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Complexities of Molecular Identification of γ-herpesviruses: Lessons from MCFV

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Abstract

The Herpesviridae family is subdivided into three subfamilies, namely α-herpersvirinae, β-herpesvirinae and γ-herpesvirinae. All members of the family are characterized by a common structure consisting of a large linear double-stranded DNA genetic core packaged into a proteic icosahedral capsid and further enclosed in a phospholipidic bilayer envelope of cellular origin. Herpesviruses are characterized, on one side, by a high stability of the genome during virus replication, however, on the other side by a high capability to change rapidly in response to natural evolutionary selecting pressure. Therefore, there is a continuous emergence and establishment of new viruses. In this contest γ-herpesviruses, whose contribution to disease outbreaks in wildlife population has often been underestimated, pose a serious problem due to their ability to cross species barriers, infect new hosts and give rise to newly emerged viruses or virus variants in reservoirs. The problem is exacerbated by the absence of vaccines and effective treatments, such as for Malignant Catarrhal Fever (MCF) in cattle or MCF-like diseases, caused by the Malignant Catarrhal Fever Viruses (MCFVs). MCFV can infect both livestock and wild animals sporadically, however when it does, it can cause clinical disease with important welfare implications, dramatic pathological changes and often has death as outcome. Due to the inability to isolate the majority of the γ-herpesviruses *in vitro*, their detection and characterization necessarily involve molecular methodologies aimed at diagnosing, identifying and resolving their phylogenetic origins and the evolutionary relationship with the host species. This information is ultimately necessary to improve the control of the disease spread, and to better identify the source of outbreaks, which can be seriously detrimental to zoological collections, especially for endangered species. This review provides an overview of the currently available methodologies applied for identification and characterization of MCFVs, critically describes benefits and disadvantages of these, recognises the gaps to be addressed and identifies future diagnostic opportunities.

KEYWORDS

Herpesviridae, γ-herpesvirus, malignant catarrhal fever virus (MCFV), Consensus Panherpes PCR, long-distance PCR, sequencing

INTRODUCTION

The Herpesviridae family encompasses viruses infecting several host species, including reptiles, birds and mammals. The viral particle is made of genetic material (large linear double-stranded deoxyribonucleic acid, DNA) packaged into a proteic icosahedral capsid, coated with a matrix of tegument proteins and further enclosed in a phospholipidic bilayer envelope. Herpesviruses cause long-lived latent and subclinical infections in the adapted hosts, serious diseases in foetuses and neonates of certain susceptible species and in immunocompromised or non-adapted hosts once the virus crosses the species barrier (Davison et al., 2009; Azab et al., 2018).

The Herpesviridae family is subdivided into three subfamilies, α-herpesvirinae, β-herpesvirinae and γ-herpesvirinae, subfamilies which evolved from a single ancestor approximately 200 million years ago and then further co-speciated with their hosts (McGeoch et al., 1995). Every single herpesvirus evolved within a specific ecological niche in the respective permissive host. Generally, α-herpesviruses are characterized by a wide and variable host range, whereas β- and γ-herpesviruses show a narrow host range, usually confined to a unique species or genus (Cohrs and Gilden, 2001). Clinically, α-herpesviruses are neurotropic, while β- and γ-herpesviruses (γHV) are immunotropic, with the additional capacity of infecting endothelial and epithelial cells.

Herpesviruses are characterized, on one side, by a high stability of the genome during virus replication, however, on the other side by a high capability to change rapidly in response to natural evolutionary selecting pressure. Therefore, there is a continuous emergence and establishment of new viruses. In this contest γ-herpesviruses, which have been often neglected, especially in regard of disease of wildlife population, pose a serious problem since they have been shown to be able to cross species barriers, infect new hosts and give rise to newly emerged viruses or virus variants in reservoirs. The problem becomes even more serious in the absence of vaccines and effective treatments, such as for Malignant Catarrhal Fever (MCF) in cattle or MCF-like disease in wild Artiodactyla, due to infection with γ-herpesviruses of the

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Malignant Catarrhal Fever Virus (MCFV) group.

Malignant Catarrhal Fever viruses and diseases

Malignant Catarrhal Fever Viruses are members of the genus Macavirus, the second most represented genus (after Rhadinovirus) for number of species of the γ-herpesvirus family. Taxonomically, γ-herpesviruses are divided into seven genera, Macavirus (e.g., Ovine γ-herpesvirus-2), Percavirus (e.g., Mustelid γ-herpesvirus-1), Lymphocryptovirus (e.g., Human γ-herpesvirus-4), Rhadinovirus (e.g., Human γ-herpesvirus-8), Bossavirus (e.g., Delphinind γ-herpesvirus-1), Manticavirus (e.g., Vombatid γ-herpesvirus-1) and Patagivirus (e.g., Vespertilionid γ-herpesvirus-3) (McGeoch et al., 2006; ICTV, 2021).

The genus Macavirus comprises two lineages of lymphotropic viruses, among which are viruses causing Malignant Catarrhal Fever (Cunha et al., 2019). Moreover, a number of other related herpesviruses, designated according to the reservoir host from which they were initially isolated, can cause MCF, such as Ibex MCFV and MCFV-white tailed deer. All MCFVs are characterised by a highly conserved sequence of their DNA polymerase and carry a characteristic 15-aminoacid antigenic peptide in the glycoprotein B (Li et al.,2001; Cunha et al., 2019). Interestingly, co-infections with two or more MCFVs are feasible (Li et al.,2001; Li et al.,2013). MCF is a systemic condition, characterised by infiltration and proliferation of infected lymphocytes (predominantly CD8+ve) in many organs, accompanied by tissue necrosis and usually leading to death of the permissive host (Gong et al., 2023). The condition is characterized by pyrexia, initially clear then mucopurulent copious nasal secretions with encrustation of nostrils, ulceration of the upper respiratory and alimentary tracts, discharge from the eyes as well as corneal opacity leading to blindness, generalized lymphadenopathy, and neurological manifestations. The outcome is the death of the diseased animal few days after the onset of the clinical symptoms, however some animals can survive for weeks showing severe welfare deterioration. Different and species-specific clinical signs have been reported according to the susceptibility of the host to the disease and the virus involved (Russell et al., 2009; Li et al., 2014; Sood et al., 2017).

