UNIVERSITA’ DEGLI STUDI DI BARI ALDO MORO

Dipartimento di Medicina Veterinaria

SCUOLA DI DOTTORATO DI RICERCA IN SANITÀ ANIMALE E ZOONOSI

XXXVII CICLO

Settore Scientifico Disciplinare VET/05

Investigating Zoonotic Potential of CRESS Viruses, Bovine Meat and Milk Factors (BMMFs), and Protoparvoviruses: Emerging Viral Threats at the Human-Animal-Environment Interface

---

Candidate: Vicenza Teresa
Matricola 765474

Coordinator: Ch.ma Prof. Maria Tempesta

Supervisor: Ch.mo Prof. Vito Martella

Dott.ssa Elisabetta Suffredini

Investigating Zoonotic Potential of CRESS Viruses, Bovine Meat and Milk Factors (BMMFs), and Protoparvoviruses: Emerging Viral Threats at the Human-Animal-Environment Interface

Sommario

1. General introduction

Emerging viral diseases pose significant threats to public health, with a substantial number of recent pandemics attributed to viral zoonotic agents originating primarily from wildlife. Since the 1980s, experts in public health and infectious diseases have raised alarms about the rise of new pathogens and the rapid evolution of existing ones, in terms of re-assortment and recombination. Notably, every viral pandemic since 1900, including those caused by HIV, Influenza, and Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), has been associated with the spillover of viruses from animals to humans (Bernstein et al., 2022; Miranda et al., 2022).

The process of pathogen spillover from animals to humans is complex and not governed by a singular pathway. Several factors influence the likelihood of spillover events, including the prevalence of pathogens among host animal species and the frequency and intensity of human-animal interactions (Plowright et al., 2017). Several studies have demonstrated that specific anthropogenic activities significantly increase the risk of zoonotic spillover. Particularly salient among these are land-use changes—most notably deforestation—the commercialization and expansion of wet markets and wildlife trade, as well as inadequate infection-control practices in livestock management. As highlighted by Gibb et al. (2020) (Gibb et al., 2020)., such factors create ecological and epidemiological conditions conducive to the emergence and cross-species transmission of pathogens. These transmission events can occur through two primary mechanisms: direct contact with reservoir species (Jacob et al., 2020) and indirect pathways, including consumption of contaminated animal products (Tei et al., 2003) or vector-mediated transmission (Gutiérrez-Bugallo et al., 2019). The complexity of these spillover events continues to challenge our scientific understanding, necessitating ongoing research into their underlying patterns (Escudero-Pérez et al., 2023).

It is important to recognize that the transmission of a microbe from an animal to a human does not inherently lead to an epidemic or, on a broader scale, a pandemic. Certain viruses that spill over may result in no illness despite infection and may not propagate easily among humans, as exemplified by the simian foamy virus (Jones-Engel et al., 2008). Conversely, some viruses can cause significant human disease but do not transmit efficiently outside specific circumstances, such as the rabies virus. Other pathogens, however, can lead to severe illness and can be transmitted between humans through various routes, which include direct contact with infectious materials (e.g., Marburg virus), exposure to respiratory secretions (e.g., Middle East Respiratory Syndrome (MERS) coronavirus), or bites from non-vertebrate vectors like mosquitoes (e.g., Dengue viruses) (Rahman et al., 2020). Even when a pathogen possesses the capacity for human-to-human transmission, additional factors such as population density and travel patterns play critical roles in determining whether an initial outbreak escalates into an epidemic or pandemic.

The Ebola epidemic in West Africa from 2014 to 2016 exemplifies the convergence of these factors leading to a public health crisis. Prior to 2014, Ebola outbreaks typically occurred in relatively rural regions of Africa often initiated by human contact with infected animals and subsequently exacerbated during funerary practices and within healthcare settings where infections spread among healthcare workers and patients. In 2014, these amplifying factors, combined with increased travel, facilitated widespread community transmission in Guinea, Liberia, and Sierra Leone. (Kamorudeen et al., 2020; Kaner & Schaack, 2016).

The recent pandemic caused by SARS-CoV-2 has highlighted the vulnerabilities in our surveillance system for preventing and tracking the emergence and spread of new and re-emerging viruses. One of the lessons gained from this experience is that employing high-throughput technological tools for the early detection and swift identification and characterization of the causative agent is crucial for mitigating the most severe impacts of any pandemic (Quer et al., 2022). Next-generation sequencing (NGS) technologies have significantly changed the landscape of viral discovery (Lauber & Seitz, 2022), allowing for an incomparable depth of analysis in biological samples (Barzon et al., 2011). The integration of innovative primer initiated protocols, as Sequence-Independent, Single-Primer Amplification (SISPA) and Rolling Circle Amplification (RCA), with NGS has allowed the detection of a wide range of viral agents responsible for human and veterinary diseases (Barzon et al., 2011; Belák et al., 2013; Blomström, 2011; Capobianchi et al., 2013; Stang et al., 2005).

Within this context, CRESS DNA viruses, belonging to the phylum *Cressdnaviricota*, have emerged as particularly significant. These viruses, characterized by their circular genomes and conserved replication-associated protein (Rep) (Krupovic et al., 2020), demonstrate remarkable ubiquity across ecological niches, infecting organisms from diatoms to vertebrates (Krupovic & Varsani, 2022).

In parallel, the discovery of Bovine Meat and Milk Factors (BMMFs), which represent viral-like particles characterized by circular DNA genomes, has further expanded the spectrum of circular DNA elements of potential biomedical relevance.

Similarly, the evolution of molecular methods allowed the identification of new viruses, including three more human parvoviruses (Bufavirus, Tusavirus, and Cutavirus), belonging to the *Protoparvovirus* genus. Parvoviruses are small single-stranded DNA viruses that infect a wide range of animals, including both vertebrates (subfamily *Parvovirinae*) and invertebrates (subfamily *Densovirinae*). Their stable, non-enveloped capsid structure makes them resistant to inactivation. Historically, human parvoviruses have been difficult to cultivate in standard cell cultures, which limited the discovery of new strains (Väisänen et al., 2017).

The ability to quickly identify new viruses also has important implications for public health, virology, and veterinary medicine, as it allows for better monitoring and control of emerging viral diseases.

This thesis investigates emerging zoonotic viruses with the aim of identifying novel viral threats to human health. The first section focuses on CRESS DNA viruses found in domestic cats, with particular attention given to circoviruses due to their potential relevance. The second section examines the characteristics and implications of Bovine Milk and Meat Factors (BMMFs). Finally, the third section explores the zoonotic potential of Protoparvoviruses.

References

Barzon, L., Lavezzo, E., Militello, V., Toppo, S., & Palù, G. (2011). Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences*, *12*(11), 7861–7884. https://doi.org/10.3390/ijms12117861

Belák, S., Karlsson, O. E., Blomström, A.-L., Berg, M., & Granberg, F. (2013). New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary Microbiology*, *165*(1–2), 95–101. https://doi.org/10.1016/j.vetmic.2013.01.022

Bernstein, A. S., Ando, A. W., Loch-Temzelides, T., Vale, M. M., Li, B. V., Li, H., Busch, J., Chapman, C. A., Kinnaird, M., Nowak, K., Castro, M. C., Zambrana-Torrelio, C., Ahumada, J. A., Xiao, L., Roehrdanz, P., Kaufman, L., Hannah, L., Daszak, P., Pimm, S. L., & Dobson, A. P. (2022). The costs and benefits of primary prevention of zoonotic pandemics. *Science Advances*, *8*(5), eabl4183. https://doi.org/10.1126/sciadv.abl4183

Blomström, A.-L. (2011). Viral metagenomics as an emerging and powerful tool in veterinary medicine. *Veterinary Quarterly*, *31*(3), 107–114. https://doi.org/10.1080/01652176.2011.604971

Capobianchi, M. R., Giombini, E., & Rozera, G. (2013). Next-generation sequencing technology in clinical virology. *Clinical Microbiology and Infection*, *19*(1), 15–22. https://doi.org/10.1111/1469-0691.12056

Escudero-Pérez, B., Lalande, A., Mathieu, C., & Lawrence, P. (2023). Host–Pathogen Interactions Influencing Zoonotic Spillover Potential and Transmission in Humans. *Viruses*, *15*(3), 599. https://doi.org/10.3390/v15030599

Gibb, R., Franklinos, L. H. V., Redding, D. W., & Jones, K. E. (2020). Ecosystem perspectives are needed to manage zoonotic risks in a changing climate. *BMJ*, m3389. https://doi.org/10.1136/bmj.m3389

Gutiérrez-Bugallo, G., Piedra, L. A., Rodriguez, M., Bisset, J. A., Lourenço-de-Oliveira, R., Weaver, S. C., Vasilakis, N., & Vega-Rúa, A. (2019). Vector-borne transmission and evolution of Zika virus. *Nature Ecology & Evolution*, *3*(4), 561–569. https://doi.org/10.1038/s41559-019-0836-z

Jacob, S. T., Crozier, I., Fischer, W. A., Hewlett, A., Kraft, C. S., Vega, M.-A. D. L., Soka, M. J., Wahl, V., Griffiths, A., Bollinger, L., & Kuhn, J. H. (2020). Ebola virus disease. *Nature Reviews Disease Primers*, *6*(1), 13. https://doi.org/10.1038/s41572-020-0147-3

Jones-Engel, L., May, C. C., Engel, G. A., Steinkraus, K. A., Schillaci, M. A., Fuentes, A., Rompis, A., Chalise, M. K., Aggimarangsee, N., Feeroz, M. M., Grant, R., Allan, J. S., Putra, A., Wandia, I. N., Watanabe, R., Kuller, L., Thongsawat, S., Chaiwarith, R., Kyes, R. C., & Linial, M. L. (2008). Diverse Contexts of Zoonotic Transmission of Simian Foamy Viruses in Asia. *Emerging Infectious Diseases*, *14*(8), 1200–1208. https://doi.org/10.3201/eid1408.071430

Kamorudeen, R. T., Adedokun, K. A., & Olarinmoye, A. O. (2020). Ebola outbreak in West Africa, 2014 – 2016: Epidemic timeline, differential diagnoses, determining factors, and lessons for future response. *Journal of Infection and Public Health*, *13*(7), 956–962. https://doi.org/10.1016/j.jiph.2020.03.014

Kaner, J., & Schaack, S. (2016). Understanding Ebola: The 2014 epidemic. *Globalization and Health*, *12*(1), 53. https://doi.org/10.1186/s12992-016-0194-4

Krupovic, M., & Varsani, A. (2022). Naryaviridae, Nenyaviridae, and Vilyaviridae: Three new families of single-stranded DNA viruses in the phylum Cressdnaviricota. *Archives of Virology*, *167*(12), 2907–2921. https://doi.org/10.1007/s00705-022-05557-w

Krupovic, M., Varsani, A., Kazlauskas, D., Breitbart, M., Delwart, E., Rosario, K., Yutin, N., Wolf, Y. I., Harrach, B., Zerbini, F. M., Dolja, V. V., Kuhn, J. H., & Koonin, E. V. (2020). *Cressdnaviricota*: A Virus Phylum Unifying Seven Families of Rep-Encoding Viruses with Single-Stranded, Circular DNA Genomes. *Journal of Virology*, *94*(12), e00582-20. https://doi.org/10.1128/JVI.00582-20

Lauber, C., & Seitz, S. (2022). Opportunities and Challenges of Data-Driven Virus Discovery. *Biomolecules*, *12*(8), 1073. https://doi.org/10.3390/biom12081073

Miranda, M. N. S., Pingarilho, M., Pimentel, V., Torneri, A., Seabra, S. G., Libin, P. J. K., & Abecasis, A. B. (2022). A Tale of Three Recent Pandemics: Influenza, HIV and SARS-CoV-2. *Frontiers in Microbiology*, *13*, 889643. https://doi.org/10.3389/fmicb.2022.889643

Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. (2017). Pathways to zoonotic spillover. *Nature Reviews Microbiology*, *15*(8), 502–510. https://doi.org/10.1038/nrmicro.2017.45

Quer, J., Colomer-Castell, S., Campos, C., Andrés, C., Piñana, M., Cortese, M. F., González-Sánchez, A., Garcia-Cehic, D., Ibáñez, M., Pumarola, T., Rodríguez-Frías, F., Antón, A., & Tabernero, D. (2022). Next-Generation Sequencing for Confronting Virus Pandemics. *Viruses*, *14*(3), 600. https://doi.org/10.3390/v14030600

Rahman, Md. T., Sobur, Md. A., Islam, Md. S., Ievy, S., Hossain, Md. J., El Zowalaty, M. E., Rahman, A. T., & Ashour, H. M. (2020). Zoonotic Diseases: Etiology, Impact, and Control. *Microorganisms*, *8*(9), 1405. https://doi.org/10.3390/microorganisms8091405

Stang, A., Korn, K., Wildner, O., & Überla, K. (2005). Characterization of Virus Isolates by Particle-Associated Nucleic Acid PCR. *Journal of Clinical Microbiology*, *43*(2), 716–720. https://doi.org/10.1128/JCM.43.2.716-720.2005

Tei, S., Kitajima, N., Takahashi, K., & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *The Lancet*, *362*(9381), 371–373. https://doi.org/10.1016/S0140-6736(03)14025-1

Väisänen, E., Fu, Y., Hedman, K., & Söderlund-Venermo, M. (2017). Human Protoparvoviruses. *Viruses*, *9*(11), 354. https://doi.org/10.3390/v9110354

2. CRESS (Circular Rep-Encoding Single-Stranded) DNA viruses

With the advent of next-generation sequencing (NGS) technologies and viral metagenomics studies, researchers have unveiled numerous viral sequences, over 60% of which exhibit low similarity to reference sequences. Thus, these have been defined as "viral dark matter," underscoring our still incomplete understanding of viral diversity. Viral metagenomics and NGS technologies enable the extraction of many sequences of viral dark matter without the need for isolation, providing a novel approach to analyzing rare and highly diversified viruses (Dai et al., 2024).(Zhu et al., 2024)

Single-stranded DNA (ssDNA) viruses with circular replication (Rep), known as CRESS-DNA viruses, members of the phylum *Cressdnaviricota*, were first described in 2012 (Rosario et al., 2012). These viruses possess circular ssDNA genomes, with sizes ranging from 1.7 to 3 kb and encode a conserved replicase (Rep protein) that initiates genome replication, through rolling circle replication (RCR) (Chandler et al., 2013). CRESS-DNA viruses are highly prevalent and infect species across the three domains of life: Archaea, Bacteria, and Eukarya. Studies in viral metagenomics have significantly expanded the number of recently described, often unclassified, viruses within the phylum *Cressdnaviricota* (Krupovic et al., 2020). To date, according to the International Committee on Taxonomy of Viruses (ICTV), this phylum encompasses two classes: i) *Repensiviricetes*, which includes plant and fungal viruses from the families *Genomoviridae*, *Geplanaviridae* and *Geminiviridae*, and ii) *Arfiviricetes*, which comprises twenty viral families (*Bacilladnaviridae, Circoviridae, Endolinaviridae, Vilyaviridae, Smacoviridae, Gandrviridae, Ouroboviridae, Draupnirviridae, Amesuviridae, Anicreviridae, Nanoviridae, Metaxyviridae, Redondoviridae, Pecoviridae, Naryaviridae, Adamaviridae, Kirkoviridae, Nenyaviridae, Kanorauviridae, Mahapunaviridae).* However, numerous related ssDNA virus groups, informally designated CRESSV1 to CRESSV6, remain unclassified (Zhu et al., 2024).

The *Cressdnaviricota* phylum includes numerous pathogenic taxa that infect eukaryotes, significantly impacting agricultural production and livelihoods. For instance *Nanoviridae, Metaxyviridae* and *Geminiviridae*, the most populous groups within the phylum, are known pathogens of economically significant crops. These viral agents are responsible for global agricultural yield reductions, resulting in substantial economic losses for farmers and agricultural industries (Fiallo-Olivé et al., 2021;

Thomas et al., 2021; Zhao et al., 2019). *Redondoviridae*, a recently identified group of CRESS DNA viruses, has been detected in the human respiratory tract and is associated with pathological conditions such as periodontitis (Abbas et al., 2021).

The family *Circoviridae* includes viruses that have a diameter of 15–25 nm and possess a covalently closed, circular, single-stranded DNA (ssDNA) genome ranging from 1.7 to 2.1 kb in length. This family is divided into two genera: *Circovirus* (CrV) and *Cyclovirus* (CyV), which contain 49 and 52 recognized species, respectively (www.ictv.global/report/circoviridae). The genome of circoviruses features an ambisense organization and includes at least two major open reading frames (ORFs) that encode for the replication-associated protein (Rep) and the capsid protein (Cap)(Rosario et al., 2017). Circoviruses of the genus *Circovirus* (CrVs) have been found in birds (Haddadmarandi et al., 2020), mammals (Bexton et al., 2015; Opriessnig et al., 2020), and fish (Lőrincz et al., 2012), while Cycloviruses (CyVs) have been identified in both vertebrates (Prades et al., 2021) and invertebrates (Dayaram et al., 2013; Padilla-Rodriguez et al., 2013). Notably, CRESS DNA viruses have frequently been found as components of the human virome. The detection of CyVs in humans was first reported in 2010 from stool samples of children with and without acute flaccid paralysis (AFP) in developing nations (Li et al., 2010). Since then, human-associated CyVs (HuACyVs) have also been found in cerebrospinal fluid (CSF) (Smits et al., 2013; Tan et al., 2013), serum (Macera et al., 2016; Sauvage et al., 2018)), and respiratory secretions (Phan et al., 2014) of both symptomatic and healthy individuals, leading to the classification of 12 distinct HuACyV species within the Cyclovirus genus (Prades et al., 2021). HuACyVs have been linked to neurological diseases, gastrointestinal issues, immunodeficiencies, and respiratory symptoms, although their pathogenicity remains uncertain. More recently, in 2022, the first human-associated CrV (HCirV-1) was identified in an immunosuppressed patient with acute hepatitis in France (Padilla-Rodriguez et al., 2013). The origins, transmission routes, and effects of these viruses on human health remain unclear, emphasizing the importance of increased awareness and further investigation into the potential of circoviruses as emerging human pathogens (Pérot et al., 2024). Circoviruses were first detected in domestic carnivores in 2012, with canine circovirus (CanineCV) identified in the serum of asymptomatic dogs in the USA (Kapoor et al., 2012). CanineCV has been linked to acute gastroenteritis, hemorrhagic diarrhea, signs of vasculitis, lymphadenitis, thrombocytopenia, neutropenia, and lymphopenia (T. R. Anderson & Slotkin, 1975; Decaro et al., 2014; Li et al., 2010).

However, the pathogenesis of CanineCV is not completely understood. Additionally, in 2021, two novel cycloviruses were detected in fecal samples from three domestic dogs with hemorrhagic gastroenteritis that tested positive for canine parvovirus (CPV-2) (Gainor et al., 2021). However, it remains unclear whether these viruses originated from dietary sources or if they actually infect dogs. Circoviruses have been frequently reported in domestic dogs, but there is limited information on their presence in domestic cats. In 2014, a feline circovirus (CrV) was identified in healthy shelter cats in Davis, California, showing 39–47% sequence identity with known circoviruses and the highest similarity to a mongoose circovirus strain (W. Zhang et al., 2014). In 2018, a new CRESS DNA virus, FeSCV, was isolated from cats with enteritis during an outbreak in Japan, and was classified into the Vilyaviridae family, unrelated to the earlier circovirus (Takano et al., 2018). In 2021, FeSCV was detected in diarrheic cats in China, where it co-infected with other enteric viruses, and a Giardia intestinalis sequence was identified within its genome (Hao et al., 2021).

Considering the ever-changing landscape of viral infections and the possibility of new strains emerging, it is crucial to conduct additional research on circoviruses to enhance early detection and monitoring in domestic carnivores. Furthermore, epidemiological studies could clarify any potential connections between the presence of circoviruses and the occurrence of diseases, as well as their distribution within canine and feline populations, which may help avert potential spillover into human communities. In **section 2.1**, we examined several feline sample collections from cats in Italy, uncovering significant genetic diversity of CRESS DNA viruses among these samples.

The existence of small circular DNA molecules in eukaryotic and prokaryotic organisms has been documented for decades. However, it remains unclear how these molecules originated and what roles they may play in specific contexts. Recently, following the discovery of small circular DNA molecules in tissues and cells associated with neurodegenerative diseases and cancer, research in this field has gained renewed momentum, leading to significant findings. Even prior to the discovery of the small circular molecules in question, it was hypothesized that a factor in the meat of domestic cattle (*Bos taurus*) could promote cancer development (Santarelli et al., 2008). The argument underpinning this assertion was that excessive consumption of red meat is associated with an increased risk of colon cancer, particularly as domestic cattle meat is often consumed undercooked. Consequently, the existence of an unknown bovine factor was proposed. This hypothesis was supported by a subsequent publication that sought to demonstrate a correlation between the rising incidence of colon carcinoma in various countries and the increasing consumption of beef (Kuhnle & Bingham, 2007). A term was thus coined to designate this mysterious factor, initially referred to as the beef factor, and later renamed the bovine meat and milk factor (BMMF). In the following years, the purported BMMFs were identified in samples from domestic cattle (serum and milk) and in samples from patients with Multiple Sclerosis, showing a significant correlation with the previously discovered and published SPHINX (Slow Progressive Hidden INfections of variable X latency) molecules isolated from cells and tissues infected with transmissible spongiform encephalopathy (TSE) (Zur Hausen, 2009, 2012). In 2019, a press conference by the German Cancer Research Center (DKFZ) garnered attention, presenting BMMFs as "factors" or "new pathogens" derived from European domestic cattle (Bos taurus) and suggesting their involvement in cancer development, after an oral intake with food. SPHINX/BMMF molecules are categorized into four groups based on nucleotide sequences in zur Hausen et al 2017 (Zur Hausen et al., 2017). Groups 1 and 2 include SPHINX1.76 and SPHINX2.34, respectively, while the third group consists of *gemycircularvirus*-related isolates from cattle serum and milk. The fourth group includes the MSSI1.162 DNA sequence from an MS patient, showing homology to a *Psychrobacter* spp. plasmid.

Consuming milk or beef products from taurine cattle, especially during infancy or early childhood, may lead to the absorption of BMMF/SPHINX, which could trigger chronic inflammatory responses in tissues such as the breast and gut. Over time, these chronic infections might result in cancers in neighbouring tissues, including colon cancer and potentially breast and prostate cancer, as suggested by various studies(Bund et al., 2021; Zur Hausen et al., 2019). This hypothesis is supported by several lines of evidence: epidemiological data connecting the incidence of specific cancers, particularly breast and colon cancer, to the consumption of taurine-derived meat or milk products (zur Hausen, 2012); the presence of SPHINX/BMMFs in taurine (De Villiers et al., 2019; Falida et al., 2017; Gunst et al., 2014; Whitley et al., 2014a); and the discovery of the SPHINX/BMMFs replication protein (Rep) in the lamina propria of colon cancer patients (De Villiers et al., 2019; Zur Hausen et al., 2019). Epidemiological data examining the connection between meat and dairy consumption from domestic cattle and cancer development, specifically colorectal and breast cancer, reveal that earlier studies suggested a link between the intake of red and processed meats and a higher risk of colorectal cancer. It's important to note that red meat encompasses meat from various warm-blooded animals, not solely cattle(Chan et al., 2011; Corpet, 2011; Huxley et al., 2009; Veettil et al., 2021). In contrast, higher consumption of milk and dairy products seems to correlate with a lower risk of colorectal cancer, and there is no evidence indicating that red meat or cow's milk increases the risk of breast cancer. A thorough review of 30,818 cases found no relationship between cheese consumption and elevated cancer mortality or incidence, including colorectal and breast cancers (X. Zhang et al., 2023). A UK study of over 500,000 subjects showed that protein intake from dairy products and milk is inversely associated with colorectal cancer, while cheese intake alone does not influence its incidence. No association was found between protein intake from milk or dairy products and breast cancer incidence (Watling et al., 2023).In summary, there is no increased risk of colon or breast cancer linked to milk or dairy products, and the association between red meat consumption and colon cancer is not specific to beef.

Studies have investigated whether SPHINX/BMMFs are present only in Bos taurus-derived food products by examining various food products and animal samples. Using specific primers, these DNA molecules were detected in milk from water buffalo, sheep, and goats. Further studies found SPHINX/BMMF DNA in a wide range of food samples, including seafood, fruits, vegetables, and baby food, as well as in animal samples from pork and chicken. SPHINX/BMMF molecules were also found in blood and feces of African and Asian cattle (Habermann et al., 2023; König et al., 2021a, 2021b, 2023a; Pohl et al., 2022).

In **section 2.2**, we present a study that analyzes various food matrices, both of bovine and non-bovine origin, as well as bovine feces, in order to investigate the presence of BMMF molecules within Italian territory. The objective is to document the spread of BMMF in Italy and contribute to a risk assessment associated with the consumption of animal products, in relation to the development of cancers potentially linked to the presence of such molecules.

