo population. The fact that only a single animal was positive may indicate interspecies transmission. However, the water buffaloes on farm 2 had no contact to cattle, only to sheep.

Four sheep were found positive for OvHV-2 by panherpes PCR and this was confirmed by OvHV-2 real-time (q)PCR. By qPCR, an additional 11 sheep were detected OvHV-2 positive. Besides the 15 sheep, none of the other animals on the three farms were found positive for OvHV-2, indicating that interspecies transmission from sheep to water buffaloes did not occur. This is underpinned by the fact that malignant catarrhal fever (MCF), a lethal clinical disease that is due to OvHV-2 infection in non-ovine hosts, had not been reported on the farms. This may be explained by the clear spatial separation of the water buffaloes from the sheep on farm 2, which is known to be an effective method for the prevention transmission of OvHV-2 and clinical MCF. We found no evidence for circulation of CpHV-2 in the three species tested, which has been reported to cause MCF in Swiss water buffaloes in a previous study by Stahel et al. (2013).

BoHV-1 and BoHV-5 were not detected in any of the animals tested, neither by PCR nor by ELISA. This was not unexpected, as Switzerland is free of BoHV-1 and BoHV-5 has so far not been reported.

Also, there was no evidence for BuHV-1 and CpHV-1 infection in the animals, two viruses which are closely related to BoHV-1 and BoHV-5. BuHV-1 has never been reported in Switzerland but seems widely distributed in water buffalo populations in other countries. Due to the serological cross-reactivity between these closely related alpha-herpesviruses, the strict control measures for BoHV-1 by serological testing may simultaneously have prevented the introduction of BoHV-5 and BuHV-1, as animals with seropositive results were not imported into Switzerland. CpHV-1 is known to circulate in Swiss goats, but the prevalence is not known as disease occurs only sporadically and thus is of minor importance.

BoHV-2 ELISA showed one water buffalo on farm 1 with positive, and one buffalo on farm 2 with questionable result. In the SNT both animals were proven to have neutralizing antibodies, at titres of 80 and 62 respectively. To the author's knowledge, this is the first report of BoHV-2, the cause of herpes mamillitis, in water buffaloes. However, no clinical signs of the disease (skin lesions and erosions on udder and tits) have been reported on the two farms. The origin of the virus is unknown. On farm 1 water buffaloes were housed with cows, making a transmission from cows to water buffaloes possible. As the cows have not been sampled for this study, no final conclusion can be drawn in this regard. Interestingly, there were no cows present on farm 2, suggesting another source of the virus, or an independent circulation with reactivation from the latent state among water buffaloes.

All animals tested were negative for pestiviruses (BVDV and BDV) by PCR, providing evidence for the absence of viremic or persistently infected animals. This was not unexpected as BVDV is being eradicated and the prevalence of BDV persistently infected sheep and goats is estimated to be very small. However, several animals were antibody positive in the ELISA. As samples were collected in 2013, shortly after the testing for BVDV antigen in the national control program had ended and the serological testing was initiated, seropositive animals were not unexpected. As the ELISA is not able to distinguish antibodies against BVDV and BDV, a differentiating SNT was performed using the BVDV strains 1a and 1h, and the BDV strain Swiss a. One ELISA positive water buffalo on farm 1 turned out seropositive for BVDV by SNT. On farm 2, three water buffaloes and one sheep were found seropositive for pestiviruses by ELISA. In the SNT the antibodies could be attributed to BVDV in two

of the water buffaloes and to BDV in the sheep. The third ELISA positive water buffalo gave an ambiguous result. Antibody titres argued for an infection with BVDV rather than with BDV. Thus, for farm 2, it seems that pestiviruses stayed with their original hosts (e.g. BVDV in bovines and BDV in sheep). As with OvHV-2, separation of the water buffaloes and the sheep may have prevented interspecies transmission. On farm 3, one water buffalo and one goat, which were seropositive in the ELISA were both shown seropositive for BVDV by SNT. Thus, on this farm interspecies transmission of BVDV may have occurred. However, it is also possible that the two species were independently infected with two different strains.

Three water buffaloes from farm 3 and one sheep from farm 2 were tested seropositive for Bluetongue Virus (BTV). No final statement can be made concerning the origin of the antibodies. They may be due to a real infection or vaccination. The specificity of the antibodies is unknown. The ELISA is able to detect antibodies of serotypes 1 to 24. As only serotype 8 has to date caused disease in Switzerland and also the BTV vaccine used during the outbreak from 2008 - 2010 was directed against this serotype, it can be hypothesized that the antibodies found in these animals were specific for serotype 8.