According to published works, just six MCFVs are known to cause disease: Alcelaphine γ-herpesvirus 1 (AlHV-1), Ovine γ-herpesvirus 2 (OvHV-2), Caprine γ-herpesvirus 2 (CpHV-2), Ibex-MCFV, MCFV-white tailed deer (WTD)/Caprine γ-herpesvirus 3 (CpHV-3), and Alcelaphine γ-herpesvirus 2 (AlHV-2). Natural hosts for these viruses are ungulates species belonging to the order Artiodactyla, however non-adapted hosts that share enclosures or are co-grazed with the natural hosts may acquire the virus (Fig. 1) from the reservoir host and become infected with MCFV directly by contact with the reservoir host or through the contaminated environment (Li et al., 2014). Due to their prevalence, the two most investigated MCFVs are AlHV-1 and OvHV-2, and this is reflected in the fact that these are the only MCFVs whose genome has been completely sequenced (Sharma et al., 2019) and for which the epidemiology is well known (Li et al., 2014). AlHV-1 infects black and blue wildebeest causing wildebeest-associated MCF (WA-MCF) particularly in cattle in Africa during the wildebeest calving season, when contaminated wildebeest secretions can come into contact with non-adapted species. Occasional infections have also been detected in game species or zoological collections worldwide (Flach et al., 2002; Li et al., 2014; Wambua et al., 2016). Conversely, OvHV-2, which is globally distributed, infects domestic and wild sheep where may be asymptomatic or cause sheep-associated MCF (SA-MCF) in cattle, deer, bison and rarely in pig and giraffe (Li et al., 2014; Cunha et al., 2019). Sheep and wildebeest can be tested for OvHV-2 and AlHV-1 pre-movement between zoos or sanctuaries, to exclude carriers, however possible infection with different MCFVs is not often taken into consideration, and this can eventually result in disease outbreaks.

Improvements required to MCFVs molecular diagnostic and phylogenesis

MCFV is distributed worldwide and infects domestic, captive

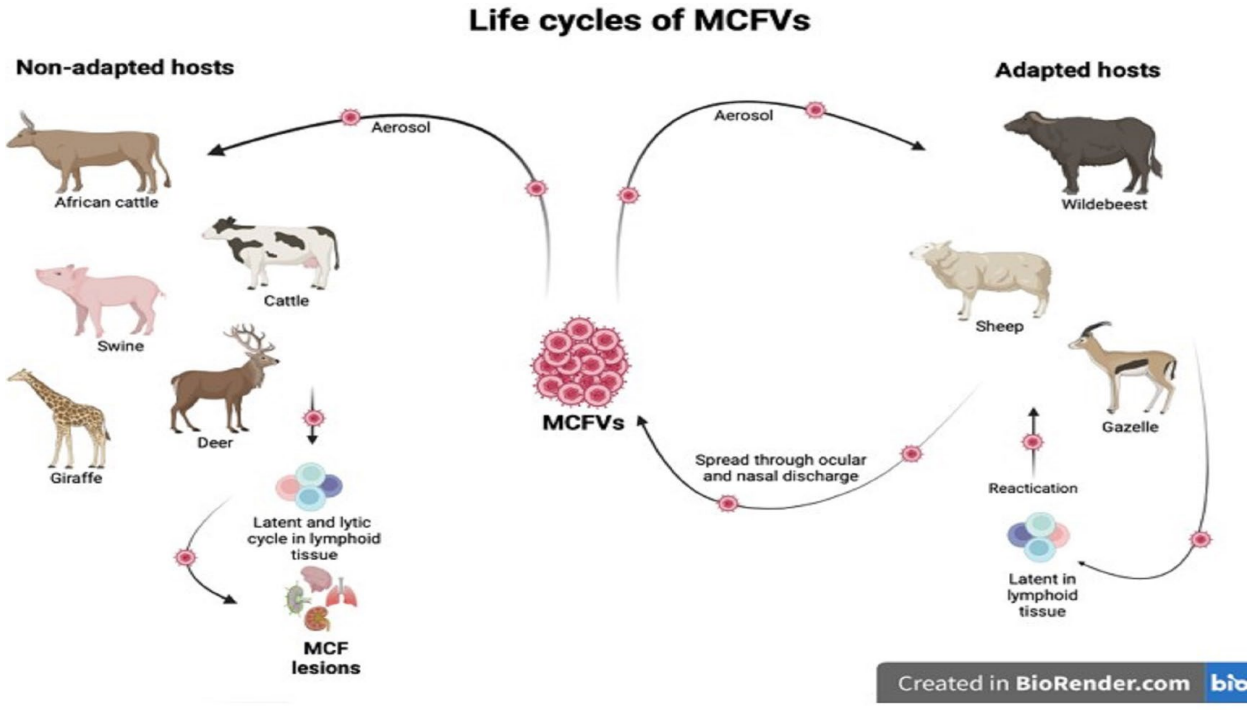

Fig. 1. Schematic representation of the life cycle of MCFV. The MCFVs infect adapted hosts through aerosol (nasal route). In these hosts the viruses establish latent infections in lymphoid tissue. During reactivation of lytic infection, the adapted hosts spread the viruses through nasal, ocular and other secretions in the environment. The MCFVs might infect the non-adapted hosts kept in the same environment (e.g. co-grazing) or through contact with fomites. In the adapted hosts, the viruses do not cause disease, whereas in the non-adapted hosts the viruses cause MCF lesions in tissues, and often the animal dies within few days from the onset of clinical signs (Li et al., 2014).

and wild Artiodactyla (Fig. 2). Since no commercial vaccines neither therapeutics are available on market, and current diagnostic methods show limitations, the prevalence of MCF is largely underestimated. Furthermore, in Europe and in the UK, there is very limited information on the molecular diversity of MCFVs in livestock and zoological collections, limiting epidemiological studies. Control of virus spread is primarily relying on disease awareness and biosecurity measures, in order to avoid contact of susceptible with non-susceptible hosts (WOAH, 2021) and therefore prevent the spread of the virus to and within susceptible zoological species and from reservoirs and carrier species to susceptible animals (e.g., from sheep to deer). The economic impact of MCF is not seen as significant in western countries, however wildebeest-associated MCF has a substantial impact in sub-Saharan Africa, where it is responsible for losses of up to 10% of the cattle population per year (Orono et al., 2019). Moreover, MCF acts as a genetic bottleneck factor that can reduce the genetic diversity in the susceptible animal species which, in the case of zoological collections, can affect species already at risk of extinction (Meteyer et al., 1989). Lastly, despite some recent publications focusing on the genetic relationships among Macaviruses (Cunha et al., 2019; Khudhair et al., 2019), the description of novel genetic variants, the discovery of phylogenetic links and the evolutionary relationships among MCFVs and with the host remains largely unknown.