References

Abbas, A., Taylor, L. J., Collman, R. G., Bushman, F. D., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Redondoviridae: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(1). https://doi.org/10.1099/jgv.0.001526

Allander, T., Tammi, M. T., Eriksson, M., Bjerkner, A., Tiveljung-Lindell, A., & Andersson, B. (2005). Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proceedings of the National Academy of Sciences*, *102*(36), 12891–12896. https://doi.org/10.1073/pnas.0504666102

Altan, E., Del Valle Mendoza, J., Deng, X., Phan, T. G., Sadeghi, M., & Delwart, E. L. (2017). Small Circular Rep-Encoding Single-Stranded DNA Genomes in Peruvian Diarrhea Virome. *Genome Announcements*, *5*(38), e00822-17. https://doi.org/10.1128/genomeA.00822-17

Altay, A., Ocal, M., Yuksek, S. K., Polat, M., Tapisiz, A., Tezer, H., Ergunay, K., Bozdayi, G., & Ahmed, K. (2016). Multicenter investigation of bufavirus in the etiology of viral central nervous system infections of adults and children. *Journal of Clinical Virology*, *82*, S57–S58. https://doi.org/10.1016/j.jcv.2016.08.113

Altay, A., Yahiro, T., Bozdayi, G., Matsumoto, T., Sahin, F., Ozkan, S., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2015). Bufavirus genotype 3 in Turkish children with severe diarrhoea. *Clinical Microbiology and Infection*, *21*(10), 965.e1-965.e4. https://doi.org/10.1016/j.cmi.2015.06.006

Anderson, M. J., Higgins, P. G., Davis, L. R., Willman, J. S., Jones, S. E., Kidd, I. M., Pattison, J. R., & Tyrrell, D. A. J. (1985). Experimental Parvoviral Infection in Humans. *Journal of Infectious Diseases*, *152*(2), 257–265. https://doi.org/10.1093/infdis/152.2.257

Anderson, T. R., & Slotkin, T. A. (1975). Maturation of the adrenal medulla—IV. Effects of morphine. *Biochemical Pharmacology*, *24*(16), 1469–1474. https://doi.org/10.1016/0006-2952(75)90020-9

Atchison, R. W., Casto, B. C., & Hammon, W. McD. (1965). Adenovirus-Associated Defective Virus Particles. *Science*, *149*(3685), 754–756. https://doi.org/10.1126/science.149.3685.754

Ayouni, S., Estienney, M., Hammami, S., Neji Guediche, M., Pothier, P., Aouni, M., Belliot, G., & De Rougemont, A. (2016). Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants. *PLOS ONE*, *11*(9), e0162255. https://doi.org/10.1371/journal.pone.0162255

Barzon, L., Lavezzo, E., Militello, V., Toppo, S., & Palù, G. (2011). Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences*, *12*(11), 7861–7884. https://doi.org/10.3390/ijms12117861

Belák, S., Karlsson, O. E., Blomström, A.-L., Berg, M., & Granberg, F. (2013). New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary Microbiology*, *165*(1–2), 95–101. https://doi.org/10.1016/j.vetmic.2013.01.022

Bernstein, A. S., Ando, A. W., Loch-Temzelides, T., Vale, M. M., Li, B. V., Li, H., Busch, J., Chapman, C. A., Kinnaird, M., Nowak, K., Castro, M. C., Zambrana-Torrelio, C., Ahumada, J. A., Xiao, L., Roehrdanz, P., Kaufman, L., Hannah, L., Daszak, P., Pimm, S. L., & Dobson, A. P. (2022). The costs and benefits of primary prevention of zoonotic pandemics. *Science Advances*, *8*(5), eabl4183. https://doi.org/10.1126/sciadv.abl4183

Bexton, S., Wiersma, L. C., Getu, S., Van Run, P. R., Verjans, G. M. G. M., Schipper, D., Schapendonk, C. M. E., Bodewes, R., Oldroyd, L., Haagmans, B. L., Koopmans, M. M. P., & Smits, S. L. (2015). Detection of Circovirus in Foxes with Meningoencephalitis, United Kingdom, 2009–2013. *Emerging Infectious Diseases*, *21*(7), 1205–1208. https://doi.org/10.3201/eid2107.150228

Blomström, A.-L. (2011). Viral metagenomics as an emerging and powerful tool in veterinary medicine. *Veterinary Quarterly*, *31*(3), 107–114. https://doi.org/10.1080/01652176.2011.604971

Bund, T., Nikitina, E., Chakraborty, D., Ernst, C., Gunst, K., Boneva, B., Tessmer, C., Volk, N., Brobeil, A., Weber, A., Heikenwalder, M., Zur Hausen, H., & De Villiers, E.-M. (2021). Analysis of chronic inflammatory lesions of the colon for BMMF Rep antigen expression and CD68 macrophage interactions. *Proceedings of the National Academy of Sciences*, *118*(12), e2025830118. https://doi.org/10.1073/pnas.2025830118

Capobianchi, M. R., Giombini, E., & Rozera, G. (2013). Next-generation sequencing technology in clinical virology. *Clinical Microbiology and Infection*, *19*(1), 15–22. https://doi.org/10.1111/1469-0691.12056

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE*, *6*(6), e20456. https://doi.org/10.1371/journal.pone.0020456

Chandler, M., De La Cruz, F., Dyda, F., Hickman, A. B., Moncalian, G., & Ton-Hoang, B. (2013). Breaking and joining single-stranded DNA: The HUH endonuclease superfamily. *Nature Reviews Microbiology*, *11*(8), 525–538. https://doi.org/10.1038/nrmicro3067

Chappuis, G., Soulier, M., & Nettleton, P. (1987). Ovine parvovirus infection in Scottish sheep. *Veterinary Record*, *121*(8), 182–182. https://doi.org/10.1136/vr.121.8.182-a

Chieochansin, T., Vutithanachot, V., Theamboonlers, A., & Poovorawan, Y. (2015). Bufavirus in fecal specimens of patients with and without diarrhea in Thailand. *Archives of Virology*, *160*(7), 1781–1784. https://doi.org/10.1007/s00705-015-2441-z

Chung, Y.-H., Jun, H. S., Son, M., Bao, M., Bae, H. Y., Kang, Y., & Yoon, J.-W. (2000). Cellular and Molecular Mechanism for Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats. *The Journal of Immunology*, *165*(5), 2866–2876. https://doi.org/10.4049/jimmunol.165.5.2866

Corpet, D. E. (2011). Red meat and colon cancer: Should we become vegetarians, or can we make meat safer? *Meat Science*, *89*(3), 310–316. https://doi.org/10.1016/j.meatsci.2011.04.009

Cossart, Y. E., Cant, B., Field, A. M., & Widdows, D. (1975). PARVOVIRUS-LIKE PARTICLES IN HUMAN SERA. *The Lancet*, *305*(7898), 72–73. https://doi.org/10.1016/S0140-6736(75)91074-0

Dai, Z., Wang, H., Xu, J., Lu, X., Ni, P., Yang, S., Shen, Q., Wang, X., Li, W., Wang, X., Zhou, C., Zhang, W., & Shan, T. (2024). Unveiling the Virome of Wild Birds: Exploring CRESS-DNA Viral Dark Matter. *Genome Biology and Evolution*, *16*(10), evae206. https://doi.org/10.1093/gbe/evae206

Dayaram, A., Potter, K. A., Moline, A. B., Rosenstein, D. D., Marinov, M., Thomas, J. E., Breitbart, M., Rosario, K., Argüello-Astorga, G. R., & Varsani, A. (2013). High global diversity of cycloviruses amongst dragonflies. *Journal of General Virology*, *94*(8), 1827–1840. https://doi.org/10.1099/vir.0.052654-0

De Villiers, E.-M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T., & Zur Hausen, H. (2019). A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. *Emerging Microbes & Infections*, *8*(1), 1205–1218. https://doi.org/10.1080/22221751.2019.1651620

Decaro, N., Martella, V., Desario, C., Lanave, G., Circella, E., Cavalli, A., Elia, G., Camero, M., & Buonavoglia, C. (2014). Genomic Characterization of a Circovirus Associated with Fatal Hemorrhagic Enteritis in Dog, Italy. *PLoS ONE*, *9*(8), e105909. https://doi.org/10.1371/journal.pone.0105909

Eilebrecht, S., Hotz-Wagenblatt, A., Sarachaga, V., Burk, A., Falida, K., Chakraborty, D., Nikitina, E., Tessmer, C., Whitley, C., Sauerland, C., Gunst, K., Grewe, I., & Bund, T. (2018). Expression and replication of virus-like circular DNA in human cells. *Scientific Reports*, *8*(1), 2851. https://doi.org/10.1038/s41598-018-21317-w

Ellerman, K. E., Richards, C. A., Guberski, D. L., Shek, W. R., & Like, A. A. (1996). Kilham Rat Virus Triggers T-Cell–Dependent Autoimmune Diabetes in Multiple Strains of Rat. *Diabetes*, *45*(5), 557–562. https://doi.org/10.2337/diab.45.5.557

Escudero-Pérez, B., Lalande, A., Mathieu, C., & Lawrence, P. (2023). Host–Pathogen Interactions Influencing Zoonotic Spillover Potential and Transmission in Humans. *Viruses*, *15*(3), 599. https://doi.org/10.3390/v15030599

Falida, K., Eilebrecht, S., Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2017). Isolation of Two Virus-Like Circular DNAs from Commercially Available Milk Samples. *Genome Announcements*, *5*(17), e00266-17. https://doi.org/10.1128/genomeA.00266-17

Fiallo-Olivé, E., Lett, J.-M., Martin, D. P., Roumagnac, P., Varsani, A., Zerbini, F. M., & Navas-Castillo, J. (2021). ICTV Virus Taxonomy Profile: Geminiviridae 2021: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(12). https://doi.org/10.1099/jgv.0.001696

Gainor, K., Malik, Y. S., & Ghosh, S. (2021). Novel Cyclovirus Species in Dogs with Hemorrhagic Gastroenteritis. *Viruses*, *13*(11), 2155. https://doi.org/10.3390/v13112155

Gibb, R., Franklinos, L. H. V., Redding, D. W., & Jones, K. E. (2020). Ecosystem perspectives are needed to manage zoonotic risks in a changing climate. *BMJ*, m3389. https://doi.org/10.1136/bmj.m3389

Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2014). Isolation of Bacterial Plasmid-Related Replication-Associated Circular DNA from a Serum Sample of a Multiple Sclerosis Patient. *Genome Announcements*, *2*(4), e00847-14. https://doi.org/10.1128/genomeA.00847-14

Gutiérrez-Bugallo, G., Piedra, L. A., Rodriguez, M., Bisset, J. A., Lourenço-de-Oliveira, R., Weaver, S. C., Vasilakis, N., & Vega-Rúa, A. (2019). Vector-borne transmission and evolution of Zika virus. *Nature Ecology & Evolution*, *3*(4), 561–569. https://doi.org/10.1038/s41559-019-0836-z

Habermann, D., Klempt, M., & Franz, C. M. A. P. (2023). Identification and Characterization of Novel SPHINX/BMMF-like DNA Sequences Isolated from Non-Bovine Foods. *Genes*, *14*(7), 1307. https://doi.org/10.3390/genes14071307

Haddadmarandi, M. R., Madani, S. A., Nili, H., & Ghorbani, A. (2020). Molecular survey of avian circoviruses in some non-psittacine birds and detection of a novel canary circovirus in a pigeon. *Iranian Journal of Veterinary Research*, *21*(1), 57–60.

Hao, X., Li, Y., Hu, X., Fu, X., Dong, J., Zhang, H., Zhou, P., & Li, S. (2021). Feline Stool-Associated Circular DNA Virus (FeSCV) in Diarrheic Cats in China. *Frontiers in Veterinary Science*, *8*, 694089. https://doi.org/10.3389/fvets.2021.694089

Huang, D.-D., Wang, W., Lu, Q.-B., Zhao, J., Guo, C.-T., Wang, H.-Y., Zhang, X.-A., Tong, Y.-G., Liu, W., & Cao, W.-C. (2015). Identification of Bufavirus-1 and Bufavirus-3 in Feces of Patients with Acute Diarrhea, China. *Scientific Reports*, *5*(1), 13272. https://doi.org/10.1038/srep13272

Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer*, *125*(1), 171–180. https://doi.org/10.1002/ijc.24343

Jacob, S. T., Crozier, I., Fischer, W. A., Hewlett, A., Kraft, C. S., Vega, M.-A. D. L., Soka, M. J., Wahl, V., Griffiths, A., Bollinger, L., & Kuhn, J. H. (2020). Ebola virus disease. *Nature Reviews Disease Primers*, *6*(1), 13. https://doi.org/10.1038/s41572-020-0147-3

Jones, M. S., Kapoor, A., Lukashov, V. V., Simmonds, P., Hecht, F., & Delwart, E. (2005). New DNA Viruses Identified in Patients with Acute Viral Infection Syndrome. *Journal of Virology*, *79*(13), 8230–8236. https://doi.org/10.1128/JVI.79.13.8230-8236.2005

Jones-Engel, L., May, C. C., Engel, G. A., Steinkraus, K. A., Schillaci, M. A., Fuentes, A., Rompis, A., Chalise, M. K., Aggimarangsee, N., Feeroz, M. M., Grant, R., Allan, J. S., Putra, A., Wandia, I. N., Watanabe, R., Kuller, L., Thongsawat, S., Chaiwarith, R., Kyes, R. C., & Linial, M. L. (2008). Diverse Contexts of Zoonotic Transmission of Simian Foamy Viruses in Asia. *Emerging Infectious Diseases*, *14*(8), 1200–1208. https://doi.org/10.3201/eid1408.071430

Kamorudeen, R. T., Adedokun, K. A., & Olarinmoye, A. O. (2020). Ebola outbreak in West Africa, 2014 – 2016: Epidemic timeline, differential diagnoses, determining factors, and lessons for future response. *Journal of Infection and Public Health*, *13*(7), 956–962. https://doi.org/10.1016/j.jiph.2020.03.014

Kaner, J., & Schaack, S. (2016). Understanding Ebola: The 2014 epidemic. *Globalization and Health*, *12*(1), 53. https://doi.org/10.1186/s12992-016-0194-4

Kapoor, A., Dubovi, E. J., Henriquez-Rivera, J. A., & Lipkin, W. I. (2012). Complete Genome Sequence of the First Canine Circovirus. *Journal of Virology*, *86*(12), 7018–7018. https://doi.org/10.1128/JVI.00791-12

Kilham, L., & Olivier, L. J. (1959). A latent virus of rats isolated in tissue culture. *Virology*, *7*(4), 428–437. https://doi.org/10.1016/0042-6822(59)90071-6

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023a). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023b). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021a). Circular Rep-Encoding Single-Stranded DNA Sequences in Milk from Water Buffaloes (Bubalus arnee f. Bubalis). *Viruses*, *13*(6), 1088. https://doi.org/10.3390/v13061088

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021b). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021c). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

Krupovic, M., & Varsani, A. (2022). Naryaviridae, Nenyaviridae, and Vilyaviridae: Three new families of single-stranded DNA viruses in the phylum Cressdnaviricota. *Archives of Virology*, *167*(12), 2907–2921. https://doi.org/10.1007/s00705-022-05557-w

Krupovic, M., Varsani, A., Kazlauskas, D., Breitbart, M., Delwart, E., Rosario, K., Yutin, N., Wolf, Y. I., Harrach, B., Zerbini, F. M., Dolja, V. V., Kuhn, J. H., & Koonin, E. V. (2020). *Cressdnaviricota*: A Virus Phylum Unifying Seven Families of Rep-Encoding Viruses with Single-Stranded, Circular DNA Genomes. *Journal of Virology*, *94*(12), e00582-20. https://doi.org/10.1128/JVI.00582-20

Kuhnle, G. G. C., & Bingham, S. A. (2007). Dietary meat, endogenous nitrosation and colorectal cancer. *Biochemical Society Transactions*, *35*(5), 1355–1357. https://doi.org/10.1042/BST0351355

Lauber, C., & Seitz, S. (2022). Opportunities and Challenges of Data-Driven Virus Discovery. *Biomolecules*, *12*(8), 1073. https://doi.org/10.3390/biom12081073

Li, L., Kapoor, A., Slikas, B., Bamidele, O. S., Wang, C., Shaukat, S., Masroor, M. A., Wilson, M. L., Ndjango, J.-B. N., Peeters, M., Gross-Camp, N. D., Muller, M. N., Hahn, B. H., Wolfe, N. D., Triki, H., Bartkus, J., Zaidi, S. Z., & Delwart, E. (2010). Multiple Diverse Circoviruses Infect Farm Animals and Are Commonly Found in Human and Chimpanzee Feces. *Journal of Virology*, *84*(4), 1674–1682. https://doi.org/10.1128/JVI.02109-09

Lőrincz, M., Dán, Á., Láng, M., Csaba, G., Tóth, Á. G., Székely, C., Cságola, A., & Tuboly, T. (2012). Novel circovirus in European catfish (Silurus glanis). *Archives of Virology*, *157*(6), 1173–1176. https://doi.org/10.1007/s00705-012-1291-1

Macera, L., Focosi, D., Vatteroni, M. L., Manzin, A., Antonelli, G., Pistello, M., & Maggi, F. (2016). Cyclovirus Vietnam DNA in immunodeficient patients. *Journal of Clinical Virology*, *81*, 12–15. https://doi.org/10.1016/j.jcv.2016.05.011

Maggi, F., Andreoli, E., Pifferi, M., Meschi, S., Rocchi, J., & Bendinelli, M. (2007). Human bocavirus in Italian patients with respiratory diseases. *Journal of Clinical Virology*, *38*(4), 321–325. https://doi.org/10.1016/j.jcv.2007.01.008

Manuelidis, L. (2011). Nuclease resistant circular DNAs copurify with infectivity in scrapie and CJD. *Journal of NeuroVirology*, *17*(2), 131–145. https://doi.org/10.1007/s13365-010-0007-0

Manuelidis, L. (2019). Prokaryotic SPHINX 1.8 REP protein is tissue-specific and expressed in human germline cells. *Journal of Cellular Biochemistry*, *120*(4), 6198–6208. https://doi.org/10.1002/jcb.27907

Matthews, P. C., Malik, A., Simmons, R., Sharp, C., Simmonds, P., & Klenerman, P. (2014). PARV4: An Emerging Tetraparvovirus. *PLoS Pathogens*, *10*(5), e1004036. https://doi.org/10.1371/journal.ppat.1004036

Mietzsch, M., McKenna, R., Väisänen, E., Yu, J. C., Ilyas, M., Hull, J. A., Kurian, J., Smith, J. K., Chipman, P., Lasanajak, Y., Smith, D., Söderlund-Venermo, M., & Agbandje-McKenna, M. (2020). Structural Characterization of Cuta- and Tusavirus: Insight into Protoparvoviruses Capsid Morphology. *Viruses*, *12*(6), 653. https://doi.org/10.3390/v12060653

Miranda, M. N. S., Pingarilho, M., Pimentel, V., Torneri, A., Seabra, S. G., Libin, P. J. K., & Abecasis, A. B. (2022). A Tale of Three Recent Pandemics: Influenza, HIV and SARS-CoV-2. *Frontiers in Microbiology*, *13*, 889643. https://doi.org/10.3389/fmicb.2022.889643

Mohanraj, U., Jokinen, M., Thapa, R. R., Paloniemi, M., Vesikari, T., Lappalainen, M., Tarkka, E., Nora-Krūkle, Z., Vilmane, A., Vettenranta, K., Mangani, C., Oikarinen, S., Fan, Y.-M., Ashorn, P., Väisänen, E., & Söderlund-Venermo, M. (2021). Human Protoparvovirus DNA and IgG in Children and Adults with and without Respiratory or Gastrointestinal Infections. *Viruses*, *13*(3), 483. https://doi.org/10.3390/v13030483

Mollerup, S., Fridholm, H., Vinner, L., Kjartansdóttir, K. R., Friis-Nielsen, J., Asplund, M., Herrera, J. A. R., Steiniche, T., Mourier, T., Brunak, S., Willerslev, E., Izarzugaza, J. M. G., Hansen, A. J., & Nielsen, L. P. (2017). Cutavirus in Cutaneous Malignant Melanoma. *Emerging Infectious Diseases*, *23*(2), 363–365. https://doi.org/10.3201/eid2302.161564

Opriessnig, T., Karuppannan, A. K., Castro, A. M. M. G., & Xiao, C.-T. (2020). Porcine circoviruses: Current status, knowledge gaps and challenges. *Virus Research*, *286*, 198044. https://doi.org/10.1016/j.virusres.2020.198044

Padilla-Rodriguez, M., Rosario, K., & Breitbart, M. (2013). Novel cyclovirus discovered in the Florida woods cockroach Eurycotis floridana (Walker). *Archives of Virology*, *158*(6), 1389–1392. https://doi.org/10.1007/s00705-013-1606-x

Pérot, P., Da Rocha, N., Farcet, M. R., Kreil, T. R., & Eloit, M. (2024). Human Circovirus is not detected in plasma pools for fractionation. *Transfusion*, *64*(1), 16–18. https://doi.org/10.1111/trf.17604

Phan, T. G., Dreno, B., Da Costa, A. C., Li, L., Orlandi, P., Deng, X., Kapusinszky, B., Siqueira, J., Knol, A.-C., Halary, F., Dantal, J., Alexander, K. A., Pesavento, P. A., & Delwart, E. (2016). A new protoparvovirus in human fecal samples and cutaneous T cell lymphomas (mycosis fungoides). *Virology*, *496*, 299–305. https://doi.org/10.1016/j.virol.2016.06.013

Phan, T. G., Luchsinger, V., Avendaño, L. F., Deng, X., & Delwart, E. (2014). Cyclovirus in nasopharyngeal aspirates of Chilean children with respiratory infections. *Journal of General Virology*, *95*(4), 922–927. https://doi.org/10.1099/vir.0.061143-0

Phan, T. G., Vo, N. P., Bonkoungou, I. J. O., Kapoor, A., Barro, N., O'Ryan, M., Kapusinszky, B., Wang, C., & Delwart, E. (2012). Acute Diarrhea in West African Children: Diverse Enteric Viruses and a Novel Parvovirus Genus. *Journal of Virology*, *86*(20), 11024–11030. https://doi.org/10.1128/JVI.01427-12

Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. (2017). Pathways to zoonotic spillover. *Nature Reviews Microbiology*, *15*(8), 502–510. https://doi.org/10.1038/nrmicro.2017.45

Pohl, S., Habermann, D., Link, E. K., Fux, R., Boldt, C. L., Franz, C. M. A. P., Hölzel, C., & Klempt, M. (2022). Detection of DNA sequences attributed to bovine meat and milk factors (BMMF/SPHINX) in food-related samples. *Food Control*, *135*, 108779. https://doi.org/10.1016/j.foodcont.2021.108779

Prades, Y., Pizarro, R., Ruiz, M., Moreno, C., Avendaño, L. F., & Luchsinger, V. (2021). Cyclovirus detection in Chilean adults with and without community-acquired pneumonia. *Journal of Medical Virology*, *93*(8), 4786–4793. https://doi.org/10.1002/jmv.27080

Qiu, J., Söderlund-Venermo, M., & Young, N. S. (2017). Human Parvoviruses. *Clinical Microbiology Reviews*, *30*(1), 43–113. https://doi.org/10.1128/CMR.00040-16

Quer, J., Colomer-Castell, S., Campos, C., Andrés, C., Piñana, M., Cortese, M. F., González-Sánchez, A., Garcia-Cehic, D., Ibáñez, M., Pumarola, T., Rodríguez-Frías, F., Antón, A., & Tabernero, D. (2022). Next-Generation Sequencing for Confronting Virus Pandemics. *Viruses*, *14*(3), 600. https://doi.org/10.3390/v14030600

Rahman, Md. T., Sobur, Md. A., Islam, Md. S., Ievy, S., Hossain, Md. J., El Zowalaty, M. E., Rahman, A. T., & Ashour, H. M. (2020). Zoonotic Diseases: Etiology, Impact, and Control. *Microorganisms*, *8*(9), 1405. https://doi.org/10.3390/microorganisms8091405

Rangarajan, S., Walsh, L., Lester, W., Perry, D., Madan, B., Laffan, M., Yu, H., Vettermann, C., Pierce, G. F., Wong, W. Y., & Pasi, K. J. (2017). AAV5–Factor VIII Gene Transfer in Severe Hemophilia A. *New England Journal of Medicine*, *377*(26), 2519–2530. https://doi.org/10.1056/NEJMoa1708483

Reuter, G., Pankovics, P., László, Z., Gáspár, G., Hui, A., Delwart, E., & Boros, Á. (2022). Human-stool-associated tusavirus (Parvoviridae) in domestic goats and sheep. *Archives of Virology*, *167*(5), 1307–1310. https://doi.org/10.1007/s00705-022-05424-8

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., & Varsani, A. (2017). Revisiting the taxonomy of the family Circoviridae: Establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Archives of Virology*, *162*(5), 1447–1463. https://doi.org/10.1007/s00705-017-3247-y

Rosario, K., Duffy, S., & Breitbart, M. (2012). A field guide to eukaryotic circular single-stranded DNA viruses: Insights gained from metagenomics. *Archives of Virology*, *157*(10), 1851–1871. https://doi.org/10.1007/s00705-012-1391-y

Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. *Nutrition and Cancer*, *60*(2), 131–144. https://doi.org/10.1080/01635580701684872

Sauvage, V., Gomez, J., Barray, A., Vandenbogaert, M., Boizeau, L., Tagny, C. T., Rakoto, O., Bizimana, P., Guitteye, H., Ciré, B. B., Soumana, H., Tchomba, J.-B. S., Caro, V., & Laperche, S. (2018). High prevalence of cyclovirus Vietnam (CyCV-VN) in plasma samples from Madagascan healthy blood donors. *Infection, Genetics and Evolution*, *66*, 9–12. https://doi.org/10.1016/j.meegid.2018.09.002

Smits, S. L., Schapendonk, C. M. E., Van Beek, J., Vennema, H., Schürch, A. C., Schipper, D., Bodewes, R., Haagmans, B. L., Osterhaus, A. D. M. E., & Koopmans, M. P. (2014). New Viruses in Idiopathic Human Diarrhea Cases, the Netherlands. *Emerging Infectious Diseases*, *20*(7). https://doi.org/10.3201/eid2007.140190

Smits, S. L., Zijlstra, E. E., Van Hellemond, J. J., Schapendonk, C. M. E., Bodewes, R., Schürch, A. C., Haagmans, B. L., & Osterhaus, A. D. M. E. (2013). Novel Cyclovirus in Human Cerebrospinal Fluid, Malawi, 2010–2011. *Emerging Infectious Diseases*, *19*(9). https://doi.org/10.3201/eid1909.130404

Stang, A., Korn, K., Wildner, O., & Überla, K. (2005). Characterization of Virus Isolates by Particle-Associated Nucleic Acid PCR. *Journal of Clinical Microbiology*, *43*(2), 716–720. https://doi.org/10.1128/JCM.43.2.716-720.2005

Takano, T., Yanai, Y., Hiramatsu, K., Doki, T., & Hohdatsu, T. (2018). Novel single-stranded, circular DNA virus identified in cats in Japan. *Archives of Virology*, *163*(12), 3389–3393. https://doi.org/10.1007/s00705-018-4020-6

Tan, L. V., Van Doorn, H. R., Nghia, H. D. T., Chau, T. T. H., Tu, L. T. P., De Vries, M., Canuti, M., Deijs, M., Jebbink, M. F., Baker, S., Bryant, J. E., Tham, N. T., BKrong, N. T. T. C., Boni, M. F., Loi, T. Q., Phuong, L. T., Verhoeven, J. T. P., Crusat, M., Jeeninga, R. E., … De Jong, M. D. (2013). Identification of a New Cyclovirus in Cerebrospinal Fluid of Patients with Acute Central Nervous System Infections. *mBio*, *4*(3), e00231-13. https://doi.org/10.1128/mBio.00231-13

Tei, S., Kitajima, N., Takahashi, K., & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *The Lancet*, *362*(9381), 371–373. https://doi.org/10.1016/S0140-6736(03)14025-1

Thomas, J. E., Gronenborn, B., Harding, R. M., Mandal, B., Grigoras, I., Randles, J. W., Sano, Y., Timchenko, T., Vetten, H. J., Yeh, H.-H., Ziebell, H., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Nanoviridae. *Journal of General Virology*, *102*(3). https://doi.org/10.1099/jgv.0.001544

Väisänen, E., Fu, Y., Hedman, K., & Söderlund-Venermo, M. (2017). Human Protoparvoviruses. *Viruses*, *9*(11), 354. https://doi.org/10.3390/v9110354

Väisänen, E., Kuisma, I., Phan, T. G., Delwart, E., Lappalainen, M., Tarkka, E., Hedman, K., & Söderlund-Venermo, M. (2014). Bufavirus in Feces of Patients with Gastroenteritis, Finland. *Emerging Infectious Diseases*, *20*(6), 1077–1079. https://doi.org/10.3201/eid2006.131674

Väisänen, E., Mohanraj, U., Kinnunen, P. M., Jokelainen, P., Al-Hello, H., Barakat, A. M., Sadeghi, M., Jalilian, F. A., Majlesi, A., Masika, M., Mwaengo, D., Anzala, O., Delwart, E., Vapalahti, O., Hedman, K., & Söderlund-Venermo, M. (2018). Global Distribution of Human Protoparvoviruses. *Emerging Infectious Diseases*, *24*(7), 1292–1299. https://doi.org/10.3201/eid2407.172128

Väisänen, E., Paloniemi, M., Kuisma, I., Lithovius, V., Kumar, A., Franssila, R., Ahmed, K., Delwart, E., Vesikari, T., Hedman, K., & Söderlund-Venermo, M. (2016). Epidemiology of two human protoparvoviruses, bufavirus and tusavirus. *Scientific Reports*, *6*(1), 39267. https://doi.org/10.1038/srep39267

Veettil, S. K., Wong, T. Y., Loo, Y. S., Playdon, M. C., Lai, N. M., Giovannucci, E. L., & Chaiyakunapruk, N. (2021). Role of Diet in Colorectal Cancer Incidence: Umbrella Review of Meta-analyses of Prospective Observational Studies. *JAMA Network Open*, *4*(2), e2037341. https://doi.org/10.1001/jamanetworkopen.2020.37341

Watling, C. Z., Kelly, R. K., Dunneram, Y., Knuppel, A., Piernas, C., Schmidt, J. A., Travis, R. C., Key, T. J., & Perez-Cornago, A. (2023). Associations of intakes of total protein, protein from dairy sources, and dietary calcium with risks of colorectal, breast, and prostate cancer: A prospective analysis in UK Biobank. *British Journal of Cancer*, *129*(4), 636–647. https://doi.org/10.1038/s41416-023-02339-2

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014a). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014b). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Yahiro, T., Wangchuk, S., Tshering, K., Bandhari, P., Zangmo, S., Dorji, T., Tshering, K., Matsumoto, T., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2014). Novel Human Bufavirus Genotype 3 in Children with Severe Diarrhea, Bhutan. *Emerging Infectious Diseases*, *20*(6), 1037–1039. https://doi.org/10.3201/eid2006.131430

Zhang, W., Li, L., Deng, X., Kapusinszky, B., Pesavento, P. A., & Delwart, E. (2014). Faecal virome of cats in an animal shelter. *Journal of General Virology*, *95*(11), 2553–2564. https://doi.org/10.1099/vir.0.069674-0

Zhang, X., Liang, S., Chen, X., Yang, J., Zhou, Y., Du, L., & Li, K. (2023). Red/processed meat consumption and non-cancer-related outcomes in humans: Umbrella review. *British Journal of Nutrition*, *130*(3), 484–494. https://doi.org/10.1017/S0007114522003415

Zhao, L., Rosario, K., Breitbart, M., & Duffy, S. (2019). Eukaryotic Circular Rep-Encoding Single-Stranded DNA (CRESS DNA) Viruses: Ubiquitous Viruses With Small Genomes and a Diverse Host Range. In *Advances in Virus Research* (Vol. 103, pp. 71–133). Elsevier. https://doi.org/10.1016/bs.aivir.2018.10.001

Zheng, L., Yuan, X., Xie, Z., Jin, Y., Gao, H., Song, J., Zhang, R., Xu, Z., Hou, Y., & Duan, Z. (2010). Human bocavirus infection in young children with acute respiratory tract infection in Lanzhou, China. *Journal of Medical Virology*, *82*(2), 282–288. https://doi.org/10.1002/jmv.21689

Zhu, P., Liu, C., Liu, G.-F., Liu, H., Xie, K.-M., Zhang, H.-S., Xu, X., Xiao, J., & Jiang, J.-Z. (2024). Unveiling CRESS DNA Virus Diversity in Oysters by Virome. *Viruses*, *16*(2), 228. https://doi.org/10.3390/v16020228

Zur Hausen, H. (2009). The search for infectious causes of human cancers: Where and why. *Virology*, *392*(1), 1–10. https://doi.org/10.1016/j.virol.2009.06.001

Zur Hausen, H. (2012). Red meat consumption and cancer: Reasons to suspect involvement of bovine infectious factors in colorectal cancer. *International Journal of Cancer*, *130*(11), 2475–2483. https://doi.org/10.1002/ijc.27413

Zur Hausen, H., Bund, T., & De Villiers, E. (2019). Specific nutritional infections early in life as risk factors for human colon and breast cancers several decades later. *International Journal of Cancer*, *144*(7), 1574–1583. https://doi.org/10.1002/ijc.31882

Zur Hausen, H., Bund, T., & De Villiers, E.-M. (2017). Infectious Agents in Bovine Red Meat and Milk and Their Potential Role in Cancer and Other Chronic Diseases. In E. Hunter & K. Bister (A c. Di), *Viruses, Genes, and Cancer* (Vol. 407, pp. 83–116). Springer International Publishing. https://doi.org/10.1007/82_2017_3

Section 2.1

Investigating the genetic diversity of CRESS DNA viruses in cats identifies a novel feline circovirus and unveils exposure of cats to canine circovirus

Adapted from:

Violetta Iris Vasinioti[a], Francesco Pellegrini[a], Alessio Buonavoglia[a], Paolo Capozza[a], Roberta Cardone[a], Georgia Diakoudi[a], Costantina Desario[a], Cristiana Catella[a], Teresa Vicenza[a], Maria Stella Lucente[a], Barbara Di Martino[b], Michele Camero[a], Gabriella Elia[a], Nicola Decaro[a], Vito Martella[a], Gianvito Lanave[a]

[a]Department of Veterinary Medicine, University of Bari Aldo Moro, S.p. per Casamassima Km 3, 70010, Valenzano, Bari, Italy

[b]Department of Veterinary Medicine, Università degli Studi di Teramo, SP18, 64100 Teramo, Italy.