In 2012, Schmallenberg Virus (SBV) emerged and spread rapidly across the Swiss ruminant population, leaving behind a high percentage of seroconverted animals. As BTV, it is mainly transmitted by vectors of the genus Culicoides ssp. Eight sheep on farm 2 and two goats on farm 3 were seropositive for SBV. However, all the water buffaloes were negative. As the vector and the transplacental route are the only ways of transmission so far known, one does not expect direct transmission of virus between small ruminants and water buffaloes, neither for SBV nor for BTV.

The Swiss cattle population is officially free of Bovine Leukaemia Virus (BLV) and as expected, all the animals were seronegative for BLV.

By Next Generation Sequencing (NGS), in 14 water buffaloes from farm 2, DNA sequences were found, that could be aligned to the only recently described genus of Gemycircularvirus (GyCV). The genus belongs to the family of Mycodnaviridae. Gemycircularviruses are small, non-enveloped viruses with a genome of circular single-stranded DNA of about 2.17kb. It was hypothesized that GyCV may infect fungi, but in the past few years, GyCV sequences have also been found in plants, faecal samples of various animal species, serum of cattle and humans as well as in brain samples of humans. So far, not much is known about these novel viruses, neither about the natural host nor about their clinical impact. In order to specifically detect GyCV DNA, PCR primers were designed and all samples were tested for GyCV DNA by real-time PCR. Interestingly, 22 of 48 water buffaloes gave a detectable signal. In these 22 samples, the 14 samples found positive by NGS were included. Among the sheep, 11 animals reacted positively. Strikingly, Gemycircularvirus was only present in samples of farm 2, namely in both water buffaloes and sheep, while it was not detected on farms 1 and 3. The fact that more GyCV-positive individuals were detected by PCR than by NGS argues for a higher sensitivity of PCR compared to NGS. While GyCV sequences have been detected in serum of a healthy cow in Germany before, it is the first report of GyCV DNA in the blood of water buffaloes and sheep – and the first report of GyCV in Switzerland. Further analyses are required to shed light on the biology and meaning of GyCV in farm animals.

Conclusions

The virome of water buffaloes:

Among the viruses, which were known to infect water buffaloes, we gained evidence for infection with BLHV (virus), BVDV (antibodies) and BTV (antibodies). BLHV in water buffaloes had previously

only been reported once, in a buffalo herd in Argentina. The present case is the first report of BLHV in Swiss water buffaloes.
In two water buffaloes we had evidence for infection with BoHV-2, which to our knowledge has not been reported before.
At first, sequences of a novel Gemycircularvirus were detected by NGS in water buffaloes on farm 2. A specific PCR survey based on this observation confirmed that GyCV sequences were detectable in an additional 8 water buffaloes as well as in 11 sheep on the same farm. Notably, this virus would have escaped our attention if we had not included NGS into our repertoire.
Importantly, we did not detect any evidence for the following infections, which are notifiable but considered eradicated from the Swiss cattle population: Bovine herpesviruses 1 and 5 (BoHV-1; BoHV-5) as well as Bovine leukaemia virus (BLV).

Inter-species transmission between water buffaloes and small ruminants:
Evidence for three viruses (BVDV, BTV, GyCV) was detected in both, the exotic water buffaloes and the domestic small ruminants, suggesting that inter-species transmission of these viruses may occur. The significance of these observations is in the notion that Water buffaloes have to be taken into account if any of those viruses should emerge in our cattle population and if their eradication would be addressed. At present, this may be the case particularly with regard to BVDV and BTV. The significance of the GyCV infection will need further evaluation.
Although we did not detect cases, it is known from the literature that water buffaloes may succumb to malignant catarrhal fever (MCF) due to either OvHV-2 or CpHV-2. The detection of these viruses among the co-housed small ruminants is, therefore, also of significance.

Technical advances:
Since sample preparation is pivotal for the detection of viruses in cell-rich material, we tested different methods for the relative enrichment of virus particles in blood samples and found a combination of filtration and nuclease treatment to be most efficient. In combination with our adapted protocol for sequence independent single primer amplification (SISPA), recovery of a wide range of spiked-in RNA and DNA viruses was possible. Furthermore, we were able to gain first experience on the bioinformatical analysis of NGS data. Importantly, the results of our own analyses were confirmed by a professional bioinformatician. Further investigations are planned to refine/adapt the method for different sample materials and animal species in order to make virome analysis by NGS available for a wide range of diagnostic questions in veterinary virology.