The currently available methods in use for the detection and characterisation of MCFVs rely on serology or on a Panherpes semi-nested PCR; however due to the nature of the epidemiology of MCF and the lack of sufficient reference sequences, there are challenges in interpreting the results. In particular, in reservoir species serological positivity is a common finding, indication of possible recurrent re-stimulation of the immune response by the virus, whereas PCR positivity is detected at lower incidence and sometime discontinuously: the same animal tested twice at different points in time might give opposite PCR results. On the contrary, in susceptible hosts, PCR positivity is frequently detected in infected animals, while serological positivity is less frequent, possibly because in many cases the animal succumbs to the disease before the immune response is detectable.

For all these reasons, improved methodological approaches are required to enhance the detection and the control of the disease in livestock and zoological collections, to advance the genetic characterization of MCFVs strains and to better pinpoint the origin of outbreaks (Li et al., 2011; Li et al., 2014; Wambua et al., 2016). An extensive molecular and genetic characterization of MCFV would be also crucial for the design and preparation of vaccines and for the set-up of improved diagnostic assays and could identify novel viruses evolved in natural hosts that may act as vectors with the potential for transmitting the disease to non-adapted hosts.

The methodologies presently applied rely on tests developed in the mid-nineties, namely the Consensus Panherpes PCR, which employs Consensus/degenerated and deoxyinosine-substituted primers, and the long-distance PCRs, still partially based on the amplification of the same target as the Consensus Panherpes PCR.

This article reviews and discusses these two approaches, identifying the challenges and examining potential solutions with innovative methodologies not used so far for MCFVs like PCR with oligonucleotides modified by insertion of locked nucleic acids and next generation sequencing.

Molecular methodologies

Few methods have been applied to detect, isolate, and identify MCFVs, and to phylogenetically characterize them (Bianchessi et al., 2022). In this section the currently applied methodologies are presented and discussed along with the innovative ones, which could address the actual challenges.

Degenerate, Consensus PCR Assays and Consensus Degenerate Hybrid Oligonucleotide Primers PCR Assays

Consensus PCR is a technique designed to amplify genetic sequences of orthologue genes from evolutionarily related organisms in order to identify new strains or species by sequenc-

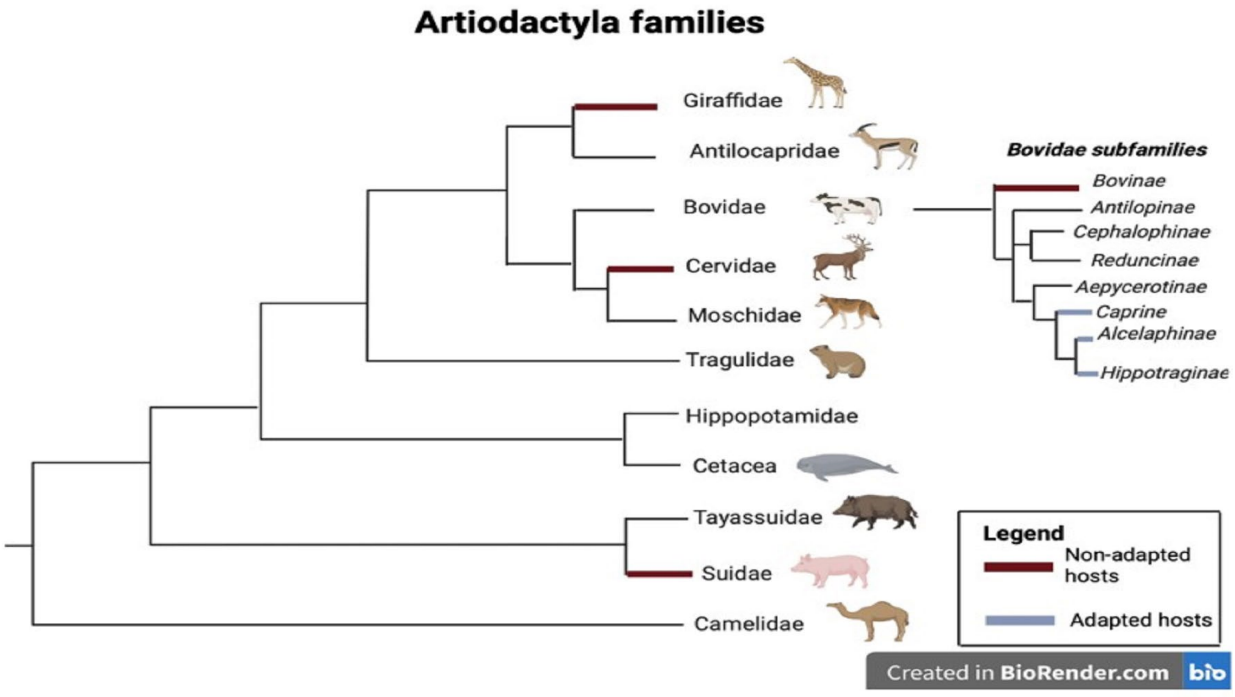

Fig. 2. Phylogenetic trees of the species infected by MCFV (Artiodactyla species). The brown lines represent the families (Giraffidae, Cervidae and Suidae) and subfamily (Bovinae) that include non-adapted hosts. The grey lines represent the subfamilies (Caprine, Alcelaphine and Hippotraginae) within the family Bovidae that include adapted hosts. The tree is not drawn to scale (Li et al., 2014).

ing and phylogenetic comparison. Primers for Consensus PCR are strategically designed in order to target very conserved sequences of orthologous genes, selected by identification and alignment of sequences already known from related organisms. Two different strategies are used for primers design (Staheli et al., 2011; Campos and Quesada, 2017). The first one relies on the use of a mixture of similar primers targeting a very conserved region, each one carrying one of the possible nucleotide variations of the conserved sequence (degenerate primers) (Rose et al., 1998; Campos and Quesada, 2017). Although not ideal for low conserved regions, and presenting possible difficulties in selecting the optimal annealing temperature for the assay (Rose et al., 1998; Staheli et al., 2011), this method represents an improvement compared to classical PCR since it allows the amplification of genes from distantly related or unknown organisms. The second approach consists in the use of a unique primer designed in order to include the nucleotide mostly represented at any codon place of the conserved sequence (Consensus primer). Although missing less similar sequences (Rose et al., 1998; Staheli et al., 2011), this method is advantageous for the isolation of very conserved homologous genes from different organisms. Basically, PCR assays with degenerate or Consensus primers are advantageous for certain purposes, like the identification of new genetic sequences highly related to known ones, but they are not ideal for low related genes.