***Research in Veterinary Science*, 2023, 161, pp. 86–95. doi: 10.1016/j.rvsc.2023.06.011**

Abstract

Circular replication-associated protein (Rep)-encoding single stranded (CRESS) DNA viruses include Circoviruses which have been found in several animal species and in human specimens. Circoviruses are associated with severe disease in pigs and birds and with respiratory and gastrointestinal disorders and systemic disease in dogs. In cats there are only a few anecdotical studies reporting CRESS DNA viruses. In this study, a total of 530 samples (361 sera, 131 stools, and 38 respiratory swabs) from cats, were screened for the presence of CRESS DNA viruses. Overall, 48 (9.0%) of 530 samples tested positive using a pan-Rep PCR. A total of 30 Rep sequences were obtained. Ten sequences of fecal origin were tightly related to each other (82.4–100% nt identity) and more distantly related to mongoose circoviruses (68.3 to 77.2% nt identity). At genome level these circoviruses dis-played the highest nt identity (74.3–78.7%) to mongoose circoviruses thus representing a novel circovirus species.

Circoviruses from different animal hosts (n = 12) and from humans (n = 8) were also identified. However, siX Rep sequences were obtained from serum samples, including canine circoviruses, a human cyclovirus and human and fish-associated CRESS DNA viruses. The presence of these viruses in the sera would imply, to various extent, virus replication in the animal host, able to sustain viremia.

Overall, these findings indicate a wide genetic diversity of CRESS DNA viruses in cats and warrant further investigations.

Keywords: Circovirus, CRESS DNA virus, Cats, Enteric samples, Sera

1. Introduction

Circular replication-associated protein (Rep)-encoding single- stranded (CRESS) DNA viruses include a group of viruses that possess circular ssDNA genomes and encode an enzyme responsible for initiating genome replication (Krupovic et al., 2020). The replication mechanism of these viruses, named rolling circle replication, is based on the conserved replicate initiation protein Rep. CRESS DNA viruses form the large phylum of *Cressdnaviricota*, are further divided into two classes, *Repensiviricetes* and *Arfiviricetes*, comprising eight virus families. Classification of CRESS DNA viruses is being continuously challenged by the discovery of a large number of diverse genome sequences in the biosphere (Kazlauskas et al., 2018, 2019). Recently, three novel viral families *Naryaviridae*, *Nenyaviridae*, and *Vilyaviridae*, associated with protozoan parasites have been established (Krupovic and Varsani, 2022).

Members of the family *Circoviridae* (circoviruses), class *Arfiviricetes*, includes nonenveloped, spherical viruses with a circular, covalently closed DNA genome ranging from 1.7 to 2.1 kb in size. The ambisense genomic organization includes two major open reading frames (ORFs) that encode the Rep and the capsid (Cap) proteins. The family *Circoviridae* includes the genera *Circovirus* and *Cyclovirus* which encompass 49 and 52 recognized species (www.ictv.global/report/circoviridae), respectively. Members of the genus *Circovirus* (CV) have been identified in various mammals, birds, and fishes. Members of the genus *Cyclovirus* (CyV) have been detected in samples from both vertebrates and invertebrates (Rosario et al., 2017).

CVs have been associated with important diseases, including post- weaning multisystemic wasting syndrome (PMWS) of pigs (Baekbo et al., 2012) and the Beak and Feather Disease (PBFD) of psittacine birds (Fogell et al., 2016).

CRESS DNA viruses have been found to be common component of human fecal virome. CyV were first discovered in a 2010 metagenomics study, in fecal samples of children from different countries, in the stools of chimpanzees and in the meat of farm animals (Li et al., 2010). Since then, human-associated CyVs have been detected in cerebrospinal fluid (CSF) (Tan et al., 2013), blood serum (Smits et al., 2013) and respiratory secretions (Phan et al., 2014) of patients with neurological disease, lower respiratory tract infections, gastrointestinal disorders but also from healthy individuals. In 2023 a novel CV has been identified on metaviromic analysis of a liver biopsy of a woman with chronic hepatitis in France. The virus established a chronic infection replicating in hepatocytes and the viral titer increased over time (Perot et al., 2023).

Information on the epidemiology of CRESS DNA viruses in cats and other felids is still limited. In 2014, a feline CyV was identified in a metagenomic study on pooled fecal samples collected from 25 clinically normal cats housed in a shelter in Davis, California, USA (Zhang et al., 2014). The complete genome of the feline CyV strain FD, shared 39–47% Rep sequence identity with previous reported CyVs. In 2018, a novel feline stool-associated CRESS DNA virus (FeSCV), was retrieved from 4 cats with enteritis during a multi-pathogen outbreak in Japan. Fourteen out of 20 cats housed together developed diarrhea and coronavirus and bocaparvovirus were also detected in the outbreak (Takano et al., 2018). Upon genome sequencing and phylogenetic analysis, FeSCV appeared as a novel CRESS DNA virus unrelated to CyV FD and subsequently classified into the family Vilyaviridae (Krupovic and Varsani, 2022).

CRESS DNA viruses have also been reported in metagenomics studies in large felids, bobcats (*Lynx rufus*) and pumas (*Puma concolor*). In total, three novel CV candidate species within the genus *Circovirus* and two different novel candidate CyV species within the genus *Cyclovirus*, were identified from scat samples (Payne et al., 2020; Cerna et al., 2023).

In this study, in order to expand the knowledge on CRESS-DNA in cats and fill in the existing information gaps, we screened collections of samples obtained from cats, including oronasal swabs, rectal swabs and serum samples, using a consensus PCR assay able to identify members of the *Circoviridae* family (Li et al., 2010).

2. Materials and methods

2.1 Origin of samples

A total of 530 archival sera, enteric (stool and rectal swabs), and respiratory (nasal and oropharyngeal swabs) samples obtained from cats, collected during 2011–2021 in accordance with the animal ethics standards of the University of Bari, Italy, were screened for circoviruses. More in detail, the collection included 38 respiratory samples (collection A), 361 sera samples (collection B) and 131 enteric samples (collection C). Collection A included nasal and oropharyngeal swab samples only from cats with respiratory signs, collected in Apulia region, Southern Italy, by veterinary practitioners of different clinics. Collections B included sera from cats collected from two different veterinary clinic laboratories located in Apulia region and tested upon request of the veterinarian practitioners after anamnesis, medical history and clinical examination. About 40% of the sera had been collected for diagnosis of infectious diseases (feline immunodeficiency virus and feline leukemia virus, feline coronavirus, toxoplasmosis, hemoplasmosis, bacterial and fungal infections) whilst the rest had been submitted to the laboratory for pre-surgical evaluation, for suspected metabolic or neoplastic dis- eases. Collection C included feces or rectal swabs collected from cats of various age with enteric signs by veterinary practitioners from different clinics in Apulia region. Additional sample collection (collection D) included 65 fecal samples from cats with enteritis in Abruzzi region, Central Italy in 2020–2021.

2.2 DNA extraction and screening for CRESS DNA virus

All samples from collections A, C, D were homogenized using 10% w/ v Dulbecco's modified Eagle's medium (DMEM) and then centrifuged at 10,000 Xg for 3 min to collect the supernatant, whilst the serum samples (collection B) were not diluted. Nucleic acids were extracted from 200µL of each sample using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) according to the manufacturer's protocol and stored in 80 ◦C until use.

All samples were screened for circoviruses with a pan-Rep PCR protocol based on a broadly reactive set of primers designed to recognize members of the *Circoviridae* family (Table 1) (Li et al., 2010). Both first- and second-round PCR protocols were performed using Platinum II Hot-Start Green PCR Master Mix (2) (Invitrogen, ThermoFisher Scientific) and the cycling conditions consisted of activation of the Hot-Start polymerase at 94 ◦C for 2 min, followed by 35 cycles of denaturation at 94 ◦C for 15 s, annealing at 52 ◦C for 15 s and extension at 68 ◦C for 15 s. One microliter of the PCR products of the first-round PCR diluted 1:100 in DEPC water was used as a template in the second-round PCR. PCR amplicons were purified and sent for direct sequencing to Eurofins Ge- nomics laboratories (Germany). Sequences of approximately 400 nucleotides (nt) were obtained and analyzed using the web-based tool FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/, accessed on 8th February 2022) (Madeira et al., 2022), using the default values to find homologous hits.

2.3 Quantitative PCR for feline CV

A quantitative real time PCR (qPCR) was designed based on the Rep sequence data obtained with the consensus pan-Rep PCR. Aligned Rep sequences generated from 10 cats were used to design primers and probe (Table 1). A total of 10 µL of sample DNA were added to the 15-µL reaction master miX (IQ SupermiX; Bio-Rad Laboratories SRL, Segrate, Italy) containing 0.6 µmol/L of each primer and 0.2 µmol/L of probe. Thermal cycling consisted of activation of iTaq DNA polymerase at 95 ◦C for 3 min, 44 cycles of denaturation at 95 ◦C for 10 s, and annealing extension at 60 ◦C for 1 min. The specificity of the assay was assessed with a panel of DNA viruses including feline panleukopenia virus (FPV), feline bufavirus, feline herpesvirus, feline-origin poxvirus. Also, a panel of circovirus-positive samples identified and characterized upon sequence analysis in this study (Table 2) was tested to rule out cross- reactivity of the primers/probe with other CRESS-DNA viruses.

2.4 Genome amplification

All the PCR products that were considered positive after gel electrophoresis were subjected to direct sequencing. The circular DNA in selected samples was enriched by multiply primed rolling cycle amplification (RCA), using the bacteriophage phi29 DNA polymerase (TempliPhi 100 amplification kit, Cytiva) and pan-Rep reverse primer CV-R1(5′-AWCCAICCRTARAARTCRTC-3′) (Table 1), according to the manufacturer's instructions (Johne et al., 2009; Wu et al., 2006). Several sets of primers were designed to recover the circular genome from different hosts using an inverse (back-to-back) PCR strategy, amplifying a fragment of approximately 1.5-2 kb. The primers were designed within the Rep gene sequence obtained with the pan-Rep PCR. The inverse PCR assays were performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe S.A.S. Saint-Germain-en-Laye, France). The thermal protocol of the first-round PCR included a first step at 94 ◦C X 2 min, followed by 35 cycles of 94 ◦C X 30 s, 60 ◦C X 30 s, and 68 ◦C X 3 min, with a final extension of 68 ◦C X 10 min. One microliter of a 1:100 dilution of the first-round PCR product was used in the second-round amplification, using outer primers. All PCR-positive products were sequenced directly (Sanger sequencing) at an external facility (Eurofins Genomics GmbH, Ebersberg, Germany) using a primer walking strategy. The list of primers used in this study is provided in Table 1.

2.5 Sequence and phylogenetic analyses

The purified PCR products with sufficient DNA concentration (> 10 ng/µL) were directly sequenced in both directions by Eurofins Genomics (Ebersberg, Germany). The obtained sequences were aligned with cognate CRESS DNA virus strains retrieved from the GenBank database by MAFFT algorithm (Katoh, 2002). The appropriate substitution model settings for the phylogenetic analysis and estimation of selection pressure on coding sequences were derived using "Find the best protein DNA/Protein Models" implemented in MEGA X version 10.0.5 software (Kumar et al., 2018). The evolutionary history was inferred by using the maximum-likelihood method, General-time reversible 6-parameter model, a discrete gamma distribution and invariant sites to model evolutionary rate differences among sites (6 categories) and supplying statistical support with 1000 replicates. Bayesian inference and neighbor joining phylogenetic approaches were also explored and the comparison of the phylogenetic outputs revealed similar topologies with slight differences in bootstrap values at the nodes of the tree. Accordingly, the maximum-likelihood tree was retained. Sequence editing, alignments and phylogenetic analyses were performed by Geneious Prime version 2021.2 (Biomatters, Auckland, New Zealand).

3. Results

3.1 Screening for DNA virus

Overall, a total of 48 (9.0%) out of 530 feline samples tested positive in the two-round pan-Rep PCR. In detail, circovirus DNA was detected in 10/361 (2.8%) sera of collection B, 32/131 (24.4%) enteric samples of collection C and 6/38 (15.8%) respiratory samples of collection A. Positive samples with a DNA concentration exceeding 10 ng/µL were subjected to direct sequencing, yielding 30 (63.8%) sequences of good quality. Sequence characterization by FASTA nucleotide online tool (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) (Madeira et al., 2022) revealed a high heterogeneity of Rep sequences, related to circoviruses found in different host species (Supplementary Table 1). A large group of sequences (n = 10) were tightly related to each other (82.4–100% nt identity) and resembled viruses detected in mongoose (68.3 to 77.2% nucleotide identity). This group of viruses was herein referred to as cat-associated mongoose-like (CAML) CVs. A spare sequence (n = 1) was similar to a bat CV. Sequences similar to CRESS DNA viruses detected in bird (n = 3), in fish (n = 2), in pig (Porcine CV 3, PCV3) (n = 2), in dog (n = 3) and in a snail (n = 1) were also identified. Interestingly, we also identified eight sequences similar to human- associated CV NG1 (n 1) and human-associated CyVs TN10 (n 2), TN12 (n 2), and PK2111 (n 3). CRESS DNA viruses other than circoviruses were not detected in the screening but in three samples, PK2111-like (Supplementary Table 1). A summary of the sequencing results is provided in Fig. 1 and Supplementary Table 1.

3.2 Screening for CAML-CVs by qPCR

The qPCR for CAML-CV was specific, since it did not recognize other feline/canine DNA viruses and also, none of the other samples positive in the pan-Rep PCR that were sequenced and characterized in our collection (Table 2). Only CAML-CVs were successfully recognized by the qPCR, with the cycle threshold (CT) ranging from 29.4 to 40.3. Using the qPCR, fecal samples from Central Italy (collection D) were also screened to assess the presence of these viruses in other geographical areas. CAML-CV was detected in 3/65 samples (4.6%) with the CT ranging from 33.4 to 39.5.

3.3 Genome amplification and sequence analysis

The circular DNA of selected samples was enriched by rolling cycle amplification (RCA) protocols. Subsequently, an inverse two-round PCR strategy with primers designed to amplify the rest of the circular genome was performed on the RCA products. The complete genome was generated successfully for 3 CAML-CVs (ITA/2019/cat/471.3, ITA/ 2019/cat/471.4 and ITA/2019/cat/471.12), for a Pigeon-like CV (ITA/ 2019/cat/518.1a) and for 3 Canine-like CVs (strains ITA/2021/cat/ 99.95, ITA/2021/cat/230.1 and ITA/2021/cat/230.3).

The genome size of the six Italian CV strains identified in this study were 1746, 2043 and 2063–2066 nt, in CAML-CVs, Pigeon-like CV and Canine-like CVs, respectively (Table 2). The genome features of the identified CVs included two major open reading frames (ORFs), located on complementary strands in opposite orientation. In the CAML-CV strains, the ORF1 (849 nt), located on the virion strand, and the ORF2 (624 nt), located on the complementary strand of the replicative form, encoded for the Rep (282 aa) and Cap (207 aa) proteins, respectively. In the Pigeon-like CV strain, the ORF1 (948 nt), located on the virion strand, and the ORF2 (834 nt), located on the complementary strand of the replicative form, encoded for the Rep (315 aa) and Cap (277 aa) proteins, respectively. In the Canine-like CV strains, the ORF1 (912 nt), located on the virion strand, and the ORF2 (813–816 nt), located on the complementary strand of the replicative form, encoded for the Rep (303 aa) and Cap (270–271 aa) proteins, respectively (Fig. 2, Table 2).

Similar to other CVs, the genome of the Italian CVs comprised two intergenic non-coding regions which were located between the start and stop codons of the replicase and capsid protein genes, respectively. The 5′ and 3′ intergenic regions were 190 and 83 nt in length in CAML-CV strains, 89 and 172 nt in length in Pigeon-like CV strain and 135 and 203 nt in length in Canine-like CV strains. The 5′-intergenic regions of CVs identified in the study contained a thermodynamically stable stem-loop, which regulates the initiation of rolling-circle replication, and the conserved mononucleotide motifs CAGTATTAC in CAML-CV strains and TAGTATTAC in Pigeon-like CV and Canine-like CV strains (Fig. 2, Table 2).

CAML-CVs displayed consistently the highest % nt identities (74.3–78.7%) to mongoose CV strain Mon-1 (MZ382570). Pigeon-like CV shared the highest % nt identity (92.9%) to Pigeon CV isolate Hebei/TS/2021 (OL901206). Canine-like CV strains ITA/2021/cat/ 230.1 and ITA/2021/cat/230.3 displayed the highest % nt identities (97.8–99%) to CanineCV strain Bari/411–13 (KJ530972) whilst strain ITA/2021/cat/99.95 displayed the highest % nt identity (97.3%) to CanineCV strain Ha13 (KF887949) (Table 2).

3.4 Phylogenetic analyses

A phylogenetic tree was constructed based on the full-length genome of CVs identified in this study along with CRESS DNA virus sequences retrieved from Genbank (Fig. 3).

The Italian CV strains were distinguishable in three different clades. CAML-CV strains ITA/2019/cat/471.3, ITA/2019/cat/471.4 and ITA/ 2019/cat/471.12 tightly clustered with CV strains Mon-1 and Mon-29 identified in 2017 from mongoose in Saint Kitts and Nevis (Gainor et al., 2021). CAML-CV strains clustered in the same clade with Calfel virus LSF45 CV 359 (ON596197), retrieved from a bobcat in California in 2011 (Cerna et al., 2023) although sharing a low % nt identity (47.6–48.4%). Pigeon CV strain ITA/2019/cat/518.1a clustered with other Pigeon CVs identified in China in 2018 and 2021 (Wang et al., 2022) with which the Italian strain shared 90.8–91% nt identity. CanineCV strains ITA/2021/cat/99.95, ITA/2021/cat/230.1 and ITA/2021/ cat/230.3 clustered in a same clade with two Italian CV strains Bari/ 411–13 (Decaro et al., 2014) and AZ663/1–13 (Zaccaria et al., 2016) and strain UCD1–1698 retrieved in USA (Li et al., 2013).

Also, a phylogenetic tree was generated on the full-length genome of Canine-like CV strains identified in this study together with cognate strains retrieved from GenBank (Fig. 4). The unrooted phylogenetic tree showed a well-distinguishable clustering of the CanineCV strains into six groups. The CanineCV strains identified in this study fell into clade 1 along with other European, Asian, and American CanineCV strains (Fig. 4) although segregating within two distinct sub-lineages.

4. Discussion

In this study, several genetically heterogeneous CRESS DNA viruses were found in cats after screening different collections of samples, i.e., stools, sera and oro-nasal swabs. Good-quality sequences were obtained from fecal samples (n=24) and from the sera (n=6) but not from the respiratory samples. Sequences resembling human-associated CV NG1 (n=1), CyV TN10 (n=2), TN12 (n=2), and an unclassified circular DNA human virus PK2111 (n=2) were identified in fecal samples. Despite several attempts, we could not generate the genome sequence of TN10-, TN12-, PK2111-like and NG1-like viruses identified in the feline samples. Interestingly, similar viruses (TN-9- and TN12-like) have been identified in the intestinal content of lizards and geckos sampled in an Italian study (Capozza et al., 2022). Accordingly, we hypothesized a dietary origin for those viruses and a possible relation with the predatory behavior of cats, since CyVs seem common in insects (Nebbak et al., 2021; Rosario et al., 2011) that are preyed by bats and by small reptiles, that, in turn, are common preys of cats. Yet, it is interesting to observe that similar viruses are present in human, feline and reptile hosts, hinting to a possible zoonotic exposure of humans to synanthropic animals.

Two sequences, retrieved from cat stools, clustered together with PCV3 CVs, indicating the possible association with the dietary consumption of PCV-infected pork or pork-containing cat food. In addition, sequences similar to CVs detected in birds (n=3) and in fish (n=2) may also reflect dietary habits of cats.

Surprisingly, CanineCV-like sequences (n=3) were also identified from feline serum samples. In several studies, it has been shown that the virome of dogs and cats may overlap, to some extent (Di Martino et al., 2016; Martella et al., 2002; Matthijnssens et al., 2011). For instance, canine parvovirus type 2 (CPV-2) originally could not replicate in cats but its variants CPV-2a, 2b and -2c are able to infect cats and cause disease (Truyen and Parrish, 1992). Also, feline panleukopenia virus can infect occasionally dogs (Diakoudi et al., 2022). In our study the CanineCV-like sequences were identified only in serum samples, a fact that might imply the ability of the virus to infect and replicate actively, thus sustaining viremia in cats. For all the 3 cat-associated CanineCV-like strains, the complete genome could be generated (Table 2), showing the highest nt identity (93.8 to 98.0% nt) to CanineCVs of clade 1, although clustering in two different sub-lineages (Fig. 4). Since the sampled cats were epidemiologically unrelated, we hypothesize that dog-to-cat transmission of CanineCV-like strains may occur sporadically but repeatedly in different settings due to the strict interactions between the two animal species, rather than the existence of a feline-adapted strain of CanineCV.

Furthermore, we identified in the sera of cats an additional 3 CRESS DNA viruses, i.e., a human-associated CyV TN12, and unclassified circular viruses identified in fish (Gudgeon strain Z374-like) (unpublished, accession number MN837846), and in human stools of patients with non-polio acute flaccid paralysis (PK2111-like) (Li et al., 2010). Interestingly, both TN12 and PK2111 viruses were also detected in the fecal samples of other cats in this study. We also identified, in our study, Rep sequences related to fish-associated CRESS DNA viruses (Fig. 1 and Supplementary Table 1). This would indicate that several CRESS DNA viruses have the ability to replicate in cats and spread systemically, although the implications of these findings for cat health remain obscure.

A large group of Rep sequences (n=10), obtained exclusively from fecal samples, segregated together and resembled, in the partial Rep sequence, CVs detected in mongoose (68.3–77.2% nt identity) and were therefore defined as CAML-CVs. In this group of CAML viruses 9 sequences shared 95.4–100% nt identity to each other, whilst a spare sequence (ITA/2019/cat/185.2.3) appeared less conserved (82.4–85.9% nt). These findings are of difficult interpretation, since the fecal samples were collected from epidemiologically unrelated cases and different geographical areas. The high sequence conservation among the various strains could imply the spread of a feline-adapted virus between cats, rather than continual/repeated exposure to viruses from other unidentified hosts. We were able to generate the complete genome sequence of 3 CAML strains. Genome-wide identity among the 3 strains was 97.6–98.3% nt, whilst the highest nt identity (74.3–78.7%) in the GenBank database was to the mongoose CV strain Mon-1 (Accession MZ382570) (Gainor et al., 2021) (Table 2), therefore with a sequence identity lower than the threshold established for designation of a novel species in the *Circoviridae* family. Thus far, based on the current ICTV classification criteria a cut-off identity of 80% nt at the genome level is used to distinguish a new species (Breitbart et al., 2017). Accordingly, we propose the CAML-CV as cat-associated circovirus-1 or, more appropriately, as feline circovirus-1 and we suggest that the virus is a novel candidate species in the *Circovirus* genus.

Interestingly, in our investigation we did not identify Rep sequences related to other CRESS DNA viruses, CyV strain FD and FeSCV, reported from the stools of cats in USA (Zhang et al., 2014) and Japan (Takano et al., 2018), respectively. Whilst CanineCV has been reported repeatedly in dogs and from several countries (Beikpour et al., 2022), viruses similar to CyV strain FD have not been confirmed/reported again after their initial discovery. However, FeSCV-like viruses have been identified in the stools of 2/10 domestic cats with diarrhea in China using a specific PCR (Hao et al., 2021). All in all, it is unclear if these were just anecdotal findings due to dietary contaminations, to occasional exposure from other animal sources, or if the viruses were able to replicate actively in the feline host. In the attempt to understand better the relevance of CAML-CVs, we therefore screened convenience collections of stool samples of cats from a distinct geographical location (collection D) using a specific qPCR. CAML-CVs were detected in 3/65 cats from Abruzzi (Italy), thus confirming that CAML-CVs are common components of feline virome and paving the way for larger structured epidemiological studies.

A major limit of our investigation was the missing history and metadata for several of the tested samples, as we mostly tested archival samples available in our laboratories. Also, this investigation was not conceived as a case/control study, so we could not infer any association with clinical signs and the viruses detected. Another limit relies on the broadly reactive primers of the pan-Rep PCR assay. The degenerated/ consensus primers were designed in 2010 on the consensus sequence from an alignment of Rep proteins from 13 representative members of the *Circoviridae* family on the basis of the sequence data available in the databases (Li et al., 2010). Therefore, the pan-Rep PCR assay could fail to detect other members of the *Circoviridae* family or could mis-detect CRESS DNA viruses of sister taxa. Metagenomic investigations using multiple strain displacement (MDA) protocol with phi29 DNA polymerase have been successfully applied for circular DNA viruses and could be used to investigate CRESS DNA viruses in different samples with an unbiased approach (Roux et al., 2016). However, consensus PCRs are more suitable for application in large-scale epidemiological investigations in terms of costs and samples processivity. The size of our study, for instance, was on average 28-fold larger, in terms of number of screened samples, than previous studies carried out in cats and large felids (Cerna et al., 2023; Payne et al., 2020; Takano et al., 2018; Zhang et al., 2014).

In conclusion, by screening nearly six hundred samples from cats, we identified a variety of CRESS DNA viruses either closely or distantly related to circular DNA viruses detected from different animal hosts, including humans. A group of CVs, termed CAML after their resemblance with mongoose CVs, was repeatedly identified in the fecal samples of cats from different geographical areas suggesting that cats could be a primary host rather than occasionally infected or exposed to this viral species and the CAML viruses were proposed as a novel species, feline CV-1 on the basis of the full-length genome sequence. Virus surveillance in domestic animals should be reinforced, in order to monitor and promptly characterize emerging and re-emerging zoonotic viruses, and to provide a baseline of virus diversity, useful for tackling future infectious emergencies. Also, the identification of animal CRESS DNA viruses, closely related to human viruses, provides useful information about their origin and ecology.
Yet, the existing knowledge regarding other circoviruses and CRESS DNA viruses that infect carnivores remains still limited.

Virus surveillance in both wild and domestic carnivores, particularly dogs and cats that live in proximity to humans should be enhanced to establish an effective monitoring framework for virus characterization and diversity that could be crucial in addressing future infectious emergencies.

Additionally, identifying animal CRESS DNA viruses and exploring their genetic similarities to human viruses offers valuable insight into their origins and ecological dynamics.

Funding

This research was supported by EU funding within the MUR PNRR EXtended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT)".

CRediT authorship contribution statement

Violetta Iris Vasinioti: Investigation, Data curation, Writing – original draft. Francesco Pellegrini: Software, Formal analysis, Investigation, Visualization. Alessio Buonavoglia: Data curation. Paolo Capozza: Resources, Data curation, Visualization. Roberta Cardone: Investigation. Georgia Diakoudi: Investigation. Costantina Desario: Investigation. Cristiana Catella: Investigation. Teresa Vicenza: Resources. Maria Stella Lucente: Investigation. Barbara Di Martino: Validation. Michele Camero: Methodology, Writing – review & editing. Gabriella Elia: Conceptualization, Supervision. Nicola Decaro: Methodology, Supervision, Project administration. Vito Martella: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. Gianvito Lanave: Methodology, Software, Data curation, Writing – review & editing, Visualization.

Declaration of Competing Interest

We declare that we have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Nucleotide sequences of cat associated CV-1 strains ITA/2019/cat/ 471.3, ITA/2019/cat/471.4 and ITA/2019/cat/471.12, cat-associated Pigeon CV strain ITA/2019/cat/518.1a and cat-associated CanineCV strains ITA/2021/cat/99.95, ITA/2021/cat/230.1 and ITA/2021/cat/ 230.3 retrieved in this study used for phylogeny were deposited in GenBank under accession nos OQ357577-OQ357583, respectively. The small Rep sequence fragments (about 350–450 nt in length) are available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online https://doi.org/10.1016/j.rvsc.2023.06.011.

Tables

Table 1: List of oligonucleotides used in this study.

Pathogen	Assay	Primers	Sequence (5’ - 3’)	Amplification size (bp)	Reference
CV	Pan-Rep PCR	CV-F1	GGIAYICCICAYYTICA RGG	500	Li et al., 2010
		CV-R1	AWCCAICCRTARAARTCRTC		
	nPCR	CV-F2	GGIAYICCICAYYTICARGGITT	400	
		CV-R2	TGYTGYTCRTAICCRTCCCACCA		
Canine-like CV	Inverse PCR	CaCV 273R	HCCCCIAGCAGGCTCAAAITGKCC	>1500	Beikpour et al., 2022
		CaCV 446F	WCTCGCGAGGSTTGCGAGASCT		
	nPCR	CaCV 165R	YTCCCCIACCTCCCGRCCACARAT		
		CaCV 548F	GCAAGAGCCGGTAYTGCATGGA		
Pigeon-like CV	Inverse PCR	PiCV 178R	TCATTGCTCTTCCGGCTTTCAC	>1500	This study
		PiCV 205F	ATCTACGTCAAGTATGGGCGTGGC		
	nPCR	PiCV 131R	CTCCGGTTTCCCTTCGCAGGAATG		
		PiCV 248F	YGATTGGTCAGMAGCCYCGTGACT		
Mongoose-like CV	Inverse PCR	MoCV 192R	ACGCATAAAGCCGGGTAATTTCT	>1500	This study
		MoCV 627F	ATTGAGAGTTTGTGATAGGTATCCCC		
	nPCR	MoCV 137R	TCAATGAATCCTTGCAAGTGGGT		
		MoCV 666F	TAAGGGCGCTTTTGTGGAATTTG		
	qRT-PCR	MoCV 81F	TCAGAAGGAGAATCAGTACCTGGA	187	This study
		MoCV 267R	CAAGCCAGCAACACAAACATAGTC		
		MoCV 135Pb	FAM-CTCAGATTTGAGTGATGCGGTGG-BHQ1		

Circovirus, member of the family Circoviridae; CV, member of the genus Circovirus; Rep, replicase protein; nPCR, nested PCR; qPCR, quantitative real time PCR.

Table 2: Genomic features of complete genomes of circoviruses sequenced in this study.

Species	Sample ID	Accession	Size (nt)	Putative Rep		Putative Cap		5' intergenic region (nt)	3' intergenic region (nt)	Loop motif (5'-3')	Identity to reference sequences	
				nt	aa	nt	aa				CV strain* (accession nr.)	nt identity %
Feline Circovirus 1 sp. [isolate 1]	ITA/2019/cat/471.3	OQ357577	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	74.3
Feline Circovirus 1 sp. [isolate 2]	ITA/2019/cat/471.4	OQ357578	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	78.7
Feline Circovirus 1 sp. [isolate 3]	ITA/2019/cat/471.12	OQ357579	1746	849	282	624	207	190	83	CAGTATTAC	mongoose circovirus strain Mon-1 (MZ382570)	78.0
Pigeon Circovirus [cat-associated isolate 1]	ITA/2019/cat/518.1a	OQ357580	2043	948	315	834	277	89	172	TAGTATTAC	Pigeon circovirus isolate Hebei/TS/2021 (OL901206)	92.9
Canine Circovirus [cat-associated isolate 1]	ITA/2021/cat/99.95	OQ357581	2066	912	303	816	271	135	203	TAGTATTAC	Canine circovirus strain Ha13 (KF887949)	97.3
Canine Circovirus [cat-associated isolate 2]	ITA/2021/cat/230.1	OQ357582	2063	912	303	813	270	135	203	TAGTATTAC	Canine circovirus strain Bari/411-13 (KJ530972)	99.0
Canine Circovirus [cat-associated isolate 3]	ITA/2021/cat/230.3	OQ357583	2063	912	303	813	270	135	203	TAGTATTAC	Canine circovirus strain Bari/411-13 (KJ530972)	97.8

nt, nucleotides; aa, amminoacids; CV, member of the genus *Circovirus*; * genome sequence with the highest identity on interrogation of European BioinformaticsInstitute database with FASTA (Madeira et al., 2022).

Figures

Fig 1: Results of genetic characterization of the CRESS-DNA viruses identified from cats based on partial Rep sequences.

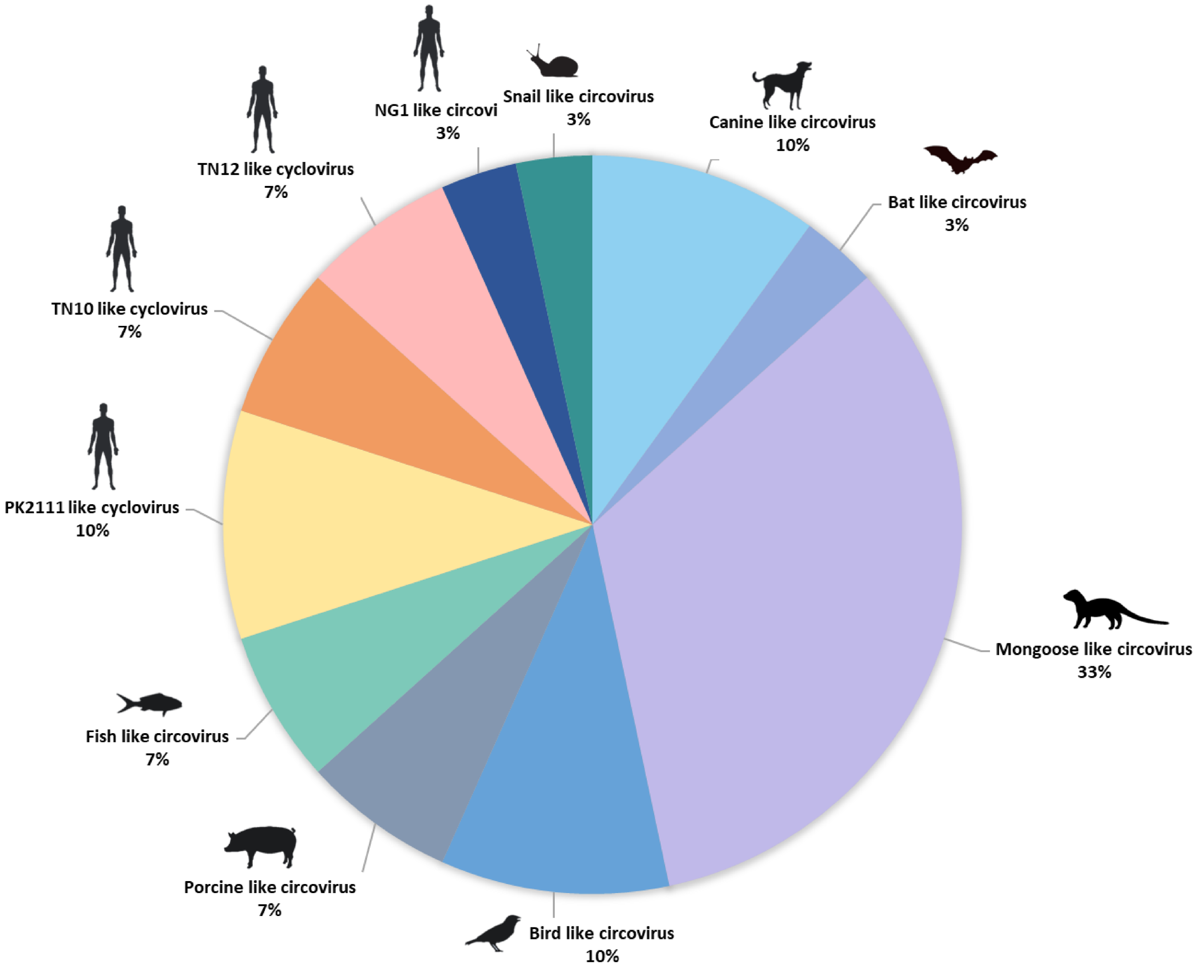
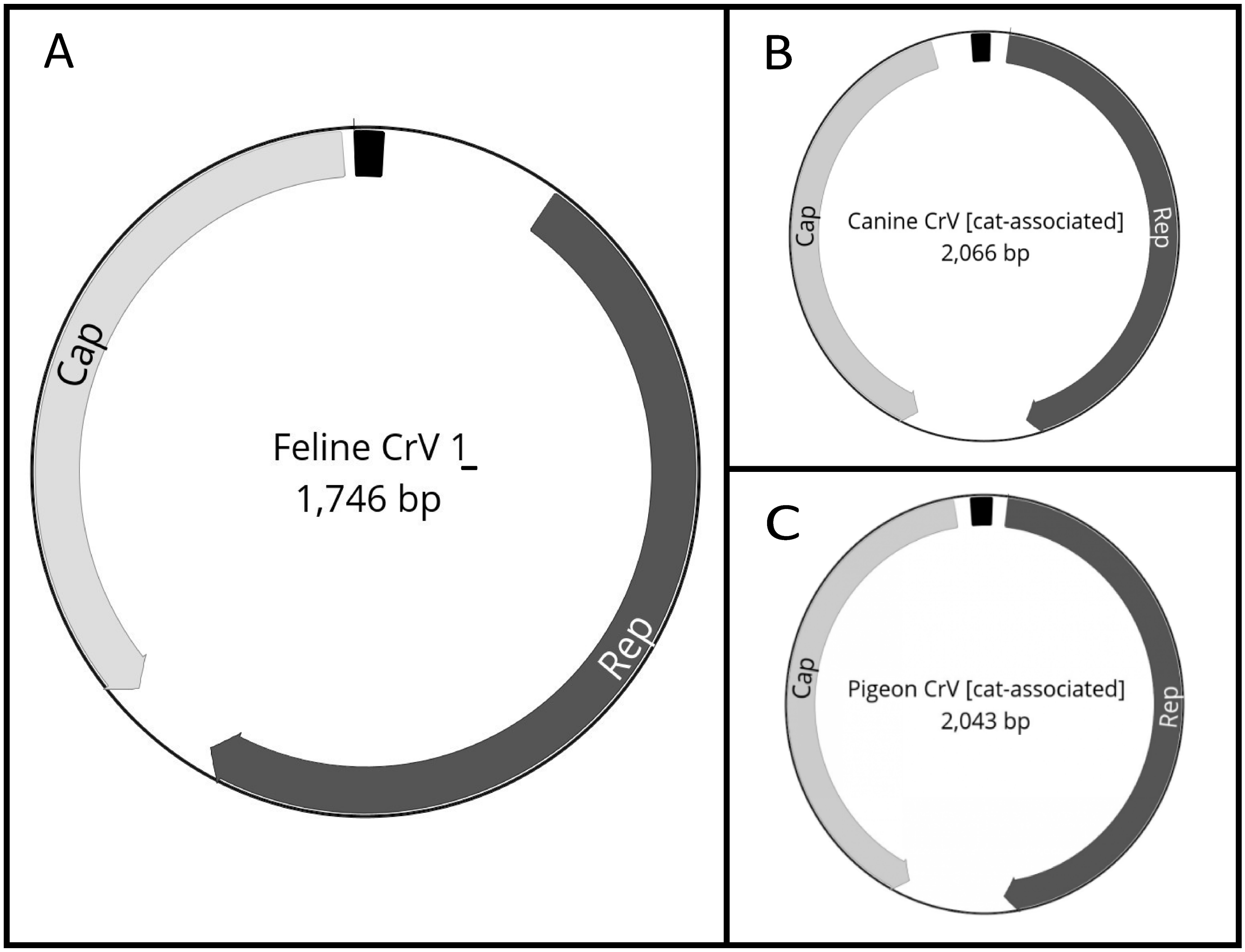


Fig 2: Genome schematic organization of the feline circoviruses sequenced in this study. The feline Circovirus-1 is shown in panel A, the cat-associated pigeon circovirus is shown in panel B and the cat-associated canine circovirus strain is shown in panel C.



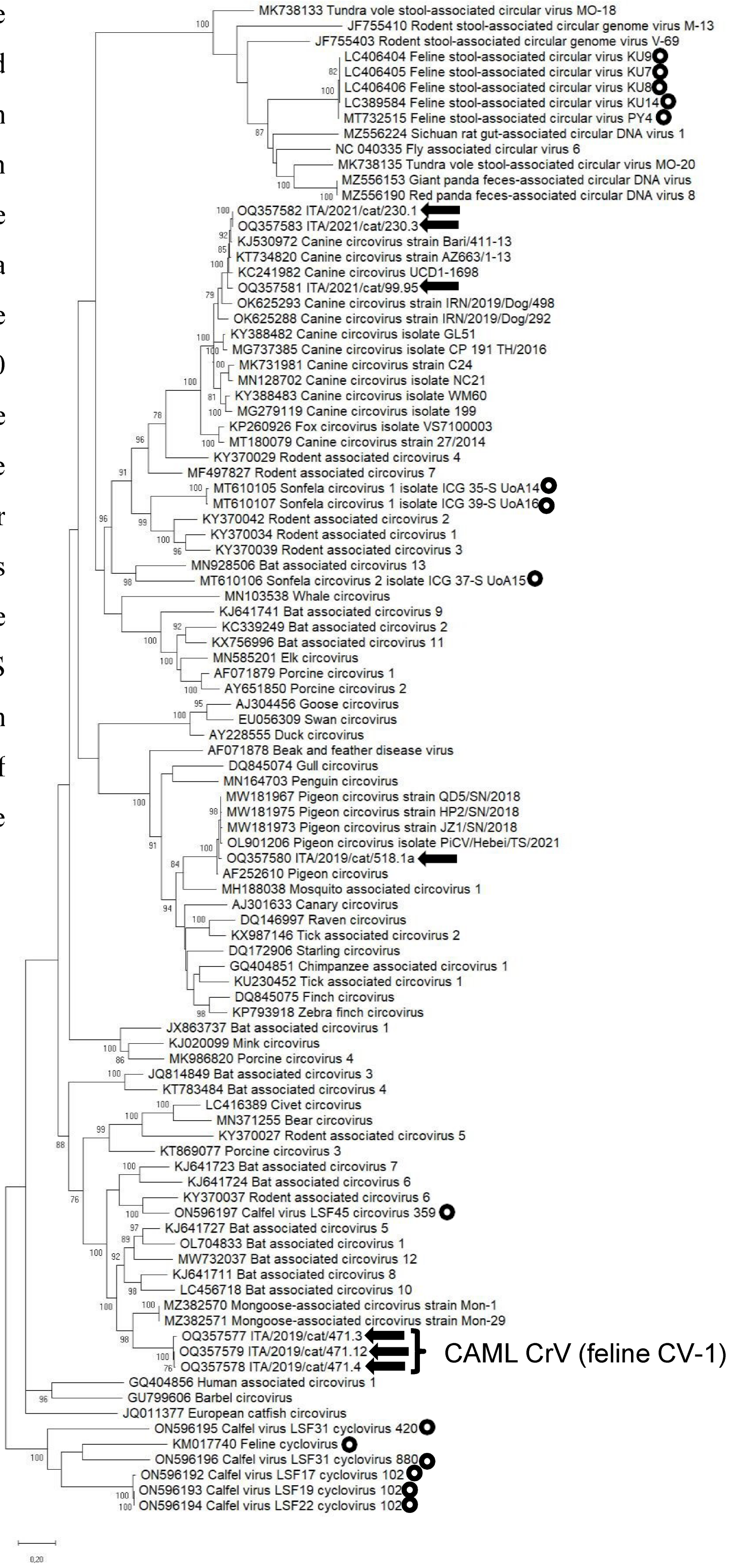


Fig 3: Full-genome-based phylogenetic tree of circoviruses identified in this study and reference strains of the Order *Cirlivirales* in the phylum *Cressdnaviricota*. The Maximum Likelihood method and General-time reversible model (six parameters) with a gamma distribution and invariable sites were used for the phylogeny. A total of 1000 bootstrap replicates were used to estimate the robustness of the individual nodes on the phylogenetic tree. Bootstrap values greater than 75% were indicated. Black arrows indicate strains detected in this study. White circles with black border indicate the CRESS DNA viruses previously identified in domestic and wild felids. Numbers of nucleotide substitutions are indicated by the scale bar.

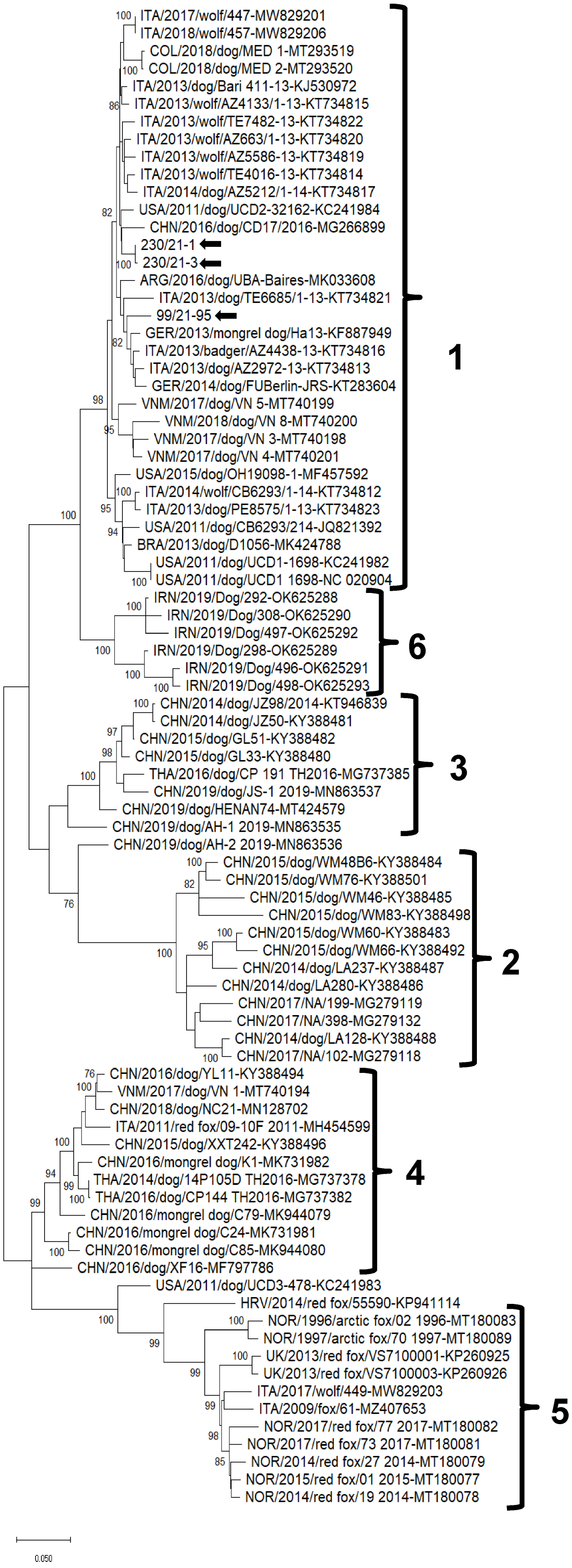


Fig 4: Full-genome-based unrooted phylogenetic tree of Canine circoviruses identified in this study and cognate strains of different genetic lineages recovered in the GenBank database. Numbers 1 to 6 indicate the phylogenetic clade (Beikpour et al., 2022; Urbani et al., 2021). The Maximum Likelihood method and General-time reversible model (six parameters) with a gamma distribution and invariable sites were used for the phylogeny. A total of 1000 bootstrap replicates were used to estimate the robustness of the individual nodes on the phylogenetic tree. Bootstrap values greater than 75% were indicated. Black arrows indicate Canine circovirus strains detected in this study. Numbers of nucleotide substitutions are indicated by the scale bar.

References

Baekbo, P., Kristensen, C.S., Larsen, L.E., 2012. Porcine Circovirus Diseases: A review of PMWS: Porcine Circovirus Diseases. Transboundary and Emerging Diseases, 59, 60–67. https://doi.org/10.1111/j.1865-1682.2011.01288.x

Beikpour, F., Ndiana, L.A., Sazmand, A., Capozza, P., Nemati, F., Pellegrini, F., Zafari, S., Zolhavarieh, S.M., Cardone, R., Faraji, R., Lanave, G., Martella, V., Decaro, N., 2022. Detection and Genomic Characterization of Canine Circovirus in Iran. Animals, 12(4), 507. https://doi.org/10.3390/ani12040507

Breitbart, M., Delwart, E., Rosario, K., Segalés, J., Varsani, A., ICTV Report Consortium, 2017. ICTV Virus Taxonomy Profile: Circoviridae. Journal of General Virology, 98(8), 1997–1998. https://doi.org/10.1099/jgv.0.000871

Capozza, P., Lanave, G., Diakoudi, G., Pellegrini, F., Cardone, R., Vasinioti, V. I., Decaro, N., Elia, G., Catella, C., Alberti, A., Bányai, K., Mendoza-Roldan, J.A., Otranto, D., Buonavoglia, C., Martella, V., 2022. Diversity of CRESS DNA Viruses in Squamates Recapitulates Hosts Dietary and Environmental Sources of Exposure. Microbiology Spectrum, 10(3), e00780-22. https://doi.org/10.1128/spectrum.00780-22

Cerna, G. M., Serieys, L.E.K., Riley, S.P.D., Richet, C., Kraberger, S., Varsani, A., 2023. A circovirus and cycloviruses identified in feces of bobcats (Lynx rufus) in California. Archives of Virology, 168(1), 23. https://doi.org/10.1007/s00705-022-05656-8

Decaro, N., Martella, V., Desario, C., Lanave, G., Circella, E., Cavalli, A., Elia, G., Camero, M., Buonavoglia, C., 2014. Genomic Characterization of a Circovirus Associated with Fatal Hemorrhagic Enteritis in Dog, Italy. PLoS ONE, 9(8), e105909. https://doi.org/10.1371/journal.pone.0105909

Di Martino, B., Di Profio, F., Melegari, I., Sarchese, V., Cafiero, M.A., Robetto, S., Aste, G., Lanave, G., Marsilio, F., Martella, V., 2016. A novel feline norovirus in diarrheic cats. Infection, Genetics and Evolution, 38, 132–137. https://doi.org/10.1016/j.meegid.2015.12.019

Diakoudi, G., Desario, C., Lanave, G., Salucci, S., Ndiana, L.A., Zarea, A.A.K., Fouad, E.A., Lorusso, A., Alfano, F., Cavalli, A., Buonavoglia, C., Martella, V., Decaro, N., 2022. Feline Panleukopenia Virus in Dogs from Italy and Egypt. Emerging Infectious Diseases, 28(9), 1933–1935. https://doi.org/10.3201/eid2809.220388

Fogell, D.J., Martin, R.O., Groombridge, J.J., 2016. Beak and feather disease virus in wild and captive parrots: An analysis of geographic and taxonomic distribution and methodological trends. Archives of Virology, 161(8), 2059–2074. https://doi.org/10.1007/s00705-016-2871-2

Gainor, K., Becker, A.A.M.J., Malik, Y.S., Ghosh, S., 2021. Detection and Complete Genome Analysis of Circoviruses and Cycloviruses in the Small Indian Mongoose (Urva auropunctata): Identification of Novel Species. Viruses, 13(9), 1700. https://doi.org/10.3390/v13091700

Hao, X., Li, Y., Hu, X., Fu, X., Dong, J., Zhang, H., Zhou, P., Li, S., 2021. Feline Stool-Associated Circular DNA Virus (FeSCV) in Diarrheic Cats in China. Front Vet Sci, 8:694089. https://10.3389/fvets.2021.694089.

Johne, R., Müller, H., Rector, A., van Ranst, M., Stevens, H., 2009. Rolling-circle amplification of viral DNA genomes using phi29 polymerase. Trends in Microbiology, 17(5), 205–211. https://doi.org/10.1016/j.tim.2009.02.004

Katoh, K., 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research, 30(14), 3059–3066. https://doi.org/10.1093/nar/gkf436

Kazlauskas, D., Varsani, A., Koonin, E.V., Krupovic, M., 2019. Multiple origins of prokaryotic and eukaryotic single-stranded DNA viruses from bacterial and archaeal plasmids. Nat Commun, 10(1):3425. https://doi.org/10.1038/s41467-019-11433-0.

Kazlauskas, D., Varsani, A., Krupovic, M., 2018. Pervasive Chimerism in the Replication-Associated Proteins of Uncultured Single-Stranded DNA Viruses. Viruses. 10(4):187. https://doi.org/10.3390/v10040187.

Krupovic, M., Varsani, A., Kazlauskas, D., Breitbart, M., Delwart, E., Rosario, K., Yutin, N., Wolf, Y.I., Harrach, B., Zerbini, F.M., Dolja, V.V., Kuhn, J.H., Koonin, E. V., 2020. Cressdnaviricota: A Virus Phylum Unifying Seven Families of Rep-Encoding Viruses with Single-Stranded, Circular DNA Genomes. Journal of Virology, 94(12), e00582-20. https://doi.org/10.1128/JVI.00582-20

Krupovic, M., Varsani, A., 2022. *Naryaviridae*, *Nenyaviridae*, and *Vilyaviridae*: three new families of single-stranded DNA viruses in the phylum *Cressdnaviricota*. Arch Virol. 167(12):2907-2921. https://doi.org/10.1007/s00705-022-05557-w.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution, 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Li, L., Kapoor, A., Slikas, B., Bamidele, O.S., Wang, C., Shaukat, S., Masroor, M. A., Wilson, M.L., Ndjango, J.-B.N., Peeters, M., Gross-Camp, N.D., Muller, M. N., Hahn, B.H., Wolfe, N.D., Triki, H., Bartkus, J., Zaidi, S.Z., Delwart, E., 2010. Multiple Diverse Circoviruses Infect Farm Animals and Are Commonly Found in Human and Chimpanzee Feces. Journal of Virology, 84(4), 1674–1682. https://doi.org/10.1128/JVI.02109-09

Li, L., McGraw, S., Zhu, K., Leutenegger, C.M., Marks, S.L., Kubiski, S., Gaffney, P., Dela Cruz Jr, F.N., Wang, C., Delwart, E., Pesavento, P.A., 2013. Circovirus in Tissues of Dogs with Vasculitis and Hemorrhage. Emerging Infectious Diseases, 19(4), 534–541. https://doi.org/10.3201/eid1904.121390

Madeira, F., Pearce, M., Tivey, A.R.N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in

2022. Nucleic Acids Research, 50(W1), W276–W279. https://doi.org/10.1093/nar/gkac240

Martella, V., Pratelli, A., Gentile, M., Buonavoglia, D., Decaro, N., Fiorente, P., Buonavoglia, C., 2002. Analysis of the capsid protein gene of a feline-like calicivirus isolated from a dog. Veterinary Microbiology, 85(4), 315–322. https://doi.org/10.1016/S0378-1135(01)00521-1

Matthijnssens, J., De Grazia, S., Piessens, J., Heylen, E., Zeller, M., Giammanco, G.M., Bányai, K., Buonavoglia, C., Ciarlet, M., Martella, V., Van Ranst, M., 2011. Multiple reassortment and interspecies transmission events contribute to the diversity of feline, canine and feline/canine-like human group A rotavirus strains. Infection, Genetics and Evolution, 11(6), 1396–1406. https://doi.org/10.1016/j.meegid.2011.05.007

Nebbak, A., Monteil-Bouchard, S., Berenger, J.-M., Almeras, L., Parola, P., Desnues, C., 2021. Virome Diversity among Mosquito Populations in a Sub-Urban Region of Marseille, France. Viruses, 13(5), 768. https://doi.org/10.3390/v13050768

Payne, N., Kraberger, S., Fontenele, R.S., Schmidlin, K., Bergeman, M.H., Cassaigne, I., Culver, M., Varsani, A., Van Doorslaer, K., 2020. Novel Circoviruses Detected in Feces of Sonoran Felids. Viruses, 12(9), 1027. https://doi.org/10.3390/v12091027

Pérot, P., Fourgeaud, J., Rouzaud, C., Regnault, B., Da Rocha, N., Fontaine, H., Le Pavec, J., Dolidon, S., Garzaro, M., Chrétien, D., Morcrette, G., Molina, T J., Ferroni, A., Leruez-Ville, M., Lortholary, O., Jamet, A., Eloit, M., 2023. Circovirus Hepatitis Infection in Heart-Lung Transplant Patient, France. Emerging Infectious Diseases, 29(2), 286–293. https://doi.org/10.3201/eid2902.221468

Phan, T.G., Luchsinger, V., Avendaño, L.F., Deng, X., Delwart, E., 2014. Cyclovirus in nasopharyngeal aspirates of Chilean children with respiratory infections. Journal of General Virology, 95(4), 922–927. https://doi.org/10.1099/vir.0.061143-0

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., Varsani, A., 2017.