In order to overcome the challenges of both degenerate and Consensus PCR assays, an additional method, was developed combining both strategies described above, the Consensus Degenerate Hybrid Oligonucleotide Primers PCR (CODEHOPs) (Rose et al., 1998). CODEHOPs strategy is based on the utilization of a mixture of primers each one containing a short degenerate core sequence at the 3′-end and a long Consensus clamp sequence at 5′-end (Rose et al., 1998; Rose, 2003; Boyce et al., 2009). Essentially, the short 3′-end core includes any possible nucleotide variation corresponding to the conserved sequence, whilst the long 5′-end clamp incorporates the nucleotides mostly represented at any codon of the conserved sequence. Primers can be designed not only starting from multiple alignment of known nucleotide sequences, but also from conserved protein regions disclosed by multiple alignment of known protein sequences somehow related to the target (Staheli et al., 2011). With this combined strategy a lower number of oligonucleotides is present in the reaction mixture and therefore the proportion of specific primers results higher; this increases specificity, especially in the early amplification steps, when the primers with the correct short 3′-end bind without mismatches to the DNA template and then the long Consensus 5′-end stabilizes the hybridization (Rose et al., 1998; Rose, 2005). The assay gains also in sensitivity because the early cycles are characterized by a high yield of specific amplicons and are then followed by cycles where the primer-template hybridization is favored by the long Consensus 5′-end (Rose et al., 1998; Rose, 2003; Rose, 2005). Ultimately, the CODEHOPs system, ensuring higher specificity and sensitivity, allows detection and amplification of genes distantly related and coding for conserved amino-acid sequences, in a pool of DNAs from various pathogens (Rose et al., 1998; Rose, 2003; Staheli et al., 2011).

The PCR strategies described above have also been applied to detect novel herpesviruses, in combination with a protein-based approach (Partin et al., 2021) thanks to a specific feature of MCFVs which are known to carry more synonymous than non-synonymous nucleotide substitutions in specific genomic regions (Davison, 2002). Therefore, it had been possible to find in all mammalian herpesviruses a core of orthologous genes coding for conserved amino-acid regions, namely the genes for the major DNA binding protein, the DNA-packaging, the catalytic subunit of DNA polymerase, the glycoprotein B (gB), the major capsid protein, the DNA helicase and the uracil-DNA glycosylase. Since both the nucleotide and the amino-acid sequences are known for these genes for numerous species of herpesviruses, phylogenetic analysis could be performed to identify target regions (McGeoch et al., 1995; Mc Geoch, 2001).

In particular, amino-acid domains conserved in the DNA polymerase were initially detected by the alignment of 40 distinct DNA polymerase sequences (Ito and Braithwaite, 1991). Three of these conserved amino-acid regions (A, B and C) were utilized to design degenerated and CODEHOP PCR primers suitable for the amplification of a short (215- to 315-bp) nucleotide sequence corresponding to the fragment interposed between B and C regions of the herpesvirus DNA polymerase (VanDevanter et al., 1996; Rose, 2005). With these primers, used in a nested PCR format, a specific amplicon was achieved and directly sequenced by Sanger method (VanDevanter et al., 1996). These universal primers were able to amplify all mammalian, avian or reptilian herpesviruses. Further investigations confirmed acceptable sensitivity levels for this method (VanDevanter et al., 1996; McGeoch, 2001), which is still considered a robust tool for the identification and characterization of both new and already known human and animal herpesviruses. Indeed, the method allowed the detection and characterisation of 7 known (5 humans and 2 animals) and 14 novel (2 humans and 12 animals) herpesvirus species in a single study (VanDevanter et al., 1996). The method was also employed to identify and characterize novel γ-herpesviruses of Artiodactyla species (Li et al., 2000; Li et al., 2003; Li et al., 2005; Partin et al., 2021). Additionally, analysis of the amino-acid sequence interposed between the B and C regions of the herpesvirus DNA polymerase proved that this sequence is individual to each different herpesvirus species (VanDevanter et al., 1996) and the phylogenetic analysis demonstrated that this correlates to the biological classification of the herpesviruses sub-families.

Nonetheless, this method may not be optimal to detect all herpesvirus species, as the amino-acid sequence utilized to generate the nucleotide sequence and then to design the primers is not necessarily conserved across all herpesvirus species and may be coded by highly degenerated triplets, resulting in inability of the primers to hybridize with similar affinity to DNA polymerase genes from herpesviruses of different species. Moreover, high degree of degeneration may cause the need of a high number of primers in the mixture, and in turn reduced amplification efficiency of the assay, with the possibility of bias due to primers affinity competition (Rose, 2005).

Consensus Panherpes PCR with Consensus/Degenerate and Deoxyinosine-Substituted Primers

The PCR method described in the previous paragraph was further improved by introducing deoxyinosine-substituted primers and incorporating deoxyinosine in all primer positions with 3- and 4-fold degeneration (Ehlers et al., 1999). Deoxyinosine (Ia) is a universal nucleoside able to hybridize with any of the four DNA bases and maintaining the double-strand bond. Therefore, deoxyinosine residues can be incorporated in highly degenerated primers in order to diminish the number of primers required in the primer mixture and to increase the yield of specific PCR amplicons. (Rossolini et al., 1994). Unfortunately, since the efficiency of deoxyinosine-substituted primers is affected by the ratio Ia residues/primer length, and by the sequence of the template, the application of this strategy may not be ideal for all virus species, even with the appropriate optimisation of annealing tempera-

tures and cycling parameters (Rossolini et al., 1994).

However, utilizing this technique and a pool of degenerate/Consensus and deoxyinosine-substituted oligonucleotides in a nested format, 16 known and three novel animal herpesvirus species were detected and characterized in a single work. This PCR method employing deoxyinosine-substituted and degenerate/Consensus primers, named Consensus Panherpes PCR, is acknowledged as a robust tool for universal identification of mammalian, avian and reptilian herpesviruses. Indeed, two new porcine γ-herpesviruses (porcine lymphotropic γ-herpesvirus type 1 and type 2), were detected with this method in samples previously considered negative (Ehlers et al., 1999). Nonetheless, even this method has drawbacks, such as limited length of the sequence amplified (few hundred nucleotides, too short for a reliable and precise phylogenetic analysis), and the difficulty to amplify all herpesvirus strains when more than one coexist in the same sample and/or in different proportions because of competition issues and the exponential trend of the PCR reaction (Prepens et al., 2007). This was proved when Porcine lymphotropic γ-herpesvirus 3 was discovered (Ehlers et al., 1999; Chmielewicz et al., 2003; Prepens et al., 2007).

High resolution multi-locus approach

An interesting approach is the one utilized to study the genetic diversity of Ovine γ-herpesvirus 2 by using a combination of conventional nested PCR assays targeting highly polymorphic loci of three different genes (Russell et al., 2014). Starting from specimens of cattle presenting MCF symptoms, three genes of OvHV-2 were amplified from each sample, Sanger sequenced and phylogenetically and evolutionarily analysed demonstrating twelve distinct viral variants. Therefore, this high-resolution multi-locus approach displays potential application in epidemiological surveys aimed at establishing the initial origins of outbreaks.

Long-Distance PCR Assay and Primer Walking

A PCR method based on a bi-genic approach, named long-distance PCR led to the discovery of Caprine γ-herpesvirus 2, a MCFV harboured in goats and transmitted by them. The long-distance PCR method is based on the possibility to combine two short PCRs, one for the glycoprotein B (gB) gene and the second one targeting the DNA polymerase gene to obtain a longer contiguous nucleotide sequence (3.6 kb) (Chmielewicz et al., 2003). In β- and γ-herpesvirus genomes the glycoprotein B gene is neighbouring and immediately upstream the DNA polymerase gene, making the long PCR possible. However, the former is less conserved than the DNA polymerase gene, therefore, it is necessary to use multiple specific degenerate or Consensus primers to amplify the gB gene, and consequently sometimes multiple attempts are needed to achieve a long sequence (Prepens at al., 2007).

Once the two short PCR amplicons are obtained from the two separate PCR assays, they are linked by a nested mid-distance PCR using forward glycoprotein B specific primers and reverse DNA polymerase specific primers to finally generate a long PCR amplicon (Chmielewicz et al., 2003; Ehlers et al., 2007; Prepens et al., 2007). Such final product undergoes sequencing by primer walking with the corresponding putative amino-acid sequences obtained in silico with bioinformatics tools. The strategy of primer walking is based on iterative successive set of sequencing rounds, thus moving along the sequence, which exploits a first PCR reaction to obtain an initial short sequence, which, after Sanger sequencing, forms the basis for the design of a new set of primers, which target the end of the first sequence. These primers are designed and employed to make the second amplicon, which is in turn sequenced and used to design a further primer for PCR and then sequencing again, thus "walking" along the entire sequence (Sverdlov and Azhikina, 2005). With this approach, starting from the PCR product of gB of Alcelaphine γ-herpesvirus 1, sequences were obtained for this and other related viruses (Chmielewicz et al., 2003). Coupling primer walking with a long PCR, 14 γ-herpesviruses (8 previously unknown) were identified in host samples from six mammalian orders (Ehlers et al., 2008). This study showed the power of long-distance PCR and the primer walking methodology for phylogenetic analysis (both at nucleic- and amino-acid levels) thereby overcoming the issues posed by the Consensus Panherpes PCR, as proved by numerous phylogenetic trees generated with significantly higher probabilities (Ehlers et al., 2008). Basically, mid-to-long-distance PCR could improve the identification and characterization of MCFVs, though its application may be limited by the specificity of the primers employed, and not being necessarily able to amplify all viruses belonging to the Herpesviridae family.

Locked Nucleic Acids Approach

In case of mixed infections, when gB and DNA polymerase genes may not originate from the same virus sequence, the above described method shows limitations (Ehlers et al., 2008). A possible solution is the use of a Locked Nucleic Acid (LNA) approach, which employs primers showing enhanced resistance to enzymatic degradation and thermal stability due to the inclusion of a modified RNA nucleoside analogue. LNA primers, added to the reaction mix, pair at high affinity with complementary nucleic acids, allowing the production of amplicons from all homologous viruses possibly present in the same sample, including the under-represented ones. The usefulness of this method has been proven for diagnostic as well as research approaches (Rodda et al., 2018; Rose et al., 2019; Mana et al., 2022).

LNA approach combined with a bi-genic DNApol/gB long-distance PCR approach has been applied to identify co-infection by multiple γ-herpesviruses in macaques and chimpanzees, where six novel herpesviruses belonging to the genera Rhadinovirus, Lymphocryptovirus and Cytomegalovirus were discovered (Prepens et al., 2007). Essentially, the combination of LNA approach with long-distance PCR and primer walking methodologies ensures the identification of long genetic sequences from multiple viruses represented in the sample and therefore enables further phylogenetic analyses and potential identification of novel viruses.

Next Generation Sequencing

The value of Next Generation Sequencing (NGS) is increasingly recognised in virology for the detection and identification of existing, new and possibly emerging viruses, even when present in the same sample. This methodology is based on the generation of short or long nucleotide sequences utilising a variety of platforms in order to provide a full-length viral genome sequence in constantly diminishing time frames, thus, accelerating virus discovery, and increasing the potential applications. Although NGS has been applied lately for detection of viruses in livestock (Lechmann et al., 2021), it has yet to be used to sequence MCFVs, probably due to limitations inherent to the technique, such as the importance of the target nucleic acid in terms of amount, purity and analytical grade.