Revisiting the taxonomy of the family Circoviridae: Establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Archives of Virology, 162(5), 1447–1463. https://doi.org/10.1007/s00705-017-3247-y

Rosario, K., Marinov, M., Stainton, D., Kraberger, S., Wiltshire, E.J., Collings, D. A., Walters, M., Martin, D.P., Breitbart, M., Varsani, A., 2011. Dragonfly cyclovirus, a novel single-stranded DNA virus discovered in dragonflies (Odonata: Anisoptera). Journal of General Virology, 92(6), 1302–1308. https://doi.org/10.1099/vir.0.030338-0

Roux, S., Solonenko, N.E., Dang, V.T., Poulos, B.T., Schwenck, S.M., Goldsmith, D.B., Coleman, M.L., Breitbart, M., Sullivan, M.B, 2016. Towards quantitative viromics for both double-stranded and single-stranded DNA viruses. PeerJ, 4, e2777. https://doi.org/10.7717/peerj.2777

Smits, S. L., Zijlstra, E.E., van Hellemond, J.J., Schapendonk, C.M.E., Bodewes, R., Schürch, A.C., Haagmans, B.L., Osterhaus, A.D.M.E., 2013. Novel Cyclovirus in Human Cerebrospinal Fluid, Malawi, 2010–2011. Emerging Infectious Diseases, 19(9). https://doi.org/10.3201/eid1909.130404

Takano, T., Yanai, Y., Hiramatsu, K., Doki, T., Hohdatsu, T., 2018. Novel single-stranded, circular DNA virus identified in cats in Japan. Archives of Virology, 163(12), 3389–3393. https://doi.org/10.1007/s00705-018-4020-6

Tan, L.V., van Doorn, H.R., Nghia, H.D.T., Chau, T.T.H., Tu, L.T.P., de Vries, M., Canuti, M., Deijs, M., Jebbink, M.F., Baker, S., Bryant, J.E., Tham, N.T., BKrong, N.T.T.C., Boni, M.F., Loi, T.Q., Phuong, L.T., Verhoeven, J.T.P., Crusat, M., Jeeninga, R. E., Schultsz, C., Chau, N.V., Hien, T.T., van der Hoek, L., Farrar, J., de Jong, M. D., 2013. Identification of a New Cyclovirus in Cerebrospinal Fluid of Patients with Acute Central Nervous System Infections. MBio, 4(3), e00231-13. https://doi.org/10.1128/mBio.00231-13

Truyen, U., Parrish, C.R., 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: Distinct host cell tropisms of each virus in vitro and in vivo. Journal of

Virology, 66(9), 5399–5408. https://doi.org/10.1128/jvi.66.9.5399-5408.1992

Urbani, L., Tryland, M., Ehrich, D., Fuglei, E., Battilani, M., Balboni, A., 2021. Ancient origin and genetic segregation of canine circovirus infecting arctic foxes (Vulpes lagopus) in Svalbard and red foxes (Vulpes vulpes) in Northern Norway. Transboundary and Emerging Diseases, 68(3). https://doi.org/10.1111/tbed.13783

Wang, H., Gao, H., Jiang, Z., Shi, L., Zhao, P., Zhang, Y., Wang, C., 2022. Molecular detection and phylogenetic analysis of pigeon circovirus from racing pigeons in Northern China. BMC Genomics, 23(1), 290. https://doi.org/10.1186/s12864-022-08425-8

Wu, L., Liu, X., Schadt, C.W., Zhou, J., 2006. Microarray-Based Analysis of Subnanogram Quantities of Microbial Community DNAs by Using Whole-Community Genome Amplification. Applied and Environmental Microbiology, 72(7), 4931–4941. https://doi.org/10.1128/AEM.02738-05

Zaccaria, G., Malatesta, D., Scipioni, G., Di Felice, E., Campolo, M., Casaccia, C., Savini, G., Di Sabatino, D., Lorusso, A., 2016. Circovirus in domestic and wild carnivores: An important opportunistic agent? Virology, 490, 69–74. https://doi.org/10.1016/j.virol.2016.01.007

Zhang, W., Li, L., Deng, X., Kapusinszky, B., Pesavento, P.A., Delwart, E., 2014. Faecal virome of cats in an animal shelter. Journal of General Virology, 95(11), 2553–2564. https://doi.org/10.1099/vir.0.069674-0

Section 2.2 (draft)

Exploring the presence of Bovine Meat and Milk Factor (BMMFs)/SPHINX DNA sequences in italian bovine and non-bovine samples

Introduction

Dietary habits, especially the consumption of red meat, have been identified as a potential risk factor for the development of cancer and neurodegenerative diseases. It had been suggested earlier that a component found in the meat of domesticated cattle (*bos taurus*) might contribute to tumor development (Santarelli et al., 2008). This idea stemmed from findings indicating that high intake of red meat correlates with a greater likelihood of colon cancer, especially considering that beef from domestic cattle is frequently eaten undercooked. Additional evidence supporting this hypothesis came from a prior study which observed a link between the rising incidence of colon cancer in multiple nations and increased beef consumption (Kuhnle & Bingham, 2007).

Epidemiological studies examining meat and dairy consumption reveal nuanced associations with cancer risk. While red and processed meat intake has been linked to increased colorectal cancer risk (Chan et al., 2011; Corpet, 2011; Huxley et al., 2009; Veettil et al., 2021), higher consumption of milk and dairy products appears to be associated with a reduced risk of colorectal cancer, with no clear evidence linking dairy intake to breast cancer risk (Watling et al., 2023; X. Zhang et al., 2023). These findings suggest that the relationship between meat and dairy consumption and cancer is complex and not solely dependent on beef.

In recent years, it has been suggested that a new class of foodborne infectious agents, called Bovine Meat and Milk Factors (BMMFs), could play a role as co-factors in the onset of malignant and degenerative diseases. BMMFs resembling Circular Replication-associated protein Encoding ssDNA (CRESS) viruses, were identified in 2014 from commercial milk samples, healthy bovine meat, bovine sera and brain tissues of humans affected by multiple sclerosis (Zur Hausen, 2009, 2012). In 2019, the German Cancer Research Center (DKFZ) highlighted BMMFs as potential "factors" or "new pathogens" originating from European cattle, implicating them in food-related carcinogenic processes.

These infectious agents seem to have a clear zoonotic nature and, according to Dr. Zur Hausen's research group, BMMFs infect humans through the diet, starting from weaning. The infection occurs in the cells of the colon lamina propria, with local spread, expression of the Rep protein, and subsequent prolonged recruitment of the macrophage population, leading to chronic inflammation.

Chronic inflammation, in turn, leads to an increase in oxidative stress and, therefore, in reactive nitrogen and oxygen species (RNS and ROS) that would induce an increase in mutations in local cells (Zur Hausen et al., 2017). This scenario could play an important role in the pathogenesis of breast and colon cancers, as well as neurodegenerative diseases such as Alzheimer's and multiple sclerosis. Furthermore, this hypothesis is corroborated by the presence of BMMF-derived proteins in various tissues such as the colon, breast, prostate gland, and brain, as well as the detection of BMMF DNA in the human colon. Conversely, the absence of BMMF sequences in tumour cells suggests their indirect involvement in carcinogenesis (De Villiers et al., 2019; Zur Hausen et al., 2017, 2019).

BMMFs are characterized by a conserved Open Reading Frame (ORF) encoding a Rep replication protein, a region rich in A-T, and a highly conserved Iteron-like region upstream of the Rep protein coding site. BMMFs are classified into 4 groups: group 1 shows high homology with the episomes Sphinx 1.76 and Sphinx 2.36, isolated from brain tissue and neuronal cell lines of patients with Creutzfeldt-Jakob (CKD) disease; group 2 shows homology with *Acinetobacter baumanii* plasmids; group 3 includes isolates with a high degree of similarity to *Gemycircularviruses*, and the last group includes those similar to plasmids of the *Psychrobacter* species (De Villiers et al., 2019; Eilebrecht et al., 2018; Zur Hausen, 2012).

The detection of SPHINX/BMMF DNA in dairy products, animal tissues, and human samples further underscores their widespread distribution (De Villiers et al., 2019; Falida et al., 2017; Gunst et al., 2014; Whitley et al., 2014b). Notably, the replication protein (Rep) of these molecules has been identified in the lamina propria of colon cancer tissues, suggesting a possible role in carcinogenesis.

Further investigations have expanded the scope of detection for SPHINX/BMMF molecules beyond Bos taurus-derived products. Using specific molecular techniques, these DNA molecules have been identified in milk from water buffalo, sheep, and goats, as well as in diverse foodstuffs including seafood, fruits, vegetables, and baby foods. Additionally, SPHINX/BMMF DNA has been found in blood and fecal samples from cattle in Africa and Asia (Habermann et al., 2023; König et al., 2021a, 2021c, 2023b; Pohl et al., 2022). These findings point toward a broad distribution of these molecules across various species and food sources, raising important questions about their potential impact on human health and disease.

The aim of this study was to investigate the presence of SPHINX/BMMFs in Italian bovine and non-bovine samples in order to assess their occurrence in Italy.

Methods

Sample collection

A total of 42 bovine faecal samples and 71 non bovine food samples, including 17 samples of raw goat milk, 4 samples of aged cheese made from raw goat milk, 21 samples of pork-based meat products, 10 samples of wild boar liver, and 20 samples of bivalve shellfish, were analized to detect the presence of SHINX/BMMF fragments. As part of an exploratory study, sampling was conducted using samples received in the National Reference Laboratory for Foodborne Viruses, Italian Institute of Health, from other research projects. Specifically, the faeces come from Emilia Romagna, the goat milk samples come from Trentino, the pork products from Abruzzo, the cheese from Trento, the wild boar liver from Umbria, and the bivalve mollusks from Veneto.

Extraction

The samples were extracted using the DNeasy Power Soil Pro Kit (Qiagen, Hilden, Germany), following manifacture's instructions.

Rolling Circle Amplification

The extracts were treated with the TempliPhi Amplification Kits (Cytiva), which allows the amplification of circular DNA, either single or double-stranded, by utilizing the properties of Phi29 DNA polymerase. Briefly, the RCA protocol involved treating 1 µl of the nucleic acids extracted from the sample with 5 µl of sample buffer for 3 minutes at 95°C. Subsequently, the samples were cooled on ice, and 0.2 µl of enzyme was added to each reaction, followed by incubation at 30°C for 20 hours. At the end of the incubation, the DNA polymerase was inactivated by raising the temperature to 65°C for 10 minutes. The treated samples were then stored at -80°C.

Gel Based PCR and Sanger sequencing

Specific Gel based PCR protocols were developed using a combination of primers described in literature. The PCRs were conducted using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The samples that tested positive in the PCR analysis were purified using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal), according to the manufacturer's instructions. They were then sequenced using the Sanger method and finally analyzed with MEGA software version 11 (Tamura K, Stecher G, and Kumar S 2021).

Results and discussion

In this study, we analyzed 42 fecal samples using specific primers for BMMF1 and BMMF2. The results, presented in Table 1, indicate that 25 samples (60%) tested positive for BMMFs. Among the positive samples, 2 (5%) were exclusively positive for BMMF1, 7 (17%) exclusively for BMMF2, while 16 samples (38%) were positive for both targets. Given the high homology of the BMMF1 group with the Sphinx 1.76 and Sphinx 2.36 episomes, additional analyses were performed on selected positive samples using gel-based PCR methods with primers from the S1C, S3B, and S5A groups, targeting the rep protein gene associated with the Sphinx episomes. The results, summarized in Table 2, demonstrate that samples testing positive for BMMF1 also exhibited positivity for the rep protein gene. Furthermore, samples 65, 66, 80, and 81, belonged to bovine matrix samples, which tested negative for both BMMF groups, were also negative for the Sphinx-associated rep protein, thereby ruling out the possibility of PCR assay inhibition and excluding the likelihood of false negatives.

Matrix	***Number of samples***	***BMMF1***	***BMMF2***	***BMMF1 & BMMF2***	***Total positives***
Bovine faeces	42	2	7	16	**25**
Raw Goat Milk	17	1	0	0	**1**
Hard Goat Cheese	4	0	0	0	**0**
Pork Meat Products	20	6	0	6	**12**
Wild Boar Liver	10	0	0	0	**0**
Bivalve Shellfish	20	0	0	0	**0**

Table 1 *Summary of results*

ID SAMPLE	**SET PRIMER BMMF1 SG1**	**SET PRIMER BMMF2**	**SET PRIMER S1C**	**SET PRIMER S3B**	**SET PRIMER S5A**
BF1	✓	✓	✓	✓	✓
BF2	✓	✓	✓	✓	✗
BF3	✓	✓	✓	✓	✓
BF4	✗	✗	✗	✗	✗
BF5	✗	✗	✗	✗	✗
BF6	✓	✓	✓	✓	✓
BF7	✓	✓	✓	✓	✓
BF8	✓	✗	✓	✗	✓
BF9	✗	✓	✓	✗	✓
BF10	✗	✗	✗	✗	✗
BF11	✗	✗	✗	✗	✗
BF12	✓	✓	✓	✓	✓

Table 2: comparison of specific sets of primers to discriminate the presence of the rep protein gene

Regarding the food matrices, only one milk sample tested positive for BMMF1, while no BMMFs were detected in the goat cheese samples. In contrast, pig samples showed a high prevalence, with 12 positive samples, 6 for BMMF1 and 6 for both targets. No positivity was observed in the wild boar samples, likely attributable to the use of liver samples instead of muscle tissue. Additionally, shellfish samples did not demonstrate the presence of BMMFs.

The phylogenetic tree depicted in Figure 1 reveals significant variability among BMMF sequences. Analyses conducted in this study identified two distinct clusters. The first cluster, designated BMMF type 1, shows affinities with sequences isolated from cow milk samples. The second cluster, defined as BMMF type 2, exhibits a close correlation with circular DNA sequences isolated from TSE Sphinx 2.36, as well as with sequences derived from commercial cow milk and the plasmid of the bacterium *Acinetobacter baumannii*. Notably, no significant differences were observed among sequences derived from different matrices in this analysis, suggesting a certain stability of the sequences across various environments.

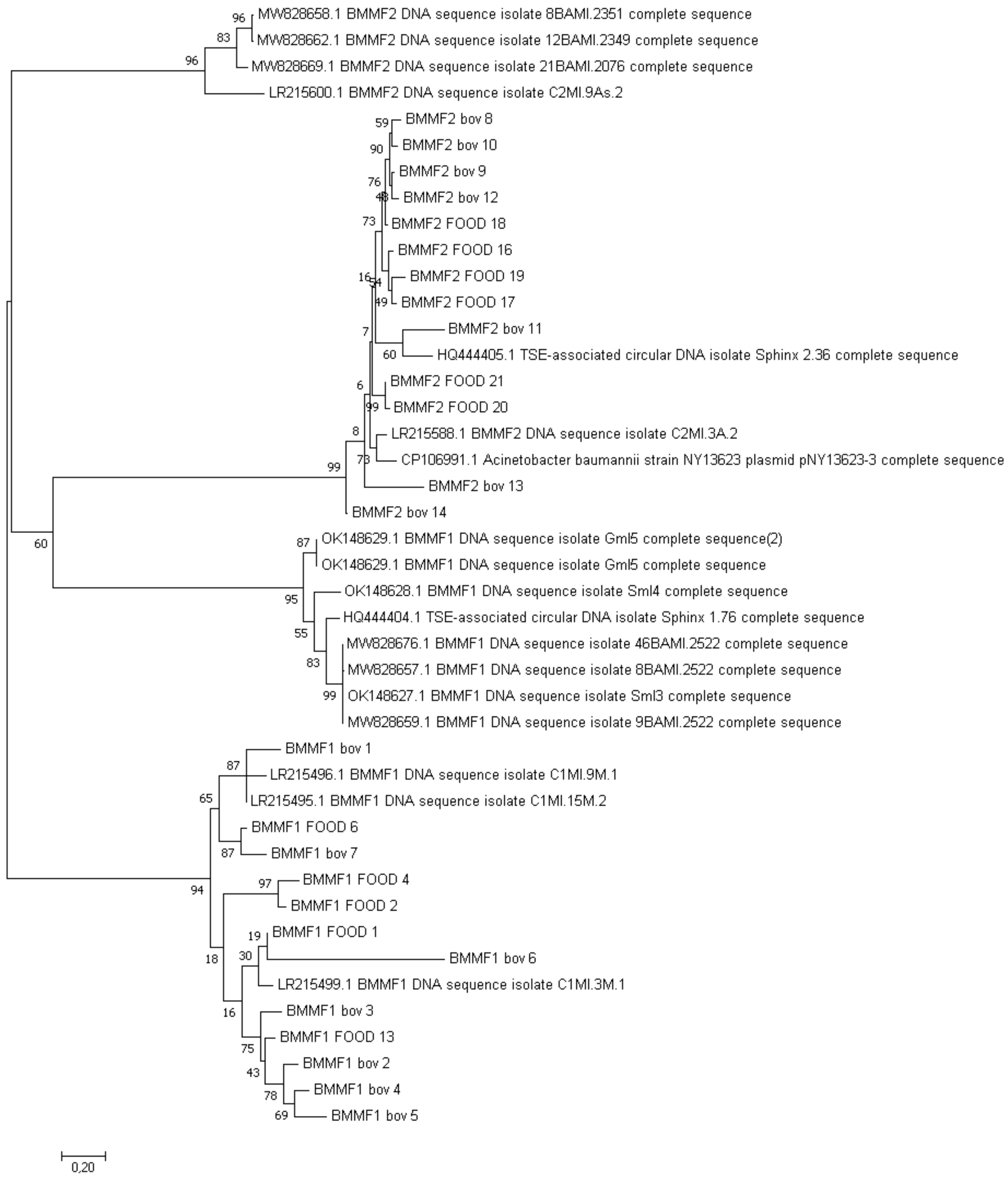


Figure .1 Phylogenetic analysis

Discussions and conclusions

In 2011, Sphinx-type episomes were described for the first time, associated with neurodegenerative diseases such as Creutzfeldt-Jakob disease and Scrapie (Manuelidis, 2011, 2019). Over time, single-stranded circular DNA molecules with homology to Sphinx episomes ranging between 66% and 98%

were isolated from bovine serum and patients with multiple sclerosis and were attributed the name Bovine Meat and Milk factors (BMMFs). In 2015, a study by Dr. zur Hausen's group (zur Hausen H, 2015) suggested that BMMFs could also be considered etiological agents of breast cancer (BC) and colorectal cancer (CRC). These hypotheses arose from the fact that in countries with higher consumption of beef and dairy products, the incidence of these cancers was high and continuously increasing. Additionally, recent studies detected the presence of the rep protein associated with BMMFs in the mucosa adjacent to tumor tissues of CRC patients, suggesting a correlation between the presence of these circular DNA molecules and the onset of CRC. However, this theory cannot be confirmed in the absence of long-term study on cancer patients in association with their diet, considering the still unclear distribution of SPHINX/BMMF in foods other than those derived from bovine.

To explore whether SPHINX/BMMF DNA is found exclusively in food products derived from *Bos taurus*, researchers expanded their analysis to include a broader range of food items and animal samples (Habermann et al., 2023; König et al., 2021c, 2021b, 2023b; Pohl et al., 2022). These investigations employed the established methods for isolating and detecting SPHINX/BMMF DNA, originally developed by the German Cancer Research Center, which involve using primers specific to SPHINX/BMMF sequences to amplify these molecules from food DNA samples enriched through Rolling Circle Amplification (RCA). Specifically, primers targeting the rep gene of the SPHINX1.76/BMMF group 1, SPHINX2.36/BMMF group 2, or related sequences were used. König et al. 2021 demonstrated that these DNA molecules were highly prevalent in milk from water buffalo, with detection rates of 83% in one herd and 100% in another. Using similar or identical primers, circular DNA resembling SPHINX1.76/BMMF group 1 was also identified in sheep and goat milk. In subsequent research, Pohl et al. 2022 found that DNA fragments with very high similarity to SPHINX1.76/BMMF group 1 or SPHINX2.23/BMMF group 2 were detected in 65% of food samples obtained from local markets, including various meats (white and red), seafood, fruits, vegetables, grains, and baby foods. Additionally, samples from individual animals—such as feces and saliva from pigs and feces from chickens—also contained these DNA fragments. Habermann et al. 2023, revealed that complete circular DNA molecules closely related to SPHINX/BMMF could be detected in foods derived from animals and plants, including pork, wild boar, chicken meat, Alaska pollock, pangasius, black tiger shrimp, apples, carrots, and sprouts from alfalfa, radish, and broccoli. Moreover, additional SPHINX/BMMF molecules had been isolated from the blood and feces of cattle from Africa and Asia.

Our study shows for the first time the presence of BMMFs group 1 and group 2 in animal-derived food products in Italy. Compared to German research groups, this pilot study conducted on Italian samples confirmed the presence of SPHINX/BMMF molecules in bovine fecal material. Compared to analogous studies, the prevalence of BMMF-positive samples in goat milk and its derivatives was significantly lower, with only one out of twenty-one samples testing positive among those analyzed. Similarly, no presence of these molecules was detected in bivalve mollusks. This finding may be attributable to both the limited number of samples examined and the absence of stratified sampling strategy, which would have ensured representation across all Italian regions. Remarkably, a high prevalence of SPHINX/BMMF molecules from groups 1 and 2 was observed in pork meat with twelve out of twenty testing positive. Although other studies have already documented the presence of these molecules, positivity rates have not been reported; in our investigation, even considering the small sample size, the percentage of positive samples remains notably high.

Considering the significant variability observed, further studies are necessary to characterize the positive samples, for example, using the NGS platform. It is also essential to expand the sampling, including samples of horsemeat and poultry, which were not included in this study, as well as samples of cow's milk and additional samples of goat milk, in order to obtain a more accurate assessment of the occurrence and risks related to milk consumption. Finally, it is necessary to collect additional samples of pork products (meat and meat derivatives) to further investigate the potential risks associated with consumption.

References

Abbas, A., Taylor, L. J., Collman, R. G., Bushman, F. D., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Redondoviridae: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(1). https://doi.org/10.1099/jgv.0.001526

Allander, T., Tammi, M. T., Eriksson, M., Bjerkner, A., Tiveljung-Lindell, A., & Andersson, B. (2005). Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proceedings of the National Academy of Sciences*, *102*(36), 12891–12896. https://doi.org/10.1073/pnas.0504666102

Altan, E., Del Valle Mendoza, J., Deng, X., Phan, T. G., Sadeghi, M., & Delwart, E. L. (2017). Small Circular Rep-Encoding Single-Stranded DNA Genomes in Peruvian Diarrhea Virome. *Genome Announcements*, *5*(38), e00822-17. https://doi.org/10.1128/genomeA.00822-17

Altay, A., Ocal, M., Yuksek, S. K., Polat, M., Tapisiz, A., Tezer, H., Ergunay, K., Bozdayi, G., & Ahmed, K. (2016). Multicenter investigation of bufavirus in the etiology of viral central nervous system infections of adults and children. *Journal of Clinical Virology*, *82*, S57–S58. https://doi.org/10.1016/j.jcv.2016.08.113

Altay, A., Yahiro, T., Bozdayi, G., Matsumoto, T., Sahin, F., Ozkan, S., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2015). Bufavirus genotype 3 in Turkish children with severe diarrhoea. *Clinical Microbiology and Infection*, *21*(10), 965.e1-965.e4. https://doi.org/10.1016/j.cmi.2015.06.006

Anderson, M. J., Higgins, P. G., Davis, L. R., Willman, J. S., Jones, S. E., Kidd, I. M., Pattison, J. R., & Tyrrell, D. A. J. (1985). Experimental Parvoviral Infection in Humans. *Journal of Infectious Diseases*, *152*(2), 257–265. https://doi.org/10.1093/infdis/152.2.257

Anderson, T. R., & Slotkin, T. A. (1975). Maturation of the adrenal medulla—IV. Effects of morphine. *Biochemical Pharmacology*, *24*(16), 1469–1474. https://doi.org/10.1016/0006-2952(75)90020-9

Atchison, R. W., Casto, B. C., & Hammon, W. McD. (1965). Adenovirus-Associated Defective Virus Particles. *Science*, *149*(3685), 754–756. https://doi.org/10.1126/science.149.3685.754

Ayouni, S., Estienney, M., Hammami, S., Neji Guediche, M., Pothier, P., Aouni, M., Belliot, G., & De Rougemont, A. (2016). Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants. *PLOS ONE*, *11*(9), e0162255. https://doi.org/10.1371/journal.pone.0162255

Barzon, L., Lavezzo, E., Militello, V., Toppo, S., & Palù, G. (2011). Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences*, *12*(11), 7861–7884. https://doi.org/10.3390/ijms12117861

Belák, S., Karlsson, O. E., Blomström, A.-L., Berg, M., & Granberg, F. (2013). New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary Microbiology*, *165*(1–2), 95–101. https://doi.org/10.1016/j.vetmic.2013.01.022

Bernstein, A. S., Ando, A. W., Loch-Temzelides, T., Vale, M. M., Li, B. V., Li, H., Busch, J., Chapman, C. A., Kinnaird, M., Nowak, K., Castro, M. C., Zambrana-Torrelio, C., Ahumada, J. A., Xiao, L., Roehrdanz, P., Kaufman, L., Hannah, L., Daszak, P., Pimm, S. L., & Dobson, A. P. (2022). The costs and benefits of primary prevention of zoonotic pandemics. *Science Advances*, *8*(5), eabl4183. https://doi.org/10.1126/sciadv.abl4183

Bexton, S., Wiersma, L. C., Getu, S., Van Run, P. R., Verjans, G. M. G. M., Schipper, D., Schapendonk, C. M. E., Bodewes, R., Oldroyd, L., Haagmans, B. L., Koopmans, M. M. P., & Smits, S. L. (2015). Detection of Circovirus in Foxes with Meningoencephalitis, United Kingdom, 2009–2013. *Emerging Infectious Diseases*, *21*(7), 1205–1208. https://doi.org/10.3201/eid2107.150228

Blomström, A.-L. (2011). Viral metagenomics as an emerging and powerful tool in veterinary medicine. *Veterinary Quarterly*, *31*(3), 107–114. https://doi.org/10.1080/01652176.2011.604971

Bund, T., Nikitina, E., Chakraborty, D., Ernst, C., Gunst, K., Boneva, B., Tessmer, C., Volk, N., Brobeil, A., Weber, A., Heikenwalder, M., Zur Hausen, H., & De Villiers, E.-M. (2021). Analysis of chronic inflammatory lesions of the colon for BMMF Rep antigen expression and CD68 macrophage interactions. *Proceedings of the National Academy of Sciences*, *118*(12), e2025830118. https://doi.org/10.1073/pnas.2025830118

Capobianchi, M. R., Giombini, E., & Rozera, G. (2013). Next-generation sequencing technology in clinical virology. *Clinical Microbiology and Infection*, *19*(1), 15–22. https://doi.org/10.1111/1469-0691.12056

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE*, *6*(6), e20456. https://doi.org/10.1371/journal.pone.0020456

Chandler, M., De La Cruz, F., Dyda, F., Hickman, A. B., Moncalian, G., & Ton-Hoang, B. (2013). Breaking and joining single-stranded DNA: The HUH endonuclease superfamily. *Nature Reviews Microbiology*, *11*(8), 525–538. https://doi.org/10.1038/nrmicro3067

Chappuis, G., Soulier, M., & Nettleton, P. (1987). Ovine parvovirus infection in Scottish sheep. *Veterinary Record*, *121*(8), 182–182. https://doi.org/10.1136/vr.121.8.182-a

Chieochansin, T., Vutithanachot, V., Theamboonlers, A., & Poovorawan, Y. (2015). Bufavirus in fecal specimens of patients with and without diarrhea in Thailand. *Archives of Virology*, *160*(7), 1781–1784. https://doi.org/10.1007/s00705-015-2441-z

Chung, Y.-H., Jun, H. S., Son, M., Bao, M., Bae, H. Y., Kang, Y., & Yoon, J.-W. (2000). Cellular and Molecular Mechanism for Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats. *The Journal of Immunology*, *165*(5), 2866–2876. https://doi.org/10.4049/jimmunol.165.5.2866

Corpet, D. E. (2011). Red meat and colon cancer: Should we become vegetarians, or can we make meat safer? *Meat Science*, *89*(3), 310–316. https://doi.org/10.1016/j.meatsci.2011.04.009

Cossart, Y. E., Cant, B., Field, A. M., & Widdows, D. (1975). PARVOVIRUS-LIKE PARTICLES IN HUMAN SERA. *The Lancet*, *305*(7898), 72–73. https://doi.org/10.1016/S0140-6736(75)91074-0

Dai, Z., Wang, H., Xu, J., Lu, X., Ni, P., Yang, S., Shen, Q., Wang, X., Li, W., Wang, X., Zhou, C., Zhang, W., & Shan, T. (2024). Unveiling the Virome of Wild Birds: Exploring CRESS-DNA Viral Dark Matter. *Genome Biology and Evolution*, *16*(10), evae206. https://doi.org/10.1093/gbe/evae206

Dayaram, A., Potter, K. A., Moline, A. B., Rosenstein, D. D., Marinov, M., Thomas, J. E., Breitbart, M., Rosario, K., Argüello-Astorga, G. R., & Varsani, A. (2013). High global diversity of cycloviruses amongst dragonflies. *Journal of General Virology*, *94*(8), 1827–1840. https://doi.org/10.1099/vir.0.052654-0

De Villiers, E.-M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T., & Zur Hausen, H. (2019). A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. *Emerging Microbes & Infections*, *8*(1), 1205–1218. https://doi.org/10.1080/22221751.2019.1651620

Decaro, N., Martella, V., Desario, C., Lanave, G., Circella, E., Cavalli, A., Elia, G., Camero, M., & Buonavoglia, C. (2014). Genomic Characterization of a Circovirus Associated with Fatal Hemorrhagic Enteritis in Dog, Italy. *PLoS ONE*, *9*(8), e105909. https://doi.org/10.1371/journal.pone.0105909

Eilebrecht, S., Hotz-Wagenblatt, A., Sarachaga, V., Burk, A., Falida, K., Chakraborty, D., Nikitina, E., Tessmer, C., Whitley, C., Sauerland, C., Gunst, K., Grewe, I., & Bund, T. (2018). Expression and replication of virus-like circular DNA in human cells. *Scientific Reports*, *8*(1), 2851. https://doi.org/10.1038/s41598-018-21317-w

Ellerman, K. E., Richards, C. A., Guberski, D. L., Shek, W. R., & Like, A. A. (1996). Kilham Rat Virus Triggers T-Cell–Dependent Autoimmune Diabetes in Multiple Strains of Rat. *Diabetes*, *45*(5), 557–562. https://doi.org/10.2337/diab.45.5.557

Escudero-Pérez, B., Lalande, A., Mathieu, C., & Lawrence, P. (2023). Host–Pathogen Interactions Influencing Zoonotic Spillover Potential and Transmission in Humans. *Viruses*, *15*(3), 599. https://doi.org/10.3390/v15030599

Falida, K., Eilebrecht, S., Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2017). Isolation of Two Virus-Like Circular DNAs from Commercially Available Milk Samples. *Genome Announcements*, *5*(17), e00266-17. https://doi.org/10.1128/genomeA.00266-17

Fiallo-Olivé, E., Lett, J.-M., Martin, D. P., Roumagnac, P., Varsani, A., Zerbini, F. M., & Navas-Castillo, J. (2021). ICTV Virus Taxonomy Profile: Geminiviridae 2021: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(12). https://doi.org/10.1099/jgv.0.001696

Gainor, K., Malik, Y. S., & Ghosh, S. (2021). Novel Cyclovirus Species in Dogs with Hemorrhagic Gastroenteritis. *Viruses*, *13*(11), 2155. https://doi.org/10.3390/v13112155

Gibb, R., Franklinos, L. H. V., Redding, D. W., & Jones, K. E. (2020). Ecosystem perspectives are needed to manage zoonotic risks in a changing climate. *BMJ*, m3389. https://doi.org/10.1136/bmj.m3389

Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2014). Isolation of Bacterial Plasmid-Related Replication-Associated Circular DNA from a Serum Sample of a Multiple Sclerosis Patient. *Genome Announcements*, *2*(4), e00847-14. https://doi.org/10.1128/genomeA.00847-14

Gutiérrez-Bugallo, G., Piedra, L. A., Rodriguez, M., Bisset, J. A., Lourenço-de-Oliveira, R., Weaver, S. C., Vasilakis, N., & Vega-Rúa, A. (2019). Vector-borne transmission and evolution of Zika virus. *Nature Ecology & Evolution*, *3*(4), 561–569. https://doi.org/10.1038/s41559-019-0836-z

Habermann, D., Klempt, M., & Franz, C. M. A. P. (2023). Identification and Characterization of Novel SPHINX/BMMF-like DNA Sequences Isolated from Non-Bovine Foods. *Genes*, *14*(7), 1307. https://doi.org/10.3390/genes14071307

Haddadmarandi, M. R., Madani, S. A., Nili, H., & Ghorbani, A. (2020). Molecular survey of avian circoviruses in some non-psittacine birds and detection of a novel canary circovirus in a pigeon. *Iranian Journal of Veterinary Research*, *21*(1), 57–60.

Hao, X., Li, Y., Hu, X., Fu, X., Dong, J., Zhang, H., Zhou, P., & Li, S. (2021). Feline Stool-Associated Circular DNA Virus (FeSCV) in Diarrheic Cats in China. *Frontiers in Veterinary Science*, *8*, 694089. https://doi.org/10.3389/fvets.2021.694089

Huang, D.-D., Wang, W., Lu, Q.-B., Zhao, J., Guo, C.-T., Wang, H.-Y., Zhang, X.-A., Tong, Y.-G., Liu, W., & Cao, W.-C. (2015). Identification of Bufavirus-1 and Bufavirus-3 in Feces of Patients with Acute Diarrhea, China. *Scientific Reports*, *5*(1), 13272. https://doi.org/10.1038/srep13272

Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer*, *125*(1), 171–180. https://doi.org/10.1002/ijc.24343

Jacob, S. T., Crozier, I., Fischer, W. A., Hewlett, A., Kraft, C. S., Vega, M.-A. D. L., Soka, M. J., Wahl, V., Griffiths, A., Bollinger, L., & Kuhn, J. H. (2020). Ebola virus disease. *Nature Reviews Disease Primers*, *6*(1), 13. https://doi.org/10.1038/s41572-020-0147-3

Jones, M. S., Kapoor, A., Lukashov, V. V., Simmonds, P., Hecht, F., & Delwart, E. (2005). New DNA Viruses Identified in Patients with Acute Viral Infection Syndrome. *Journal of Virology*, *79*(13), 8230–8236. https://doi.org/10.1128/JVI.79.13.8230-8236.2005

Jones-Engel, L., May, C. C., Engel, G. A., Steinkraus, K. A., Schillaci, M. A., Fuentes, A., Rompis, A., Chalise, M. K., Aggimarangsee, N., Feeroz, M. M., Grant, R., Allan, J. S., Putra, A., Wandia, I. N., Watanabe, R., Kuller, L., Thongsawat, S., Chaiwarith, R., Kyes, R. C., &

Linial, M. L. (2008). Diverse Contexts of Zoonotic Transmission of Simian Foamy Viruses in Asia. *Emerging Infectious Diseases*, *14*(8), 1200–1208. https://doi.org/10.3201/eid1408.071430

Kamorudeen, R. T., Adedokun, K. A., & Olarinmoye, A. O. (2020). Ebola outbreak in West Africa, 2014 – 2016: Epidemic timeline, differential diagnoses, determining factors, and lessons for future response. *Journal of Infection and Public Health*, *13*(7), 956–962. https://doi.org/10.1016/j.jiph.2020.03.014

Kaner, J., & Schaack, S. (2016). Understanding Ebola: The 2014 epidemic. *Globalization and Health*, *12*(1), 53. https://doi.org/10.1186/s12992-016-0194-4

Kapoor, A., Dubovi, E. J., Henriquez-Rivera, J. A., & Lipkin, W. I. (2012). Complete Genome Sequence of the First Canine Circovirus. *Journal of Virology*, *86*(12), 7018–7018. https://doi.org/10.1128/JVI.00791-12

Kilham, L., & Olivier, L. J. (1959). A latent virus of rats isolated in tissue culture. *Virology*, *7*(4), 428–437. https://doi.org/10.1016/0042-6822(59)90071-6

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023a). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023b). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021a). Circular Rep-Encoding Single-Stranded DNA Sequences in Milk from Water Buffaloes (Bubalus arnee f. Bubalis). *Viruses*, *13*(6), 1088. https://doi.org/10.3390/v13061088

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021b). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021c). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

Krupovic, M., & Varsani, A. (2022). Naryaviridae, Nenyaviridae, and Vilyaviridae: Three new families of single-stranded DNA viruses in the phylum Cressdnaviricota. *Archives of Virology*, *167*(12), 2907–2921. https://doi.org/10.1007/s00705-022-05557-w

Krupovic, M., Varsani, A., Kazlauskas, D., Breitbart, M., Delwart, E., Rosario, K., Yutin, N., Wolf, Y. I., Harrach, B., Zerbini, F. M., Dolja, V. V., Kuhn, J. H., & Koonin, E. V. (2020). *Cressdnaviricota*: A Virus Phylum Unifying Seven Families of Rep-Encoding Viruses with Single-Stranded, Circular DNA Genomes. *Journal of Virology*, *94*(12), e00582-20. https://doi.org/10.1128/JVI.00582-20

Kuhnle, G. G. C., & Bingham, S. A. (2007). Dietary meat, endogenous nitrosation and colorectal cancer. *Biochemical Society Transactions*, *35*(5), 1355–1357. https://doi.org/10.1042/BST0351355

Lauber, C., & Seitz, S. (2022). Opportunities and Challenges of Data-Driven Virus Discovery. *Biomolecules*, *12*(8), 1073. https://doi.org/10.3390/biom12081073

Li, L., Kapoor, A., Slikas, B., Bamidele, O. S., Wang, C., Shaukat, S., Masroor, M. A., Wilson, M. L., Ndjango, J.-B. N., Peeters, M., Gross-Camp, N. D., Muller, M. N., Hahn, B. H., Wolfe, N. D., Triki, H., Bartkus, J., Zaidi, S. Z., & Delwart, E. (2010). Multiple Diverse Circoviruses Infect Farm Animals and Are Commonly Found in Human and Chimpanzee Feces. *Journal of Virology*, *84*(4), 1674–1682. https://doi.org/10.1128/JVI.02109-09

Lőrincz, M., Dán, Á., Láng, M., Csaba, G., Tóth, Á. G., Székely, C., Cságola, A., & Tuboly, T. (2012). Novel circovirus in European catfish (Silurus glanis). *Archives of Virology*, *157*(6), 1173–1176. https://doi.org/10.1007/s00705-012-1291-1

Macera, L., Focosi, D., Vatteroni, M. L., Manzin, A., Antonelli, G., Pistello, M., & Maggi, F. (2016). Cyclovirus Vietnam DNA in immunodeficient patients. *Journal of Clinical Virology*, *81*, 12–15. https://doi.org/10.1016/j.jcv.2016.05.011

Maggi, F., Andreoli, E., Pifferi, M., Meschi, S., Rocchi, J., & Bendinelli, M. (2007). Human bocavirus in Italian patients with respiratory diseases. *Journal of Clinical Virology*, *38*(4), 321–325. https://doi.org/10.1016/j.jcv.2007.01.008

Manuelidis, L. (2011). Nuclease resistant circular DNAs copurify with infectivity in scrapie and CJD. *Journal of NeuroVirology*, *17*(2), 131–145. https://doi.org/10.1007/s13365-010-0007-0

Manuelidis, L. (2019). Prokaryotic SPHINX 1.8 REP protein is tissue-specific and expressed in human germline cells. *Journal of Cellular Biochemistry*, *120*(4), 6198–6208. https://doi.org/10.1002/jcb.27907

Matthews, P. C., Malik, A., Simmons, R., Sharp, C., Simmonds, P., & Klenerman, P. (2014). PARV4: An Emerging Tetraparvovirus. *PLoS Pathogens*, *10*(5), e1004036. https://doi.org/10.1371/journal.ppat.1004036

Mietzsch, M., McKenna, R., Väisänen, E., Yu, J. C., Ilyas, M., Hull, J. A., Kurian, J., Smith, J. K., Chipman, P., Lasanajak, Y., Smith, D., Söderlund-Venermo, M., & Agbandje-McKenna, M. (2020). Structural Characterization of Cuta- and Tusavirus: Insight into Protoparvoviruses Capsid Morphology. *Viruses*, *12*(6), 653. https://doi.org/10.3390/v12060653

Miranda, M. N. S., Pingarilho, M., Pimentel, V., Torneri, A., Seabra, S. G., Libin, P. J. K., & Abecasis, A. B. (2022). A Tale of Three Recent Pandemics: Influenza, HIV and SARS-CoV-2. *Frontiers in Microbiology*, *13*, 889643. https://doi.org/10.3389/fmicb.2022.889643

Mohanraj, U., Jokinen, M., Thapa, R. R., Paloniemi, M., Vesikari, T., Lappalainen, M., Tarkka, E., Nora-Krūkle, Z., Vilmane, A., Vettenranta, K., Mangani, C., Oikarinen, S., Fan, Y.-M., Ashorn, P., Väisänen, E., & Söderlund-Venermo, M. (2021). Human Protoparvovirus DNA and IgG in Children and Adults with and without Respiratory or Gastrointestinal Infections. *Viruses*, *13*(3), 483. https://doi.org/10.3390/v13030483

Mollerup, S., Fridholm, H., Vinner, L., Kjartansdóttir, K. R., Friis-Nielsen, J., Asplund, M., Herrera, J. A. R., Steiniche, T., Mourier, T., Brunak, S., Willerslev, E., Izarzugaza, J. M. G., Hansen, A. J., & Nielsen, L. P. (2017). Cutavirus in Cutaneous Malignant Melanoma. *Emerging Infectious Diseases*, *23*(2), 363–365. https://doi.org/10.3201/eid2302.161564

Opriessnig, T., Karuppannan, A. K., Castro, A. M. M. G., & Xiao, C.-T. (2020). Porcine circoviruses: Current status, knowledge gaps and challenges. *Virus Research*, *286*, 198044. https://doi.org/10.1016/j.virusres.2020.198044

Padilla-Rodriguez, M., Rosario, K., & Breitbart, M. (2013). Novel cyclovirus discovered in the Florida woods cockroach Eurycotis floridana (Walker). *Archives of Virology*, *158*(6), 1389–1392. https://doi.org/10.1007/s00705-013-1606-x

Pérot, P., Da Rocha, N., Farcet, M. R., Kreil, T. R., & Eloit, M. (2024). Human Circovirus is not detected in plasma pools for fractionation. *Transfusion*, *64*(1), 16–18. https://doi.org/10.1111/trf.17604

Phan, T. G., Dreno, B., Da Costa, A. C., Li, L., Orlandi, P., Deng, X., Kapusinszky, B., Siqueira, J., Knol, A.-C., Halary, F., Dantal, J., Alexander, K. A., Pesavento, P. A., & Delwart, E. (2016). A new protoparvovirus in human fecal samples and cutaneous T cell lymphomas (mycosis fungoides). *Virology*, *496*, 299–305. https://doi.org/10.1016/j.virol.2016.06.013

Phan, T. G., Luchsinger, V., Avendaño, L. F., Deng, X., & Delwart, E. (2014). Cyclovirus in nasopharyngeal aspirates of Chilean children with respiratory infections. *Journal of General Virology*, *95*(4), 922–927. https://doi.org/10.1099/vir.0.061143-0

Phan, T. G., Vo, N. P., Bonkoungou, I. J. O., Kapoor, A., Barro, N., O'Ryan, M., Kapusinszky, B., Wang, C., & Delwart, E. (2012). Acute Diarrhea in West African Children: Diverse Enteric Viruses and a Novel Parvovirus Genus. *Journal of Virology*, *86*(20), 11024–11030. https://doi.org/10.1128/JVI.01427-12

Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. (2017). Pathways to zoonotic spillover. *Nature Reviews Microbiology*, *15*(8), 502–510. https://doi.org/10.1038/nrmicro.2017.45

Pohl, S., Habermann, D., Link, E. K., Fux, R., Boldt, C. L., Franz, C. M. A. P., Hölzel, C., & Klempt, M. (2022). Detection of DNA sequences attributed to bovine meat and milk factors (BMMF/SPHINX) in food-related samples. *Food Control*, *135*, 108779. https://doi.org/10.1016/j.foodcont.2021.108779

Prades, Y., Pizarro, R., Ruiz, M., Moreno, C., Avendaño, L. F., & Luchsinger, V. (2021). Cyclovirus detection in Chilean adults with and without community-acquired pneumonia. *Journal of Medical Virology*, *93*(8), 4786–4793. https://doi.org/10.1002/jmv.27080

Qiu, J., Söderlund-Venermo, M., & Young, N. S. (2017). Human Parvoviruses. *Clinical Microbiology Reviews*, *30*(1), 43–113. https://doi.org/10.1128/CMR.00040-16

Quer, J., Colomer-Castell, S., Campos, C., Andrés, C., Piñana, M., Cortese, M. F., González-Sánchez, A., Garcia-Cehic, D., Ibáñez, M., Pumarola, T., Rodríguez-Frías, F., Antón, A., & Tabernero, D. (2022). Next-Generation Sequencing for Confronting Virus Pandemics. *Viruses*, *14*(3), 600. https://doi.org/10.3390/v14030600

Rahman, Md. T., Sobur, Md. A., Islam, Md. S., Ievy, S., Hossain, Md. J., El Zowalaty, M. E., Rahman, A. T., & Ashour, H. M. (2020). Zoonotic Diseases: Etiology, Impact, and Control. *Microorganisms*, *8*(9), 1405. https://doi.org/10.3390/microorganisms8091405

Rangarajan, S., Walsh, L., Lester, W., Perry, D., Madan, B., Laffan, M., Yu, H., Vettermann, C., Pierce, G. F., Wong, W. Y., & Pasi, K. J. (2017). AAV5–Factor VIII Gene Transfer in

Severe Hemophilia A. *New England Journal of Medicine*, *377*(26), 2519–2530. https://doi.org/10.1056/NEJMoa1708483

Reuter, G., Pankovics, P., László, Z., Gáspár, G., Hui, A., Delwart, E., & Boros, Á. (2022). Human-stool-associated tusavirus (Parvoviridae) in domestic goats and sheep. *Archives of Virology*, *167*(5), 1307–1310. https://doi.org/10.1007/s00705-022-05424-8

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., & Varsani, A. (2017). Revisiting the taxonomy of the family Circoviridae: Establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Archives of Virology*, *162*(5), 1447–1463. https://doi.org/10.1007/s00705-017-3247-y

Rosario, K., Duffy, S., & Breitbart, M. (2012). A field guide to eukaryotic circular single-stranded DNA viruses: Insights gained from metagenomics. *Archives of Virology*, *157*(10), 1851–1871. https://doi.org/10.1007/s00705-012-1391-y

Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. *Nutrition and Cancer*, *60*(2), 131–144. https://doi.org/10.1080/01635580701684872

Sauvage, V., Gomez, J., Barray, A., Vandenbogaert, M., Boizeau, L., Tagny, C. T., Rakoto, O., Bizimana, P., Guitteye, H., Ciré, B. B., Soumana, H., Tchomba, J.-B. S., Caro, V., & Laperche, S. (2018). High prevalence of cyclovirus Vietnam (CyCV-VN) in plasma samples from Madagascan healthy blood donors. *Infection, Genetics and Evolution*, *66*, 9–12. https://doi.org/10.1016/j.meegid.2018.09.002

Smits, S. L., Schapendonk, C. M. E., Van Beek, J., Vennema, H., Schürch, A. C., Schipper, D., Bodewes, R., Haagmans, B. L., Osterhaus, A. D. M. E., & Koopmans, M. P. (2014). New Viruses in Idiopathic Human Diarrhea Cases, the Netherlands. *Emerging Infectious Diseases*, *20*(7). https://doi.org/10.3201/eid2007.140190

Smits, S. L., Zijlstra, E. E., Van Hellemond, J. J., Schapendonk, C. M. E., Bodewes, R., Schürch, A. C., Haagmans, B. L., & Osterhaus, A. D. M. E. (2013). Novel Cyclovirus in Human

Cerebrospinal Fluid, Malawi, 2010–2011. *Emerging Infectious Diseases*, *19*(9). https://doi.org/10.3201/eid1909.130404

Stang, A., Korn, K., Wildner, O., & Überla, K. (2005). Characterization of Virus Isolates by Particle-Associated Nucleic Acid PCR. *Journal of Clinical Microbiology*, *43*(2), 716–720. https://doi.org/10.1128/JCM.43.2.716-720.2005

Takano, T., Yanai, Y., Hiramatsu, K., Doki, T., & Hohdatsu, T. (2018). Novel single-stranded, circular DNA virus identified in cats in Japan. *Archives of Virology*, *163*(12), 3389–3393. https://doi.org/10.1007/s00705-018-4020-6

Tan, L. V., Van Doorn, H. R., Nghia, H. D. T., Chau, T. T. H., Tu, L. T. P., De Vries, M., Canuti, M., Deijs, M., Jebbink, M. F., Baker, S., Bryant, J. E., Tham, N. T., BKrong, N. T. T. C., Boni, M. F., Loi, T. Q., Phuong, L. T., Verhoeven, J. T. P., Crusat, M., Jeeninga, R. E., … De Jong, M. D. (2013). Identification of a New Cyclovirus in Cerebrospinal Fluid of Patients with Acute Central Nervous System Infections. *mBio*, *4*(3), e00231-13. https://doi.org/10.1128/mBio.00231-13

Tei, S., Kitajima, N., Takahashi, K., & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *The Lancet*, *362*(9381), 371–373. https://doi.org/10.1016/S0140-6736(03)14025-1

Thomas, J. E., Gronenborn, B., Harding, R. M., Mandal, B., Grigoras, I., Randles, J. W., Sano, Y., Timchenko, T., Vetten, H. J., Yeh, H.-H., Ziebell, H., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Nanoviridae. *Journal of General Virology*, *102*(3). https://doi.org/10.1099/jgv.0.001544

Väisänen, E., Fu, Y., Hedman, K., & Söderlund-Venermo, M. (2017). Human Protoparvoviruses. *Viruses*, *9*(11), 354. https://doi.org/10.3390/v9110354

Väisänen, E., Kuisma, I., Phan, T. G., Delwart, E., Lappalainen, M., Tarkka, E., Hedman, K., & Söderlund-Venermo, M. (2014). Bufavirus in Feces of Patients with Gastroenteritis, Finland. *Emerging Infectious Diseases*, *20*(6), 1077–1079. https://doi.org/10.3201/eid2006.131674

Väisänen, E., Mohanraj, U., Kinnunen, P. M., Jokelainen, P., Al-Hello, H., Barakat, A. M., Sadeghi, M., Jalilian, F. A., Majlesi, A., Masika, M., Mwaengo, D., Anzala, O., Delwart, E., Vapalahti, O., Hedman, K., & Söderlund-Venermo, M. (2018). Global Distribution of Human Protoparvoviruses. *Emerging Infectious Diseases*, *24*(7), 1292–1299. https://doi.org/10.3201/eid2407.172128

Väisänen, E., Paloniemi, M., Kuisma, I., Lithovius, V., Kumar, A., Franssila, R., Ahmed, K., Delwart, E., Vesikari, T., Hedman, K., & Söderlund-Venermo, M. (2016). Epidemiology of two human protoparvoviruses, bufavirus and tusavirus. *Scientific Reports*, *6*(1), 39267. https://doi.org/10.1038/srep39267

Veettil, S. K., Wong, T. Y., Loo, Y. S., Playdon, M. C., Lai, N. M., Giovannucci, E. L., & Chaiyakunapruk, N. (2021). Role of Diet in Colorectal Cancer Incidence: Umbrella Review of Meta-analyses of Prospective Observational Studies. *JAMA Network Open*, *4*(2), e2037341. https://doi.org/10.1001/jamanetworkopen.2020.37341

Watling, C. Z., Kelly, R. K., Dunneram, Y., Knuppel, A., Piernas, C., Schmidt, J. A., Travis, R. C., Key, T. J., & Perez-Cornago, A. (2023). Associations of intakes of total protein, protein from dairy sources, and dietary calcium with risks of colorectal, breast, and prostate cancer: A prospective analysis in UK Biobank. *British Journal of Cancer*, *129*(4), 636–647. https://doi.org/10.1038/s41416-023-02339-2

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014a). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014b). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and

Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Yahiro, T., Wangchuk, S., Tshering, K., Bandhari, P., Zangmo, S., Dorji, T., Tshering, K., Matsumoto, T., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2014). Novel Human Bufavirus Genotype 3 in Children with Severe Diarrhea, Bhutan. *Emerging Infectious Diseases*, *20*(6), 1037–1039. https://doi.org/10.3201/eid2006.131430

Zhang, W., Li, L., Deng, X., Kapusinszky, B., Pesavento, P. A., & Delwart, E. (2014). Faecal virome of cats in an animal shelter. *Journal of General Virology*, *95*(11), 2553–2564. https://doi.org/10.1099/vir.0.069674-0

Zhang, X., Liang, S., Chen, X., Yang, J., Zhou, Y., Du, L., & Li, K. (2023). Red/processed meat consumption and non-cancer-related outcomes in humans: Umbrella review. *British Journal of Nutrition*, *130*(3), 484–494. https://doi.org/10.1017/S0007114522003415

Zhao, L., Rosario, K., Breitbart, M., & Duffy, S. (2019). Eukaryotic Circular Rep-Encoding Single-Stranded DNA (CRESS DNA) Viruses: Ubiquitous Viruses With Small Genomes and a Diverse Host Range. In *Advances in Virus Research* (Vol. 103, pp. 71–133). Elsevier. https://doi.org/10.1016/bs.aivir.2018.10.001

Zheng, L., Yuan, X., Xie, Z., Jin, Y., Gao, H., Song, J., Zhang, R., Xu, Z., Hou, Y., & Duan, Z. (2010). Human bocavirus infection in young children with acute respiratory tract infection in Lanzhou, China. *Journal of Medical Virology*, *82*(2), 282–288. https://doi.org/10.1002/jmv.21689

Zhu, P., Liu, C., Liu, G.-F., Liu, H., Xie, K.-M., Zhang, H.-S., Xu, X., Xiao, J., & Jiang, J.-Z. (2024). Unveiling CRESS DNA Virus Diversity in Oysters by Virome. *Viruses*, *16*(2), 228. https://doi.org/10.3390/v16020228

Zur Hausen, H. (2009). The search for infectious causes of human cancers: Where and why. *Virology*, *392*(1), 1–10. https://doi.org/10.1016/j.virol.2009.06.001

Zur Hausen, H. (2012). Red meat consumption and cancer: Reasons to suspect involvement of bovine infectious factors in colorectal cancer. *International Journal of Cancer*, *130*(11), 2475–2483. https://doi.org/10.1002/ijc.27413

Zur Hausen, H., Bund, T., & De Villiers, E. (2019). Specific nutritional infections early in life as risk factors for human colon and breast cancers several decades later. *International Journal of Cancer*, *144*(7), 1574–1583. https://doi.org/10.1002/ijc.31882

Zur Hausen, H., Bund, T., & De Villiers, E.-M. (2017). Infectious Agents in Bovine Red Meat and Milk and Their Potential Role in Cancer and Other Chronic Diseases. In E. Hunter & K. Bister (A c. Di), *Viruses, Genes, and Cancer* (Vol. 407, pp. 83–116). Springer International Publishing. https://doi.org/10.1007/82_2017_3

3. Human protoparvoviruses

The earliest identified parvovirus, derived from the Latin term "*parvus*" meaning "small," was the Kilham rat virus (KRV), isolated in 1959 from a rat tumor (Kilham & Olivier, 1959). KRV has been extensively characterized, revealing its widespread prevalence among rodent populations and its capacity to induce fetal mortality or autoimmune diabetes in rats (Chung et al., 2000; Ellerman et al., 1996). In 1965, Atchinson et al.(Atchison et al., 1965) isolated small virus-like particles from rhesus monkey kidney cell cultures infected with simian adenovirus type 15 (SV15), and called adenovirus-associated virus (AAV). Subsequent research identified AAV as a parvovirus; further studies demonstrated that AAV could infect humans without causing disease, highlighting its potential utility in gene therapy (Qiu et al., 2017; Rangarajan et al., 2017).

Human parvovirus B19 (B19V) was first identified in human sera in 1975 (Cossart et al., 1975). B19V infections are highly prevalent, particularly among pediatric populations, characterized by *erythema infectiosum*, commonly referred to as fifth disease (M. J. Anderson et al., 1985). The International Committee on Taxonomy of Viruses (ICTV) officially recognized the family Parvoviridae in 1975.