Examples of application of NGS to non MCFV γ-herpesviruses successful in generating full-genome sequence are reported in literature, such as for Bovine herpesvirus 4 (amplified in vitro and purified) (Gagnon et al., 2017), Kaposi sarcoma herpesvirus from enriched samples (Cornejo Castro et al., 2020) and Human Epstein–Barr virus from nasopharyngeal carcinoma specimens (Liu et al., 2011). Herpesviruses with significant impact in the veterinary field were lately identified by mining livestock genome (e.g. bovine γ-HV-4 and 6, suid γ-herpesvirus 3 and 4 and gallid α-herpesviruses 1, 2 and 3) (Bovo et al., 2022), but in all the cases the host genome sequence was known and therefore could be subtracted bioinformatically, and also the viral sequences obtained could be verified with the reference sequences already available in Gene Bank.

Since no permissive cell culture protocol or lines are available for MCFVs, is very difficult to obtain an adequate amount of high quality DNA; NGS requires a complex processing of the sample, with substantial loss of material during the initial steps, is still costly and necessitates sophisticated computational resources to assemble, annotate and analyse the massive quantity of outputs generated. Until these issues are addressed it will be difficult to apply NGS to MCFVs. Therefore, it is advisable to improve strategies aimed at increasing the efficiency, reducing the errors, maximizing the reproducibility and ensuring a reviewing output management while approaching these methodologies.

CONCLUSION AND PERSPECTIVES

Until now, the molecular methodology most used for the identification and characterization of the γ-herpesviruses responsible for MCF in livestock, wild and zoological species is the Consensus Panherpesvirus PCR, which employs primers that allow the amplification of the DNA polymerase gene of all viruses belonging to the Herpesviridae family with the advantage of comprehensiveness. The drawback of this method is represented by the limited length of the amplicon generated, that could result in inaccurate identification or ambiguous phylogenetic outputs.

Therefore, lately, as to address this drawback, mid-to-long-distance PCR methods have been developed, connecting the glycoprotein B gene with the DNA polymerase gene and therefore allowing the amplification of a longer sequence and the achievement of more accurate phylogenetic analyses. This approach, involving γ-herpesvirus universal primers, resulted in the identification and characterization of different Macaviruses. The drawbacks are represented by potential false negative results due to lower sensitivity than the Consensus Panherpes PCR, and unsuitability for samples containing more than one herpesvirus species. More recent methods, like the Locked Nucleic Acids or Next Generation Sequencing may be promising for the detection and the characterization of MCFVs once the presently recognized issues will be addressed.

More research is needed for validation of the molecular tools applied to MCFVs as well as other γ-herpesviruses to obtain information on novel genetic variants, phylogenetic links and evolutionary relationships, and finally help to develop diagnostic tests and vaccines to limit the spread and improve the surveillance capability.

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CONFLICT OF INTEREST

The authors declare no actual or potential conflict of interest.

REFERENCES

Azab, W., Dayaram, A., Greenwood, A.D., Osterrieder, N., 2018. How host specific are herpesviruses? Lessons from herpesviruses infecting wild and endangered Mammals. Ann. Rev. Virol. 5, 53–68. https://doi.org/10.1146/annurev-virology-092917-043227.

Bianchessi, L., Rocchi, M.S., Maley, M., Piccinini, R., Turin, L., 2022. Molecular tools to identify and characterize Malignant Catarrhal Fever viruses (MCFV) of ruminants and captive Artiodactyla. Viruses 14, 2697. https://doi.org/10.3390/v14122697.

Bovo, S., Schiavo, G., Bolner, M., Ballan, M., Fontanesi, L., 2022. Mining livestock genome datasets for an unconventional characterization of animal DNA viromes. Genomics 114, 110312. https://doi.org/10.1016/j.ygeno.2022.110312.

Boyce, R., Chilana, P., Rose, T.M., 2009. ICODEHOP: a new interactive program for designing COnsensus-DEgenerate hybrid oligonucleotide primers from multiply aligned protein sequences. Nucleic Acids Res. 37, W222–W228. https://doi.org/10.1093/nar/gkp379.

Campos, M.J., Quesada, A., 2017. Strategies to improve efficiency and specificity of degenerate primers in PCR. In: PCR, Domingues, L. Ed., Methods in Molecular Biology, Springer New York, New York, NY, USA, pp. 75–85. ISBN 978-1-4939-7059-9.

Chmielewicz, B., Goltz, M., Franz, T., Bauer, C., Brema, S., Ellerbrok, H., Beckmann, S., Rziha, H.-J., Lahrmann, K.-H., Romero, C., Ehlers. B., 2003. A novel porcine gammaherpesvirus. Virology 308, 317–329. https://doi.org/10.1016/S0042-6822(03)00006-0.

Cohrs, R.J., Gilden, D.H., 2001. Human herpesvirus latency. Brain Pathol. 11, 465-474. https://doi.org/10.1111/j.1750-3639.2001.tb00415.x.

Cornejo Castro, E.M., Marshall, V., Lack, J., Lurain, K., Immonen, T., Labo, N., Fisher, N.C., Ramaswami, R., Polizzotto, M.N., Keele, B.F., Yarchoan, R., Uldrick, S.T., Whitby, D., 2020. Dual infection and recombination of Kaposi sarcoma herpesvirus revealed by whole-genome sequence analysis of effusion samples. Virus Evol. 6, veaa047. https://doi.org/10.1093/ve/veaa047.

Cunha, C.W., Slater, O.M., Macbeth, B., Duignan, P.J., Warren, A., Highland, M.A., Li, H, 2019. Domestic sheep and bighorn sheep carry distinct gammaherpesviruses belonging to the genus Macavirus. Virus Res. 272, 197729. https://doi.org/10.1016/j.virusres.2019.197729.

Davison, A.J., 2002. Evolution of the herpesviruses. Vet. Microbiol. 86, 69–88. https://doi.org/10.1016/S0378-1135(01)00492-8.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order Herpesvirales. Arch. Virol. 154, 171–177. https://doi.org/10.1007/s00705-008-0278-4.

Ehlers, B., Borchers, K., Grund, C., Frölich, K., Ludwig, H., Buhk, H.-J., 1999. Detection of new DNA polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and deoxyinosine-substituted primers. Virus Genes 18, 211–220. https://doi.org/10.1023/A:1008064118057.