In 2005, human bocavirus 1 (HBoV1) and PARV4 were discovered via viral metagenomics. HBoV1 was first isolated from respiratory samples of a child with respiratory illness and is strongly linked to respiratory infections, especially in children (Allander et al., 2005; Jones et al., 2005; Maggi et al., 2007; Zheng et al., 2010). Additional bocaviruses (HBoV2-4) were identified in stool samples, with tentative associations to gastrointestinal disease; however, their clinical significance remains less clear. PARV4 was initially found in plasma from an intravenous drug user with acute viral symptoms; its infections often co-occur with other viruses like HIV, HBV and HCV, but its pathogenic role and symptomatology are not fully understood (Jones et al., 2005; Matthews et al., 2014).

In 2012, three additional putative human pathogenic parvoviruses, belonging to *Protoparvirus* genus, bufavirus (BuV), cutavirus (CuV) and tusavirus (TuV) were identified through genomic studies (Phan et al., 2012, 2014, 2016). Protoparvoviruses possess non-enveloped icosahedral capsids with diameters approximately ranging from 18 to 26 nm. Their genomes comprise single-stranded linear DNA molecules spanning 4 to 6 kilobases, each containing terminal palindromic sequences of 100–500 base pairs that adopt duplex hairpin conformations. The viral genome encodes two primary open reading frames (ORFs): one for the structural capsid proteins (VPs) and another for non-structural proteins (NSs) (Väisänen et al., 2017).

The **bufavirus** (BuV) was first identified in fecal samples from children under 5 years of age suffering from diarrhea and negative for rotavirus antigens. In this initial investigation, two distinct genotypes, BuV1 and BuV2, were recognized. In 2014, genotype BuV3 was subsequently isolated from fecal samples of children with diarrhea in Bhutan (Phan et al., 2016). Subsequently, the presence of BuV has been documented across various geographic regions, including Tunisia, Thailand, Turkey, China, Peru, and Finland, in both pediatric and adult populations; among the pediatric groups, BuV DNA prevalence in fecal samples has generally been low, ranging from 0.3% to 4.1%. All positive BuV DNA belonged to genotypes 1 and 3, except for a single case from Burkina Faso where genotype BuV2 was identified, suggesting an extensive geographic distribution of these genotypes. Additionally, in six studies, BuV DNA detection showed higher concentrations during colder months, with most positive samples collected between September and April (Altan et al., 2017; Altay et al., 2015; Ayouni et al., 2016; Chieochansin et al., 2015; Huang et al., 2015; Smits et al., 2014; Väisänen et al., 2014; Yahiro et al., 2014). Beyond fecal analysis, BuV DNA has also been investigated in nasal swabs from children and cerebrospinal fluid (CSF) samples from adults and children. In Finland, 955 nasal swabs collected from children diagnosed with acute gastroenteritis (AGE), acute respiratory infections (ARI), or both were analysed, and BuV DNA was detected at low copy levels in a single sample from a child with both conditions. However, the corresponding fecal sample from this child tested negative for BuV DNA, indicating a possible nonspecific presence or limited involvement of the virus in respiratory samples (Altay et al., 2016; Väisänen et al., 2016, 2017).

In 2016, **cutavirus** (CuV) was identified through viral metagenomic techniques in two diarrheic fecal samples out of 245 from Brazilian children, as well as in a sample from Botswana. The nearly complete genome sequence of CuV, of 4.4 kb (GenBank accession #KT868811), revealed the presence of two main open reading frames (ORFs) corresponding to the NS1 and VP1 genes, with amino acid identities of 76% and 82%, respectively, compared to the phylogenetically closest parvovirus, BuV2 (Phan et al., 2016; Väisänen et al., 2017). These findings suggest that CuV represented a distinct species within the protoparvovirus genus. Additionally, in silico analysis of existing next-generation sequencing (NGS) libraries identified CuV DNA retrospectively in two skin biopsies from French patients with cutaneous T-cell lymphoma (CTCL), thereby naming the virus cutavirus. Subsequently, CuV DNA was also detected via NGS in malignant skin lesions of a Danish patient with melanoma (Mollerup et al., 2017; Phan et al., 2016).

The **tusavirus** was first identified in 2014 in an 18-month-old Tunisian child suffering from diarrhea of unknown etiology, through next-generation sequencing (NGS) analysis (Phan et al., 2014). Infections with tusavirus appeared to be characterized by gastrointestinal symptoms, as also evidenced in two Finnish adults (Mohanraj et al., 2021). Serological studies conducted recently in five countries (Finland, the United States, Iran, Iraq, and Kenya) assessed the prevalence of protoparvoviruses in humans, revealing a low level of seropositivity in the general population (Väisänen et al., 2018). Conversely, earlier serological studies in the United Kingdom detected a high prevalence of ovine parvoviruses in British and Scottish sheep flocks, although the genetic nature of this parvovirus remained unknown (Chappuis et al., 1987). In 2022, a study identified the genome of a parvovirus genetically related to human stool-associated tusavirus, in a high percentage of fecal samples from goats (17.8%) and sheep (25.5%) in Hungary. This finding led to the hypothesis that animals could represent a potential zoonotic source of tusavirus infections in humans (Reuter et al., 2022). Supporting this hypothesis, Mietzsch and colleagues compared the sequence and capsid structure of tusavirus in human and ovine samples, defining this virus as a "hybrid" with molecular characteristics intermediate between primate and non-primate viruses. Additionally, it was observed that tusavirus exhibited a broader glycan-binding affinity compared to human cutavirus, based on its pattern of interaction with cellular sialic acid receptors (Mietzsch et al., 2020; Mohanraj et al., 2021). These findings suggested that tusavirus might have a wider host range than previously thought.

The clinical significance of tusavirus infections in humans and animals remains to be clarified; therefore, it is essential to deepen the understanding of this novel parvovirus's biology to better comprehend its epidemiological role and potential impact on public health.

In **section 3.1**, four abstracts presented at the Congress of the Italian Society of Virology (SIV) in 2022, 2023, 2024, and 2025 are introduced. Briefly, since data on the prevalence and distribution of protoparvoviruses in Italy were unavailable, we explored the circulation of these viruses in various matrices, including bivalve mollusks, wastewater, and animal feces.

A preliminary investigation conducted on 43 bivalve mollusk samples collected between January and April 2022 in Campania detected BuV, TuV, and CuV in 9% of the samples, suggesting environmental contamination and a potential risk of human exposure through seafood consumption.

Meanwhile, we carried out a molecular analysis on environmental samples collected from three wastewater treatment plants (WTPs). Of the 51 samples analyzed, 30 tested positive for the three targets. Specifically, 22% were positive for BuV, 29% for CuV, and 43% for TuV. The positive samples were subsequently characterized in 2024 through Sanger sequencing and phylogenetic analysis. Results indicated that the BuV sequence obtained, when compared to those in GenBank, could belong to genotypes 1 or 3. The CuV sequence showed high identity with a skin biopsy sequence from France and clustered with various skin biopsy and fecal samples from France, Denmark, Brazil, and China. Finally, the TuV sequences divided into two clusters with 92% identity, mixing sequences from human and ovine feces, supporting the hypothesis of a potential zoonotic nature of TuV.

To further investigate the zoonotic potential of TuV, a broader study was conducted, including 848 ovine fecal samples (both feces and swabs), 56 food samples, and 300 wastewater samples. The study found that 7.9% of animal fecal samples and 37.7% of urban wastewater samples tested positive for TuV, with significant regional variation (e.g., 59% in Abruzzo and only 11% in Sicily). No food samples tested positive. RT-nested PCR detected the virus in 14 animal samples and 41 wastewater samples, with some mixed results. Phylogenetic analysis identified two main clusters of sequences: one of animal and environmental origin and the other of ovine and caprine origin, confirming a genetic distance between them (up to 81.1% nucleotide identity). Nearly complete sequences were obtained from some samples, contributing to the understanding of the genetic diversity of the virus.

References

Abbas, A., Taylor, L. J., Collman, R. G., Bushman, F. D., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Redondoviridae: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(1). https://doi.org/10.1099/jgv.0.001526

Allander, T., Tammi, M. T., Eriksson, M., Bjerkner, A., Tiveljung-Lindell, A., & Andersson, B. (2005). Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proceedings of the National Academy of Sciences*, *102*(36), 12891–12896. https://doi.org/10.1073/pnas.0504666102

Altan, E., Del Valle Mendoza, J., Deng, X., Phan, T. G., Sadeghi, M., & Delwart, E. L. (2017). Small Circular Rep-Encoding Single-Stranded DNA Genomes in Peruvian Diarrhea Virome. *Genome Announcements*, *5*(38), e00822-17. https://doi.org/10.1128/genomeA.00822-17

Altay, A., Ocal, M., Yuksek, S. K., Polat, M., Tapisiz, A., Tezer, H., Ergunay, K., Bozdayi, G., & Ahmed, K. (2016). Multicenter investigation of bufavirus in the etiology of viral central nervous system infections of adults and children. *Journal of Clinical Virology*, *82*, S57–S58. https://doi.org/10.1016/j.jcv.2016.08.113

Altay, A., Yahiro, T., Bozdayi, G., Matsumoto, T., Sahin, F., Ozkan, S., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2015). Bufavirus genotype 3 in Turkish children with severe diarrhoea. *Clinical Microbiology and Infection*, *21*(10), 965.e1-965.e4. https://doi.org/10.1016/j.cmi.2015.06.006

Anderson, M. J., Higgins, P. G., Davis, L. R., Willman, J. S., Jones, S. E., Kidd, I. M., Pattison, J. R., & Tyrrell, D. A. J. (1985). Experimental Parvoviral Infection in Humans. *Journal of Infectious Diseases*, *152*(2), 257–265. https://doi.org/10.1093/infdis/152.2.257

Anderson, T. R., & Slotkin, T. A. (1975). Maturation of the adrenal medulla—IV. Effects of morphine. *Biochemical Pharmacology*, *24*(16), 1469–1474. https://doi.org/10.1016/0006-2952(75)90020-9

Atchison, R. W., Casto, B. C., & Hammon, W. McD. (1965). Adenovirus-Associated Defective Virus Particles. *Science*, *149*(3685), 754–756. https://doi.org/10.1126/science.149.3685.754

Ayouni, S., Estienney, M., Hammami, S., Neji Guediche, M., Pothier, P., Aouni, M., Belliot, G., & De Rougemont, A. (2016). Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants. *PLOS ONE*, *11*(9), e0162255. https://doi.org/10.1371/journal.pone.0162255

Barzon, L., Lavezzo, E., Militello, V., Toppo, S., & Palù, G. (2011). Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences*, *12*(11), 7861–7884. https://doi.org/10.3390/ijms12117861

Belák, S., Karlsson, O. E., Blomström, A.-L., Berg, M., & Granberg, F. (2013). New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary Microbiology*, *165*(1–2), 95–101. https://doi.org/10.1016/j.vetmic.2013.01.022

Bernstein, A. S., Ando, A. W., Loch-Temzelides, T., Vale, M. M., Li, B. V., Li, H., Busch, J., Chapman, C. A., Kinnaird, M., Nowak, K., Castro, M. C., Zambrana-Torrelio, C., Ahumada, J. A., Xiao, L., Roehrdanz, P., Kaufman, L., Hannah, L., Daszak, P., Pimm, S. L., & Dobson, A. P. (2022). The costs and benefits of primary prevention of zoonotic pandemics. *Science Advances*, *8*(5), eabl4183. https://doi.org/10.1126/sciadv.abl4183

Bexton, S., Wiersma, L. C., Getu, S., Van Run, P. R., Verjans, G. M. G. M., Schipper, D., Schapendonk, C. M. E., Bodewes, R., Oldroyd, L., Haagmans, B. L., Koopmans, M. M. P., & Smits, S. L. (2015). Detection of Circovirus in Foxes with Meningoencephalitis, United Kingdom, 2009–2013. *Emerging Infectious Diseases*, *21*(7), 1205–1208. https://doi.org/10.3201/eid2107.150228

Blomström, A.-L. (2011). Viral metagenomics as an emerging and powerful tool in veterinary medicine. *Veterinary Quarterly*, *31*(3), 107–114. https://doi.org/10.1080/01652176.2011.604971

Bund, T., Nikitina, E., Chakraborty, D., Ernst, C., Gunst, K., Boneva, B., Tessmer, C., Volk, N., Brobeil, A., Weber, A., Heikenwalder, M., Zur Hausen, H., & De Villiers, E.-M. (2021). Analysis of chronic inflammatory lesions of the colon for BMMF Rep antigen expression and CD68 macrophage interactions. *Proceedings of the National Academy of Sciences*, *118*(12), e2025830118. https://doi.org/10.1073/pnas.2025830118

Capobianchi, M. R., Giombini, E., & Rozera, G. (2013). Next-generation sequencing technology in clinical virology. *Clinical Microbiology and Infection*, *19*(1), 15–22. https://doi.org/10.1111/1469-0691.12056

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE*, *6*(6), e20456. https://doi.org/10.1371/journal.pone.0020456

Chandler, M., De La Cruz, F., Dyda, F., Hickman, A. B., Moncalian, G., & Ton-Hoang, B. (2013). Breaking and joining single-stranded DNA: The HUH endonuclease superfamily. *Nature Reviews Microbiology*, *11*(8), 525–538. https://doi.org/10.1038/nrmicro3067

Chappuis, G., Soulier, M., & Nettleton, P. (1987). Ovine parvovirus infection in Scottish sheep. *Veterinary Record*, *121*(8), 182–182. https://doi.org/10.1136/vr.121.8.182-a

Chieochansin, T., Vutithanachot, V., Theamboonlers, A., & Poovorawan, Y. (2015). Bufavirus in fecal specimens of patients with and without diarrhea in Thailand. *Archives of Virology*, *160*(7), 1781–1784. https://doi.org/10.1007/s00705-015-2441-z

Chung, Y.-H., Jun, H. S., Son, M., Bao, M., Bae, H. Y., Kang, Y., & Yoon, J.-W. (2000). Cellular and Molecular Mechanism for Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats. *The Journal of Immunology*, *165*(5), 2866–2876. https://doi.org/10.4049/jimmunol.165.5.2866

Corpet, D. E. (2011). Red meat and colon cancer: Should we become vegetarians, or can we make meat safer? *Meat Science*, *89*(3), 310–316. https://doi.org/10.1016/j.meatsci.2011.04.009

Cossart, Y. E., Cant, B., Field, A. M., & Widdows, D. (1975). PARVOVIRUS-LIKE PARTICLES IN HUMAN SERA. *The Lancet*, *305*(7898), 72–73. https://doi.org/10.1016/S0140-6736(75)91074-0

Dai, Z., Wang, H., Xu, J., Lu, X., Ni, P., Yang, S., Shen, Q., Wang, X., Li, W., Wang, X., Zhou, C., Zhang, W., & Shan, T. (2024). Unveiling the Virome of Wild Birds: Exploring CRESS-DNA Viral Dark Matter. *Genome Biology and Evolution*, *16*(10), evae206. https://doi.org/10.1093/gbe/evae206

Dayaram, A., Potter, K. A., Moline, A. B., Rosenstein, D. D., Marinov, M., Thomas, J. E., Breitbart, M., Rosario, K., Argüello-Astorga, G. R., & Varsani, A. (2013). High global diversity of cycloviruses amongst dragonflies. *Journal of General Virology*, *94*(8), 1827–1840. https://doi.org/10.1099/vir.0.052654-0

De Villiers, E.-M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T., & Zur Hausen, H. (2019). A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. *Emerging Microbes & Infections*, *8*(1), 1205–1218. https://doi.org/10.1080/22221751.2019.1651620

Decaro, N., Martella, V., Desario, C., Lanave, G., Circella, E., Cavalli, A., Elia, G., Camero, M., & Buonavoglia, C. (2014). Genomic Characterization of a Circovirus Associated with Fatal Hemorrhagic Enteritis in Dog, Italy. *PLoS ONE*, *9*(8), e105909. https://doi.org/10.1371/journal.pone.0105909

Eilebrecht, S., Hotz-Wagenblatt, A., Sarachaga, V., Burk, A., Falida, K., Chakraborty, D., Nikitina, E., Tessmer, C., Whitley, C., Sauerland, C., Gunst, K., Grewe, I., & Bund, T. (2018). Expression and replication of virus-like circular DNA in human cells. *Scientific Reports*, *8*(1), 2851. https://doi.org/10.1038/s41598-018-21317-w

Ellerman, K. E., Richards, C. A., Guberski, D. L., Shek, W. R., & Like, A. A. (1996). Kilham Rat Virus Triggers T-Cell–Dependent Autoimmune Diabetes in Multiple Strains of Rat. *Diabetes*, *45*(5), 557–562. https://doi.org/10.2337/diab.45.5.557

Escudero-Pérez, B., Lalande, A., Mathieu, C., & Lawrence, P. (2023). Host–Pathogen Interactions Influencing Zoonotic Spillover Potential and Transmission in Humans. *Viruses*, *15*(3), 599. https://doi.org/10.3390/v15030599

Falida, K., Eilebrecht, S., Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2017). Isolation of Two Virus-Like Circular DNAs from Commercially Available Milk Samples. *Genome Announcements*, *5*(17), e00266-17. https://doi.org/10.1128/genomeA.00266-17

Fiallo-Olivé, E., Lett, J.-M., Martin, D. P., Roumagnac, P., Varsani, A., Zerbini, F. M., & Navas-Castillo, J. (2021). ICTV Virus Taxonomy Profile: Geminiviridae 2021: This article is part of the ICTV Virus Taxonomy Profiles collection. *Journal of General Virology*, *102*(12). https://doi.org/10.1099/jgv.0.001696

Gainor, K., Malik, Y. S., & Ghosh, S. (2021). Novel Cyclovirus Species in Dogs with Hemorrhagic Gastroenteritis. *Viruses*, *13*(11), 2155. https://doi.org/10.3390/v13112155

Gibb, R., Franklinos, L. H. V., Redding, D. W., & Jones, K. E. (2020). Ecosystem perspectives are needed to manage zoonotic risks in a changing climate. *BMJ*, m3389. https://doi.org/10.1136/bmj.m3389

Gunst, K., Zur Hausen, H., & De Villiers, E.-M. (2014). Isolation of Bacterial Plasmid-Related Replication-Associated Circular DNA from a Serum Sample of a Multiple Sclerosis Patient. *Genome Announcements*, *2*(4), e00847-14. https://doi.org/10.1128/genomeA.00847-14

Gutiérrez-Bugallo, G., Piedra, L. A., Rodriguez, M., Bisset, J. A., Lourenço-de-Oliveira, R., Weaver, S. C., Vasilakis, N., & Vega-Rúa, A. (2019). Vector-borne transmission and evolution of Zika virus. *Nature Ecology & Evolution*, *3*(4), 561–569. https://doi.org/10.1038/s41559-019-0836-z

Habermann, D., Klempt, M., & Franz, C. M. A. P. (2023). Identification and Characterization of Novel SPHINX/BMMF-like DNA Sequences Isolated from Non-Bovine Foods. *Genes*, *14*(7), 1307. https://doi.org/10.3390/genes14071307

Haddadmarandi, M. R., Madani, S. A., Nili, H., & Ghorbani, A. (2020). Molecular survey of avian circoviruses in some non-psittacine birds and detection of a novel canary circovirus in a pigeon. *Iranian Journal of Veterinary Research*, *21*(1), 57–60.

Hao, X., Li, Y., Hu, X., Fu, X., Dong, J., Zhang, H., Zhou, P., & Li, S. (2021). Feline Stool-Associated Circular DNA Virus (FeSCV) in Diarrheic Cats in China. *Frontiers in Veterinary Science*, *8*, 694089. https://doi.org/10.3389/fvets.2021.694089

Huang, D.-D., Wang, W., Lu, Q.-B., Zhao, J., Guo, C.-T., Wang, H.-Y., Zhang, X.-A., Tong, Y.-G., Liu, W., & Cao, W.-C. (2015). Identification of Bufavirus-1 and Bufavirus-3 in Feces of

Patients with Acute Diarrhea, China. *Scientific Reports*, *5*(1), 13272. https://doi.org/10.1038/srep13272

Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer*, *125*(1), 171–180. https://doi.org/10.1002/ijc.24343

Jacob, S. T., Crozier, I., Fischer, W. A., Hewlett, A., Kraft, C. S., Vega, M.-A. D. L., Soka, M. J., Wahl, V., Griffiths, A., Bollinger, L., & Kuhn, J. H. (2020). Ebola virus disease. *Nature Reviews Disease Primers*, *6*(1), 13. https://doi.org/10.1038/s41572-020-0147-3

Jones, M. S., Kapoor, A., Lukashov, V. V., Simmonds, P., Hecht, F., & Delwart, E. (2005). New DNA Viruses Identified in Patients with Acute Viral Infection Syndrome. *Journal of Virology*, *79*(13), 8230–8236. https://doi.org/10.1128/JVI.79.13.8230-8236.2005

Jones-Engel, L., May, C. C., Engel, G. A., Steinkraus, K. A., Schillaci, M. A., Fuentes, A., Rompis, A., Chalise, M. K., Aggimarangsee, N., Feeroz, M. M., Grant, R., Allan, J. S., Putra, A., Wandia, I. N., Watanabe, R., Kuller, L., Thongsawat, S., Chaiwarith, R., Kyes, R. C., & Linial, M. L. (2008). Diverse Contexts of Zoonotic Transmission of Simian Foamy Viruses in Asia. *Emerging Infectious Diseases*, *14*(8), 1200–1208. https://doi.org/10.3201/eid1408.071430

Kamorudeen, R. T., Adedokun, K. A., & Olarinmoye, A. O. (2020). Ebola outbreak in West Africa, 2014 – 2016: Epidemic timeline, differential diagnoses, determining factors, and lessons for future response. *Journal of Infection and Public Health*, *13*(7), 956–962. https://doi.org/10.1016/j.jiph.2020.03.014

Kaner, J., & Schaack, S. (2016). Understanding Ebola: The 2014 epidemic. *Globalization and Health*, *12*(1), 53. https://doi.org/10.1186/s12992-016-0194-4

Kapoor, A., Dubovi, E. J., Henriquez-Rivera, J. A., & Lipkin, W. I. (2012). Complete Genome Sequence of the First Canine Circovirus. *Journal of Virology*, *86*(12), 7018–7018. https://doi.org/10.1128/JVI.00791-12

Kilham, L., & Olivier, L. J. (1959). A latent virus of rats isolated in tissue culture. *Virology*, *7*(4), 428–437. https://doi.org/10.1016/0042-6822(59)90071-6

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023a). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Frölich, K., Jandowsky, A., Knauf-Witzens, T., Langner, C., Dietrich, R., Märtlbauer, E., & Didier, A. (2023b). First Insights into the Occurrence of Circular Single-Stranded DNA Genomes in Asian and African Cattle. *Animals*, *13*(9), 1492. https://doi.org/10.3390/ani13091492

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021a). Circular Rep-Encoding Single-Stranded DNA Sequences in Milk from Water Buffaloes (Bubalus arnee f. Bubalis). *Viruses*, *13*(6), 1088. https://doi.org/10.3390/v13061088

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021b). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

König, M.-T., Fux, R., Link, E., Sutter, G., Märtlbauer, E., & Didier, A. (2021c). Identification and Characterization of Circular Single-Stranded DNA Genomes in Sheep and Goat Milk. *Viruses*, *13*(11), 2176. https://doi.org/10.3390/v13112176

Krupovic, M., & Varsani, A. (2022). Naryaviridae, Nenyaviridae, and Vilyaviridae: Three new families of single-stranded DNA viruses in the phylum Cressdnaviricota. *Archives of Virology*, *167*(12), 2907–2921. https://doi.org/10.1007/s00705-022-05557-w

Krupovic, M., Varsani, A., Kazlauskas, D., Breitbart, M., Delwart, E., Rosario, K., Yutin, N., Wolf, Y. I., Harrach, B., Zerbini, F. M., Dolja, V. V., Kuhn, J. H., & Koonin, E. V. (2020). *Cressdnaviricota*: A Virus Phylum Unifying Seven Families of Rep-Encoding Viruses with Single-Stranded, Circular DNA Genomes. *Journal of Virology*, *94*(12), e00582-20. https://doi.org/10.1128/JVI.00582-20

Kuhnle, G. G. C., & Bingham, S. A. (2007). Dietary meat, endogenous nitrosation and colorectal cancer. *Biochemical Society Transactions*, *35*(5), 1355–1357. https://doi.org/10.1042/BST0351355

Lauber, C., & Seitz, S. (2022). Opportunities and Challenges of Data-Driven Virus Discovery. *Biomolecules*, *12*(8), 1073. https://doi.org/10.3390/biom12081073

Li, L., Kapoor, A., Slikas, B., Bamidele, O. S., Wang, C., Shaukat, S., Masroor, M. A., Wilson, M. L., Ndjango, J.-B. N., Peeters, M., Gross-Camp, N. D., Muller, M. N., Hahn, B. H., Wolfe, N. D., Triki, H., Bartkus, J., Zaidi, S. Z., & Delwart, E. (2010). Multiple Diverse Circoviruses Infect Farm Animals and Are Commonly Found in Human and Chimpanzee Feces. *Journal of Virology*, *84*(4), 1674–1682. https://doi.org/10.1128/JVI.02109-09

Lőrincz, M., Dán, Á., Láng, M., Csaba, G., Tóth, Á. G., Székely, C., Cságola, A., & Tuboly, T. (2012). Novel circovirus in European catfish (Silurus glanis). *Archives of Virology*, *157*(6), 1173–1176. https://doi.org/10.1007/s00705-012-1291-1

Macera, L., Focosi, D., Vatteroni, M. L., Manzin, A., Antonelli, G., Pistello, M., & Maggi, F. (2016). Cyclovirus Vietnam DNA in immunodeficient patients. *Journal of Clinical Virology*, *81*, 12–15. https://doi.org/10.1016/j.jcv.2016.05.011

Maggi, F., Andreoli, E., Pifferi, M., Meschi, S., Rocchi, J., & Bendinelli, M. (2007). Human bocavirus in Italian patients with respiratory diseases. *Journal of Clinical Virology*, *38*(4), 321–325. https://doi.org/10.1016/j.jcv.2007.01.008

Manuelidis, L. (2011). Nuclease resistant circular DNAs copurify with infectivity in scrapie and CJD. *Journal of NeuroVirology*, *17*(2), 131–145. https://doi.org/10.1007/s13365-010-0007-0

Manuelidis, L. (2019). Prokaryotic SPHINX 1.8 REP protein is tissue-specific and expressed in human germline cells. *Journal of Cellular Biochemistry*, *120*(4), 6198–6208. https://doi.org/10.1002/jcb.27907

Matthews, P. C., Malik, A., Simmons, R., Sharp, C., Simmonds, P., & Klenerman, P. (2014). PARV4: An Emerging Tetraparvovirus. *PLoS Pathogens*, *10*(5), e1004036. https://doi.org/10.1371/journal.ppat.1004036

Mietzsch, M., McKenna, R., Väisänen, E., Yu, J. C., Ilyas, M., Hull, J. A., Kurian, J., Smith, J. K., Chipman, P., Lasanajak, Y., Smith, D., Söderlund-Venermo, M., & Agbandje-McKenna, M. (2020). Structural Characterization of Cuta- and Tusavirus: Insight into Protoparvoviruses Capsid Morphology. *Viruses*, *12*(6), 653. https://doi.org/10.3390/v12060653

Miranda, M. N. S., Pingarilho, M., Pimentel, V., Torneri, A., Seabra, S. G., Libin, P. J. K., & Abecasis, A. B. (2022). A Tale of Three Recent Pandemics: Influenza, HIV and SARS-CoV-2. *Frontiers in Microbiology*, *13*, 889643. https://doi.org/10.3389/fmicb.2022.889643

Mohanraj, U., Jokinen, M., Thapa, R. R., Paloniemi, M., Vesikari, T., Lappalainen, M., Tarkka, E., Nora-Krūkle, Z., Vilmane, A., Vettenranta, K., Mangani, C., Oikarinen, S., Fan, Y.-M., Ashorn, P., Väisänen, E., & Söderlund-Venermo, M. (2021). Human Protoparvovirus DNA and IgG in Children and Adults with and without Respiratory or Gastrointestinal Infections. *Viruses*, *13*(3), 483. https://doi.org/10.3390/v13030483

Mollerup, S., Fridholm, H., Vinner, L., Kjartansdóttir, K. R., Friis-Nielsen, J., Asplund, M., Herrera, J. A. R., Steiniche, T., Mourier, T., Brunak, S., Willerslev, E., Izarzugaza, J. M. G., Hansen, A. J., & Nielsen, L. P. (2017). Cutavirus in Cutaneous Malignant Melanoma. *Emerging Infectious Diseases*, *23*(2), 363–365. https://doi.org/10.3201/eid2302.161564