Ehlers, B., Dural, G., Yasmum, N., Lembo, T., de Thoisy, B., Ryser-Degiorgis, M.-P., Ulrich, R.G., McGeoch, D.J., 2008. Novel mammalian herpesviruses and lineages within the Gammaherpesvirinae: cospeciation and interspecies transfer. J. Virol. 82, 3509–3516. https://doi.org/10.1128/JVI.02646-07.

Ehlers, B., Küchler, J., Yasmum, N., Dural, G., Voigt, S., Schmidt-Chanasit, J., Jäkel, T., Matuschka, F.-R., Richter, D., Essbauer, S., Hughes, D.J., Summers, C., Bennett, M., Stewart, J.P., Ulrich, R.G., 2007. Identification of novel rodent herpesviruses, including the first gammaherpesvirus of Mus Musculus. J. Virol 81, 8091–8100. https://doi.org/10.1128/JVI.00255-07.

Ehlers, B., Ulrich, S., Goltz, M., 1999. Detection of two novel porcine herpesviruses with high similarity to gammaherpesviruses. J. Gen. Virol 80, 971–978. https://doi.org/10.1099/0022-1317-80-4-971.

Flach, E.J., Reid, H., Pow, I., Klemt, A., 2002. Gamma herpesvirus carrier status of captive artiodactyls. Res. Vet. Sci. 73, 93–99. https://doi.org/10.1016/S0034-5288(02)00094-2.

Gagnon, C.A., Traesel, C.K., Music, N., Laroche, J., Tison, N., Auger, J.P., Music, S., Provost, C., Bellehumeur, C., Abrahamyan, L., Carman, S., DesCôteaux, L., Charette, S.J., 2017. Whole genome sequencing of a Canadian bovine Gammaherpesvirus 4 strain and the possible link between the viral infection and respiratory and reproductive clinical manifestations in dairy cattle. Front. Vet. Sci. 4, 92. https://doi.org/10.3389/fvets.2017.00092.

Gong, M., Myster, F., van Campe, W., Roels, S., Mostin, L., van den Berg, T., Vanderplasschen, A., Dewals, B.G., 2023. Wildebeest-derived Malignant Catarrhal Fever: a bovine peripheral T cell lymphoma caused by cross-species transmission of Alcelaphine Gammaherpesvirus 1. Viruses 15, 526. https://doi.org/10.3390/v15020526.

ICTV (International Committee on Taxonomy of Viruses). Virus Taxonomy: 2021 Release. EC 53, Online, July 2021. Email Ratification March

2022 (MSL #37). https://talk.ictvonline.org/taxonomy/ [accessed on 1 March 2023].

Ito, J., Braithwaite, D.K., 1991. Compilation and alignment of DNA polymerase sequences. Nucleic Acids Res. 19, 4045–4057. https://doi.org/10.1093/nar/19.15.4045.

Khudhair, Y.I., Ayyez, H.N., Hussain, M.H., 2019. Phylogenetic analysis of ovine herpes virus-2 (OHV-2) in malignant catarrhal fever infected cattle in Al-Qadisiyah governorate of Iraq. Iraqi J. Vet. Sci. 33, 51–58.

Lechmann, J., Ackermann, M., Kaiser, V., Bachofen, C., 2021. Viral infections shared between water buffaloes and small ruminants in Switzerland. J. Vet. Diagn. Invest. 33, 894–905. https://doi.org/10.1177/10406387211027131.

Li, H., Cunha, C.W., Abbitt, B., deMaar, T.W., Lenz, S.D., Hayes, J.R., Taus, N.S., 2013. Goats are a potential reservoir for the herpesvirus (MCFV-WTD), causing malignant catarrhal fever in deer. J. Zoo Wildl. Med. 44, 484–486. https://doi.org/10.1638/2012-0087R.1.

Li, H., Cunha, C.W., Taus, N.S., 2011. Malignant Catarrhal Fever: understanding molecular diagnostics in context of epidemiology. Int. J. Mol. Sci. 12, 6881–6893. https://doi.org/10.3390/ijms12106881.

Li, H., Cunha, C.W., Taus, N.S., Knowles, D.P., 2014. Malignant Catarrhal Fever: inching toward understanding. Annu. Rev. Anim. Biosci. 2, 209–233. https://doi.org/10.1146/annurev-animal-022513-114156.

Li, H., Dyer, N., Keller, J., Crawford, T.B., 2000. Newly recognized herpesvirus causing Malignant Catarrhal Fever in White-Tailed deer (Odocoileus Virginianus). J. Clin. Microbiol. 38, 1313–1318. https://doi.org/10.1128/JCM.38.4.1313-1318.2000.

Li, H., Gailbreath, K., Bender, L.C., West, K., Keller, J., Crawford, T.B., 2003. Evidence of three new members of Malignant Catarrhal Fever Virus group in Muskox (Ovibos Moschatus), Nubian Ibex (Capra Nubiana), and Gemsbok (Oryx Gazella). J. Wildl. Dis. 39, 875–880. https://doi.org/10.7589/0090-3558-39.4.875.

Li, H., Gailbreath, K., Flach, E.J., Taus, N.S., Cooley, J., Keller, J., Russell, G.C., Knowles, D.P., Haig, D.M., Oaks, J.L., Traul, D.L., Crawford, T.B., 2005. A novel subgroup of Rhadinoviruses in ruminants. J. Gen. Virol. 86, 3021–3026. https://doi.org/10.1099/vir.0.81238-0.

Li, H., Keller, J., Knowles, D.P., Crawford, T.B., 2001. Recognition of another member of the Malignant Catarrhal Fever Virus group: an endemic gammaherpesvirus in domestic goats. J. Gen. Virol. 82, 227–232. https://doi.org/10.1099/0022-1317-82-1-227.

Liu, P., Fang, X., Feng, Z., Guo, Y.M., Peng, R.J., Liu, T., Huang, Z., Feng, Y., Sun, X., Xiong. Z., Guo, X., Pang, S., Wang, B., Lv, X., Feng, F., Li, D., Chen, L., Feng, Q., Huang, W., Zeng, M., Bei, J.,Zhang, Y., Zeng, Y., 2011. Direct sequencing and characterization of a clinical isolate of Epstein-Barr virus from nasopharyngeal carcinoma tissue by using next-generation sequencing technology. J Virol. 85, 11291–11299. https://doi.org/10.1128/JVI.00823-11.

Mana, T., Bhattacharya, B., Lahiri, H., Mukhopadhyay, R., 2022. XNAs: a troubleshooter for nucleic acid sensing. ACS Omega 7, 15296–15307. https://doi.org/10.1021/acsomega.2c00581.

McGeoch, D.J., 2001. Molecular evolution of the γ–Herpesvirinae. Philos. Trans. Royal Soc. London Ser. B 356, 421–435. https://doi.org/10.1098/rstb.2000.0775.

McGeoch, D.J., Cook, S., Dolan, A., Jamieson, F.E., Telford, E.A.R., 1995. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J. Mol. Biol. 247, 443–458. https://doi.org/10.1006/jmbi.1995.0152.

McGeoch, D.J., Rixon, F.J., Davison, A.J., 2006. Topics in herpesvirus genomics and evolution. Virus Res. 117, 90–104. https://doi.org/10.1016/j.virusres.2006.01.002.

Meteyer, C.U., Gonzales, B.J., Heuschele, W.P., Howard, E.B, 1989. Epidemiologic and pathologic aspects of an epizootic of malignant catarrhal fever in exotic hoofstock. J. Wildl. Dis. 25, 280–286. https://doi.org/10.7589/0090-3558-25.2.280.

Orono, S.A., Gitao, G.C., Mpatswenumugabo, J.P., Chepkwony, M., Mutisya, C., Okoth, E., Bronsvoort, B.M.C., Russell, G.C., Nene, V., Cook, E.A.J., 2019. Field validation of clinical and laboratory diagnosis of wildebeest associated malignant catarrhal fever in cattle. BMC Vet. Res. 15, 69. https://doi.org/10.1186/s12917-019-1818-8.

Partin, T.G., Schrenzel, M.D., Braun, J., Witte, C.L., Kubiski, S.V., Lee, J., Rideout, B.A., 2021. Herpesvirus surveillance and discovery in zoo-housed ruminants. PLoS ONE 16, e0246162. https://doi.org/10.1371/journal.pone.0246162.

Prepens, S., Kreuzer, K.-A., Leendertz, F., Nitsche, A., Ehlers, B., 2007. Discovery of herpesviruses in multi-infected primates using locked nucleic acids (LNA) and a bigenic PCR approach. Virol. J. 4, 84. https://doi.org/10.1186/1743-422X-4-84.

Rodda, A.E., Parker, B.J., Spencer, A., Corrie, S.R., 2018. Extending circulating tumor DNA analysis to ultralow abundance mutations: techniques and challenges. ACS Sens. 3, 540–560. https://doi.org/10.1021/acssensors.7b00953.

Rose, T., 2003. CODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res. 31, 3763–3766. https://doi.org/10.1093/nar/gkg524.

Rose, T.M., 2005. CODEHOP-mediated PCR - a powerful technique for the identification and characterization of viral genomes. Virol. J. 2, 20. https://doi.org/10.1186/1743-422X-2-20.

Rose, K.M., Alves Ferreira-Bravo, I., Li, M., Craigie, R., Ditzler, M.A., Holliger, P., DeStefano, J.J., 2019. Selection of 2′-deoxy-2′-fluoroarabino nucleic acid (FANA) aptamers that bind HIV-1 integrase with picomolar affinity. ACS Chem. Biol. 14, 2166–2175. https://doi.org/10.1021/acschembio.9b00237.

Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M., Henikoff, S., 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. 26, 1628–1635. https://doi.org/10.1093/nar/26.7.1628.

Rossolini, G.M., Cresti, S., Ingianni, A., Cattani, P., Riccio, M.L., Satta, G., 1994. Use of deoxyinosine-containing primers vs degenerate primers for polymerase chain reaction based on ambiguous sequence information. Mol. Cell. Probes 8, 91–98. https://doi.org/10.1006/mcpr.1994.1013.

Russell, G.C., Scholes, S.F., Twomey, D.F., Courtenay, A.E., Grant, D.M., Lamond, B., Norris, D., Willoughby, K., Haig, D.M., Stewart, J.P., 2014. Analysis of the genetic diversity of ovine herpesvirus 2 in samples from livestock with malignant catarrhal fever. Vet. Microbiol. 172, 63–71. https://doi.org/10.1016/j.vetmic.2014.04.011.

Russell, G.C., Stewart, J.P., Haig, D.M., 2009. Malignant Catarrhal Fever: a review. Vet. J. 179, 324–335. https://doi.org/10.1016/j.tvjl.2007.11.007.

Sharma, B., Parul, S., Basak, G., Mishra, R., 2019. Malignant catarrhal fever (MCF): an emerging threat. J. Entomol. Zool. Stud. 7, 26–32.

Sood, R., Kumar, N., Bhatia, S., 2017. Malignant Catarrhal Fever. In: Emerging and Re-emerging Infectious Diseases of Livestock, Bayry, J. Ed., Springer International Publishing, Berlin/Heidelberg, Germany, pp. 347–362. ISBN 978-3-319-47424-3.

Staheli, J.P., Boyce, R., Kovarik, D., Rose, T.M. 2011. CODEHOP PCR and CODEHOP PCR primer design. In: PCR Protocols, Park, D.J. Ed., Methods in Molecular Biology, Humana Press, Totowa, NJ, USA, pp. 57–73. ISBN 978-1-60761-943-7.

Sverdlov, E., Azhikina, T., 2005. Primer walking. Wiley Online Library. https://doi.org/10.1038/npg.els.0005382 [accessed 1 March 2023].

VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose, T.M., 1996. Detection and analysis of diverse herpesviral species by Consensus primer PCR. J. Clin. Microbiol. 34, 1666–1671. https://doi.org/10.1128/jcm.34.7.1666-1671.1996.

Wambua, L., Wambua, P.N., Ramogo, A.M., Mijele, D., Otiende, M.Y., 2016. Wildebeest- associated Malignant Catarrhal Fever: perspectives for integrated control of a lymphoproliferative disease of cattle in sub-Saharan Africa. Arch. Virol. 161, 1–10. https://doi.org/10.1007/s00705-015-2617-6.

WOAH (World Organization for Animal Health), 2021. Malignant Catarrhal Fever. https://www.woah.org/app/uploads/2021/03/malignant-catharral-fever.pdf [accessed 1 March 2023].