Opriessnig, T., Karuppannan, A. K., Castro, A. M. M. G., & Xiao, C.-T. (2020). Porcine circoviruses: Current status, knowledge gaps and challenges. *Virus Research*, *286*, 198044. https://doi.org/10.1016/j.virusres.2020.198044

Padilla-Rodriguez, M., Rosario, K., & Breitbart, M. (2013). Novel cyclovirus discovered in the Florida woods cockroach Eurycotis floridana (Walker). *Archives of Virology*, *158*(6), 1389–1392. https://doi.org/10.1007/s00705-013-1606-x

Pérot, P., Da Rocha, N., Farcet, M. R., Kreil, T. R., & Eloit, M. (2024). Human Circovirus is not detected in plasma pools for fractionation. *Transfusion*, *64*(1), 16–18. https://doi.org/10.1111/trf.17604

Phan, T. G., Dreno, B., Da Costa, A. C., Li, L., Orlandi, P., Deng, X., Kapusinszky, B., Siqueira, J., Knol, A.-C., Halary, F., Dantal, J., Alexander, K. A., Pesavento, P. A., & Delwart, E. (2016). A new protoparvovirus in human fecal samples and cutaneous T cell lymphomas (mycosis fungoides). *Virology*, *496*, 299–305. https://doi.org/10.1016/j.virol.2016.06.013

Phan, T. G., Luchsinger, V., Avendaño, L. F., Deng, X., & Delwart, E. (2014). Cyclovirus in nasopharyngeal aspirates of Chilean children with respiratory infections. *Journal of General Virology*, *95*(4), 922–927. https://doi.org/10.1099/vir.0.061143-0

Phan, T. G., Vo, N. P., Bonkoungou, I. J. O., Kapoor, A., Barro, N., O'Ryan, M., Kapusinszky, B., Wang, C., & Delwart, E. (2012). Acute Diarrhea in West African Children: Diverse Enteric Viruses and a Novel Parvovirus Genus. *Journal of Virology*, *86*(20), 11024–11030. https://doi.org/10.1128/JVI.01427-12

Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. (2017). Pathways to zoonotic spillover. *Nature Reviews Microbiology*, *15*(8), 502–510. https://doi.org/10.1038/nrmicro.2017.45

Pohl, S., Habermann, D., Link, E. K., Fux, R., Boldt, C. L., Franz, C. M. A. P., Hölzel, C., & Klempt, M. (2022). Detection of DNA sequences attributed to bovine meat and milk factors

(BMMF/SPHINX) in food-related samples. *Food Control*, *135*, 108779. https://doi.org/10.1016/j.foodcont.2021.108779

Prades, Y., Pizarro, R., Ruiz, M., Moreno, C., Avendaño, L. F., & Luchsinger, V. (2021). Cyclovirus detection in Chilean adults with and without community-acquired pneumonia. *Journal of Medical Virology*, *93*(8), 4786–4793. https://doi.org/10.1002/jmv.27080

Qiu, J., Söderlund-Venermo, M., & Young, N. S. (2017). Human Parvoviruses. *Clinical Microbiology Reviews*, *30*(1), 43–113. https://doi.org/10.1128/CMR.00040-16

Quer, J., Colomer-Castell, S., Campos, C., Andrés, C., Piñana, M., Cortese, M. F., González-Sánchez, A., Garcia-Cehic, D., Ibáñez, M., Pumarola, T., Rodríguez-Frías, F., Antón, A., & Tabernero, D. (2022). Next-Generation Sequencing for Confronting Virus Pandemics. *Viruses*, *14*(3), 600. https://doi.org/10.3390/v14030600

Rahman, Md. T., Sobur, Md. A., Islam, Md. S., Ievy, S., Hossain, Md. J., El Zowalaty, M. E., Rahman, A. T., & Ashour, H. M. (2020). Zoonotic Diseases: Etiology, Impact, and Control. *Microorganisms*, *8*(9), 1405. https://doi.org/10.3390/microorganisms8091405

Rangarajan, S., Walsh, L., Lester, W., Perry, D., Madan, B., Laffan, M., Yu, H., Vettermann, C., Pierce, G. F., Wong, W. Y., & Pasi, K. J. (2017). AAV5–Factor VIII Gene Transfer in Severe Hemophilia A. *New England Journal of Medicine*, *377*(26), 2519–2530. https://doi.org/10.1056/NEJMoa1708483

Reuter, G., Pankovics, P., László, Z., Gáspár, G., Hui, A., Delwart, E., & Boros, Á. (2022). Human-stool-associated tusavirus (Parvoviridae) in domestic goats and sheep. *Archives of Virology*, *167*(5), 1307–1310. https://doi.org/10.1007/s00705-022-05424-8

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., & Varsani, A. (2017). Revisiting the taxonomy of the family Circoviridae: Establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Archives of Virology*, *162*(5), 1447–1463. https://doi.org/10.1007/s00705-017-3247-y

Rosario, K., Duffy, S., & Breitbart, M. (2012). A field guide to eukaryotic circular single-stranded DNA viruses: Insights gained from metagenomics. *Archives of Virology*, *157*(10), 1851–1871. https://doi.org/10.1007/s00705-012-1391-y

Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. *Nutrition and Cancer*, *60*(2), 131–144. https://doi.org/10.1080/01635580701684872

Sauvage, V., Gomez, J., Barray, A., Vandenbogaert, M., Boizeau, L., Tagny, C. T., Rakoto, O., Bizimana, P., Guitteye, H., Ciré, B. B., Soumana, H., Tchomba, J.-B. S., Caro, V., & Laperche, S. (2018). High prevalence of cyclovirus Vietnam (CyCV-VN) in plasma samples from Madagascan healthy blood donors. *Infection, Genetics and Evolution*, *66*, 9–12. https://doi.org/10.1016/j.meegid.2018.09.002

Smits, S. L., Schapendonk, C. M. E., Van Beek, J., Vennema, H., Schürch, A. C., Schipper, D., Bodewes, R., Haagmans, B. L., Osterhaus, A. D. M. E., & Koopmans, M. P. (2014). New Viruses in Idiopathic Human Diarrhea Cases, the Netherlands. *Emerging Infectious Diseases*, *20*(7). https://doi.org/10.3201/eid2007.140190

Smits, S. L., Zijlstra, E. E., Van Hellemond, J. J., Schapendonk, C. M. E., Bodewes, R., Schürch, A. C., Haagmans, B. L., & Osterhaus, A. D. M. E. (2013). Novel Cyclovirus in Human Cerebrospinal Fluid, Malawi, 2010–2011. *Emerging Infectious Diseases*, *19*(9). https://doi.org/10.3201/eid1909.130404

Stang, A., Korn, K., Wildner, O., & Überla, K. (2005). Characterization of Virus Isolates by Particle-Associated Nucleic Acid PCR. *Journal of Clinical Microbiology*, *43*(2), 716–720. https://doi.org/10.1128/JCM.43.2.716-720.2005

Takano, T., Yanai, Y., Hiramatsu, K., Doki, T., & Hohdatsu, T. (2018). Novel single-stranded, circular DNA virus identified in cats in Japan. *Archives of Virology*, *163*(12), 3389–3393. https://doi.org/10.1007/s00705-018-4020-6

Tan, L. V., Van Doorn, H. R., Nghia, H. D. T., Chau, T. T. H., Tu, L. T. P., De Vries, M., Canuti, M., Deijs, M., Jebbink, M. F., Baker, S., Bryant, J. E., Tham, N. T., BKrong, N. T. T. C., Boni, M. F., Loi, T. Q., Phuong, L. T., Verhoeven, J. T. P., Crusat, M., Jeeninga, R. E., … De Jong, M. D. (2013). Identification of a New Cyclovirus in Cerebrospinal Fluid of Patients with Acute Central Nervous System Infections. *mBio*, *4*(3), e00231-13. https://doi.org/10.1128/mBio.00231-13

Tei, S., Kitajima, N., Takahashi, K., & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *The Lancet*, *362*(9381), 371–373. https://doi.org/10.1016/S0140-6736(03)14025-1

Thomas, J. E., Gronenborn, B., Harding, R. M., Mandal, B., Grigoras, I., Randles, J. W., Sano, Y., Timchenko, T., Vetten, H. J., Yeh, H.-H., Ziebell, H., & ICTV Report Consortium. (2021). ICTV Virus Taxonomy Profile: Nanoviridae. *Journal of General Virology*, *102*(3). https://doi.org/10.1099/jgv.0.001544

Väisänen, E., Fu, Y., Hedman, K., & Söderlund-Venermo, M. (2017). Human Protoparvoviruses. *Viruses*, *9*(11), 354. https://doi.org/10.3390/v9110354

Väisänen, E., Kuisma, I., Phan, T. G., Delwart, E., Lappalainen, M., Tarkka, E., Hedman, K., & Söderlund-Venermo, M. (2014). Bufavirus in Feces of Patients with Gastroenteritis, Finland. *Emerging Infectious Diseases*, *20*(6), 1077–1079. https://doi.org/10.3201/eid2006.131674

Väisänen, E., Mohanraj, U., Kinnunen, P. M., Jokelainen, P., Al-Hello, H., Barakat, A. M., Sadeghi, M., Jalilian, F. A., Majlesi, A., Masika, M., Mwaengo, D., Anzala, O., Delwart, E., Vapalahti, O., Hedman, K., & Söderlund-Venermo, M. (2018). Global Distribution of Human Protoparvoviruses. *Emerging Infectious Diseases*, *24*(7), 1292–1299. https://doi.org/10.3201/eid2407.172128

Väisänen, E., Paloniemi, M., Kuisma, I., Lithovius, V., Kumar, A., Franssila, R., Ahmed, K., Delwart, E., Vesikari, T., Hedman, K., & Söderlund-Venermo, M. (2016). Epidemiology of two human protoparvoviruses, bufavirus and tusavirus. *Scientific Reports*, *6*(1), 39267. https://doi.org/10.1038/srep39267

Veettil, S. K., Wong, T. Y., Loo, Y. S., Playdon, M. C., Lai, N. M., Giovannucci, E. L., & Chaiyakunapruk, N. (2021). Role of Diet in Colorectal Cancer Incidence: Umbrella Review of Meta-analyses of Prospective Observational Studies. *JAMA Network Open*, *4*(2), e2037341. https://doi.org/10.1001/jamanetworkopen.2020.37341

Watling, C. Z., Kelly, R. K., Dunneram, Y., Knuppel, A., Piernas, C., Schmidt, J. A., Travis, R. C., Key, T. J., & Perez-Cornago, A. (2023). Associations of intakes of total protein, protein from dairy sources, and dietary calcium with risks of colorectal, breast, and prostate cancer: A prospective analysis in UK Biobank. *British Journal of Cancer*, *129*(4), 636–647. https://doi.org/10.1038/s41416-023-02339-2

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014a). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H., & De Villiers, E.-M. (2014b). Novel Replication-Competent Circular DNA Molecules from Healthy Cattle Serum and Milk and Multiple Sclerosis-Affected Human Brain Tissue. *Genome Announcements*, *2*(4), e00849-14. https://doi.org/10.1128/genomeA.00849-14

Yahiro, T., Wangchuk, S., Tshering, K., Bandhari, P., Zangmo, S., Dorji, T., Tshering, K., Matsumoto, T., Nishizono, A., Söderlund-Venermo, M., & Ahmed, K. (2014). Novel Human Bufavirus Genotype 3 in Children with Severe Diarrhea, Bhutan. *Emerging Infectious Diseases*, *20*(6), 1037–1039. https://doi.org/10.3201/eid2006.131430

Zhang, W., Li, L., Deng, X., Kapusinszky, B., Pesavento, P. A., & Delwart, E. (2014). Faecal virome of cats in an animal shelter. *Journal of General Virology*, *95*(11), 2553–2564. https://doi.org/10.1099/vir.0.069674-0

Zhang, X., Liang, S., Chen, X., Yang, J., Zhou, Y., Du, L., & Li, K. (2023). Red/processed meat consumption and non-cancer-related outcomes in humans: Umbrella review. *British Journal of Nutrition*, *130*(3), 484–494. https://doi.org/10.1017/S0007114522003415

Zhao, L., Rosario, K., Breitbart, M., & Duffy, S. (2019). Eukaryotic Circular Rep-Encoding Single-Stranded DNA (CRESS DNA) Viruses: Ubiquitous Viruses With Small Genomes and a Diverse Host Range. In *Advances in Virus Research* (Vol. 103, pp. 71–133). Elsevier. https://doi.org/10.1016/bs.aivir.2018.10.001

Zheng, L., Yuan, X., Xie, Z., Jin, Y., Gao, H., Song, J., Zhang, R., Xu, Z., Hou, Y., & Duan, Z. (2010). Human bocavirus infection in young children with acute respiratory tract infection in Lanzhou, China. *Journal of Medical Virology*, *82*(2), 282–288. https://doi.org/10.1002/jmv.21689

Zhu, P., Liu, C., Liu, G.-F., Liu, H., Xie, K.-M., Zhang, H.-S., Xu, X., Xiao, J., & Jiang, J.-Z. (2024). Unveiling CRESS DNA Virus Diversity in Oysters by Virome. *Viruses*, *16*(2), 228. https://doi.org/10.3390/v16020228

Zur Hausen, H. (2009). The search for infectious causes of human cancers: Where and why. *Virology*, *392*(1), 1–10. https://doi.org/10.1016/j.virol.2009.06.001

Zur Hausen, H. (2012). Red meat consumption and cancer: Reasons to suspect involvement of bovine infectious factors in colorectal cancer. *International Journal of Cancer*, *130*(11), 2475–2483. https://doi.org/10.1002/ijc.27413

Zur Hausen, H., Bund, T., & De Villiers, E. (2019). Specific nutritional infections early in life as risk factors for human colon and breast cancers several decades later. *International Journal of Cancer*, *144*(7), 1574–1583. https://doi.org/10.1002/ijc.31882

Zur Hausen, H., Bund, T., & De Villiers, E.-M. (2017). Infectious Agents in Bovine Red Meat and Milk and Their Potential Role in Cancer and Other Chronic Diseases. In E. Hunter & K. Bister (A c. Di), *Viruses, Genes, and Cancer* (Vol. 407, pp. 83–116). Springer International Publishing. https://doi.org/10.1007/82_2017_3

Section 3.1

Exploring the circulation of protoparvovirus through environmental surveillance

T Vicenza[1], Carolina Veneri[2], Pamela Mancini[2], Giusy Bonanno Ferraro[2], Claudia Del Giudice[2], Marcello Iaconelli[2], L. Orlandi[1], A Boni[1], S Di Pasquale[1], L Cozzi[1], YTR Proroga[3], E. Suffredini[1], G. La Rosa[2]
[1] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, National Reference Laboratory for Foodborne Viruses, Rome, Italy
[2] Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy
[3] Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici (NA), Italy

Protoparvovirus is a genus of non-enveloped, ssDNA viruses within the Parvoviridae family. Two species, bufavirus (BuV) and tusavirus (TuV), described in 2012 and 2014 respectively, have been reported in association with gastroenteritis in children, while a third species, cutavirus (CuV), has been detected in both faecal specimens and skin biopsies, and has been later associated with the development of cutaneous T-cell lymphoma. Although BuV has been previously investigated in a group of children displaying symptoms of gastroenteritis, data on the prevalence and distribution of protoparvoviruses are currently unavailable in Italy. Aim of this preliminary study was exploring the circulation of BuV, TuV and CuV in the population through environmental surveillance.

A total of 51 samples (24h composite) were collected monthly between February 2021 and March 2023 from three wastewater treatment plants (WTPs) in Rome. Samples were concentrated by PEG precipitation and nucleic acids were extracted according to a standardized protocol and analysis was undertaken by real-time qPCR using previously described primers/probes. For target quantification, an in vitro synthetized template was used, and the quality of the analysis was ensured using recovery and inhibition controls.

Thirty samples (59%) tested positive for at least one of the viruses. Specifically, BuV was detected in 11 samples (22%), TuV in 22 (43%) and CuV in 15 (29%). Viral concentrations in positive samples were generally low and, considering WTP daily flow rates and contributing population, the median excretion was estimated to be 2.2×10^2 (range 5.5×10^1 - 4.3×10^2) genome copies / inhabitant*die for BuV, 4.5×10^2 (range 5.2×10^1 - 2.6×10^3) for TuV, and 3.5×10^2 (range 1.2×10^2 - 5.6×10^2) for CuV. Although no clear seasonal trend was observed in viral occurrence, most of the positive samples and higher TuV concentrations were obtained in samples collected beween late winter and early summer (February to July).

The results of this waterwater surveillance study highlights a significant ciruclation of BuV, TuV and CuV within the population. However, further studies are needed to define the potential impact of these viruses on human health.

Occurrence of protoparvoviruses in bivalve mollusks: a preliminary study

A Mancusi[2], T Vicenza[1], O Dimaro[2], A Boni[1], E Suffredini[1], YTR Proroga[2]

[1] Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici (NA), Italy
[2] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, National Reference Laboratory for Foodborne Viruses, Rome, Italy

Protoparviruses are small, non-enveloped, ssDNA viruses within the family Parvoviridae. The constant progress in metagenomic sequencing has led, in the last decade, to the discovery of three members of the Protoparvovirus genus infecting humans: i) bufavirus (BuV), first detected in 2012 in the diarrhoeic faeces of children from Burkina Faso; ii) tusavirus (TuV), discovered in 2014 in the faeces of a Tunisian child with diarrhea of unknown origin; iii) cutavirus (CuV), reported in 2016 in faecal and cutaneous specimens and since then associated with cutaneous T-cell lymphoma. Studies on the occurrence of these protoparvoviruses has been undertaken in some EU countries, but data on their circulation in the Italian population are scarce. Bivalve mollusks are filter feeder animals which may accumulate to significant levels microorganisms present in their seawater growing areas. Due to this, they are often used for indirect monitoring of environmental contaminants and/or of microorganisms originating by the discharge of wastewater into the sea. Aim of this study was a preliminary investigation of the occurrence of protoparvoviruses in bivalve mollusks, as a marker of their circulation in the population.
A total of 43 bivalve bivalve shellfish samples (mussels, *Mytilus galloprovincialis*), collected between January and April 2022 in 15 production areas of the Region of Campania, were tested. Samples were prepared by dissection and lysis of the digestive tissue, according to ISO 15216. Detection of BuV, TuV and CuV was performed by real-time PCR using previously described primers/probes (Väisänen et al., 2014; Väisänen et al., 2019). The three protoparvoviruses were detected in 4 samples (9%). In detail, BuV and CuV occurred in 2 samples, respectively, while TuV was detect in one of the samples already positive for BuV. Positive samples were all characterized by high *Cq* values (>38) and had been all collected between the end of February and first part of March. Interestingly, both CuV-positive samples had been taken in concecutive samplings from the same shellfish growing area, indicating either a steady or a recurring contamination. Further and more extensive studies are required to assess the circulation of these parvoviruses at national level.

Väisänen E, Kuisma I, Phan TG, et al. Bufavirus in Feces of Patients with Gastroenteritis, Finland. Emerg Infect Dis. 2014;20(6):1077-1079.
Väisänen E, Fu Y, Koskenmies S, et al. Cutavirus DNA in Malignant and Nonmalignant Skin of Cutaneous T-Cell Lymphoma and Organ Transplant Patients but Not of Healthy Adults. Clin Infect Dis 2019;68(11):1904-1910.

Molecular characterization of protoparvovirus from urban wastewater

T Vicenza[1], C Veneri[2], P Mancini[2], G Bonanno Ferraro[2], M Iaconelli[2], L. Orlandi[1], S Di Pasquale[1], L Cozzi[1], G. La Rosa[2], E. Suffredini[1], A Boni[1]

[1] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, National Reference Laboratory for Foodborne Viruses, Rome, Italy
[2] National Center for Water Safety (CeNSia), Istituto Superiore di Sanità, Rome, Italy

In the last decade, advancements in metagenomic sequencing have led to the detection of three human-infecting Protoparvoviruses within the Parvoviridae family: bufavirus (BuV), tusavirus (TuV), and cutavirus (CuV). BuV was first found in 2012 in the feces of children in Burkina Faso, TuV in 2014 in a Tunisian child with unexplained diarrhoea, and CuV in 2016 in faecal and skin samples linked to cutaneous T-cell lymphoma. The presence of these protoparvoviruses has been studied in some EU countries, but there is limited information on their prevalence in the Italian population. In a preliminary study, we investigated the precence of these Protoparvoviruses in samples from wastewater treatment plants (WTPs) in Rome. Samples were concentrated by PEG precipitation, and nucleic acids were extracted following a standardized protocol. Analysis was carried out using real-time qPCR with specific primers and probes and showed the presence of at least one of the viruses in 59% of the samples. Specifically, BuV was present in 11 samples (22%), TuV in 22 samples (43%), and CuV in 15 samples (29%).

In this study, an attempt was made to characterize the viruses in the positive samples using nested PCR with specific primers designed to amplify the BuV NS1, CuV VP1, and TuV NS1 regions [1, 2, 3]. Subsequently, these samples were subjected to Sanger sequencing and phylogenetic analysis. Amplification was obtained for BuV in one sample, for TuV in four samples and for CuV in one sample. Comparison of the sequence obtained with sequences available in GenBank showed that the BuV detected belonged to genotype 1 or 3 (100% identity to genotype 1 sequence KM580347 and 99.5% identity to genotype 3 sequence AB982218), but complete discrimination was not possible due to the high identity of the two genotypes in the sequenced region. The sequence obtained from the CuV-positive sample showed the highest identity with a skin biopsy from France (98.1%; KT868815) and clustered with several sequences from skin and stool samples from France, Denmark, Brazil, and China. Finally, the four TuV sequences clearly differentiated into two clusters with 92% identity. In BLAST analysis, these sequences intermixed with sequences from human and ovine faecal samples, supporting the hypothesis that this virus may have a zoonotic potential. Further studies are required to provide a complete genome characterization of protoparvovirus viral strains occurring in the human population.

[1] Phan TG, Vo NP, Bonkoungou IJO, et al. Acute diarrhea in west African children: diverse enteric viruses and a novel Parvovirus genus. J Virol. doi: 10.1128/JVI.01427-12
[2] Phan TG, Dreno B, da Costa AC, et al. A new protoparvovirus in human fecal samples and cutaneous T cell lymphomas (mycosis fungoides). Virology. doi:10.1016/j.virol.2016.06.013
[3] Phan TG, Sdiri-Loulizi K, Aouni M, et al. New Parvovirus in child with unexplained diarrhea, Tunisia. EID. doi:10.3201/eid2011.140428

Exploring occurrence and genetic diversity of tusavirus in animals, food and environmental matrices

Authors:
Vicenza T. [1], Boni A. [1], Suffredini E. [1]
La Rosa G. [2], C. Veneri. [2],
V. Sarchese[3], F. Di Profio[3], B. Di Martino[3]
V. Martella[4], G. Lanave[4], F. Pellegrini[4]

† These authors equally contributed

Affiliations:
[1] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy
[2] National Center for Water Safety (CeNSia), Istituto Superiore di Sanità, Rome, Italy
[3] Department of Veterinary Medicine, University of Teramo, Italy
[4] Department of Veterinary Medicine, University of Bari Aldo Moro, Italy

Aim: Tusavirus (TuV) are non-enveloped, ssDNA viruses within the Parvoviridae family, first described in 2014 in a Tunisian child with unexplained diarrhoea. Following their first reporting, these viruses have been reported in multiple countries and, more recently, in animal hosts (goat and sheeps). To improve the understanding of TuV circulation and to assess the hypothesis of their possible zoonotic transmission, a study on TuV occurrence and genetic diversity in animal, food and environmental matrices was undertaken.

Methods: Animal, food and environmental samples were included in the study to explore occurrence of Tusavirus in livestock, foods of animal origin and humans. Overall, 848 fecal samples (stools or rectal swabs) were collected from ovicaprines in farms or at slaughtering in the Regions of Abruzzo, Molise, Apulia, Basilicata and Calabria, 56 food samples (sheep and goat milk, meat and meat products of bovine and swine origin) were collected from different Italian regions including Abruzzo, Latium, and the autonomous province of Bolzano, and 300 urban wastewater samples were taken from major cities of Latium, Piedmont, Sicily and Bolzano province. Samples were treated with matrix-specific protocol to extract viral nucleic acids and presence of TuV was assessed by real-time RT-PCR according to Vaisanen et al., 2019 [ref]. Characterization of positive samples was attempted by nested RT-PCR [ref] followed by Sanger sequencing (NS1 gene). Full genome sequencing using an Artic-like strategy and MinION ONT platform was performed on selected samples.

Results: Sixty-seven (7.9%) animal fecal samples and 113 (37.7%) urban wastewater tested positive for TuV. Significant regional differences in virus occurrence were detected, e.g. TuV were present in 59%, 53%, 50%, 21% and 11% of wastewater samples from Abruzzo, Piedmont, Bolzano, Latium and Sicily, respectively, and uneven distribution of positive samples was also evident for animal samples. No food sample tested positive for TuV. Nested RT-PCR amplification was achieved for 14 animal fecal samples and for 41 wastewater samples (4 of which with mixed electropherograms). Phylogenetic analysis of the fragment showed clustering of sequences into two major clusters supported by high bootstrap values, with one cluster encompassing both sequences of animal and environmental origin, and the other including sequences from ovine and caprine samples. Complete or almost complete genome sequence was obtained for three animal samples and two wastewater samples, and phylogenetic analysis confirmed genetic distance between the two major clusters (nucleotide identity as low as 81.1%).

Conclusions: This study highlights a significant occurrence of Tusavirus in ovicaprines and in urban wastewater samples (representing the communities of the collected areas). Furthermore it provides molecular characterization of the strains circulating in both the animal and human populations, providing new information on the genetic diversity of this virus.

4. Conclusion

The comprehensive exploration undertaken in this thesis underscores the critical importance of advancing our understanding of emerging and re-emerging viral pathogens, particularly those with zoonotic potential. As demonstrated through the multifaceted analysis of CRESS DNA viruses, BMMF molecules, and human protoparvoviruses, the landscape of viral diversity is vast and continually expanding, driven by analytical innovations such as next-generation sequencing and viral metagenomics.

The study of CRESS DNA viruses, especially circoviruses, highlights their ubiquity across species and environments, as well as their potential to infect domestic animals like cats and dogs. The detection of these viruses in feline populations and the genetic diversity uncovered in Italian samples emphasize the necessity for ongoing epidemiological surveillance. Such efforts are vital to clarify their pathogenic roles, assess risks of spillover, and develop effective control measures. Moreover, the identification of circoviruses in human-associated samples and in various environmental matrices raises critical questions about their zoonotic potential and possible contributions to disease processes, including neurological and gastrointestinal disorders.

Similarly, the investigation into BMMF molecules presents an intriguing hypothesis linking dietary exposure to bovine-derived products with chronic inflammatory states and carcinogenesis. The widespread presence of BMMFs in food, water, and animal samples suggests a complex web of transmission pathways that merit further exploration. While epidemiological data have yet to definitively establish causality, the potential association between these molecules and cancers such as colorectal and breast cancer underscores the importance of comprehensive risk assessments and the development of molecular detection strategies to monitor their prevalence.

Finally, the analysis of human protoparvoviruses expands our awareness of the diversity and distribution of these small DNA viruses. The discovery of novel genotypes, along with evidence of their presence in environmental, animal, and human samples, highlights the dynamic nature of parvovirus evolution and host interactions. The detection of tusavirus in animal feces and environmental samples, along with phylogenetic analyses suggesting zoonotic links, reinforces the necessity for integrated One Health approaches. These approaches should aim to elucidate transmission pathways, assess pathogenicity, and evaluate potential public health impacts, especially given the low seroprevalence yet widespread detection of these viruses.

Viral emergence is a multifactorial process influenced by ecological, anthropogenic, and biological factors. Human activities such as wildlife trade, deforestation, intensive farming, and environmental contamination are pivotal in facilitating spillover events. The recent SARS-CoV-2 pandemic exemplifies how rapidly a zoonotic virus can adapt and disseminate globally, highlighting the urgent need for robust surveillance systems, early detection capabilities, and international collaboration. The integration of advanced molecular techniques and bioinformatics has provided unprecedented insights into viral diversity, evolution, and epidemiology. These tools are essential for identifying potential zoonotic threats before they escalate into outbreaks. Continued research into the molecular biology of these viruses, their transmission dynamics, and their ecological niches will be instrumental in developing predictive models and preventive strategies.

In conclusion, this thesis underscores the imperative for a proactive, multidisciplinary approach to viral surveillance, encompassing wildlife, domestic animals, humans, and the environment. Strengthening our understanding of viral ecology and evolution will aid in early detection, risk assessment, and the development of targeted interventions to mitigate the impact of emerging viral diseases. As the global community faces ongoing and future pandemics, fostering collaboration across scientific disciplines, public health, veterinary medicine, and policy-making will be vital to safeguard health and resilience in an increasingly interconnected world.