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Innovative approaches in diagnosing and characterizing emerging viruses

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Preface

The original research works reported and discussed in this thesis were carried out at the Department of Veterinary Medicine, University of Bari. The thesis is written in as “by publication” setup and features accepted and under-evaluation manuscripts of the selected topic. The author contributed to the analyses, experiments and writing of all reported scientific papers.

General introduction

Viral infectious diseases are a significant threat to world's public health and significant economic implications (Misu et al., 2023). Viruses account for a significant proportion of emerging infectious diseases (EIDs), and the majority (about 80%) are zoonotic in origin (Morse et al., 2012). Among zoonotic viruses, Ebolavirus (Hussein, 2023), human immunodeficiency virus (HIV) (Russotto et al., 2022), Hendra and Nipah virus (Eaton et al., 2006) , hepatitis E virus (Park et al., 2016), severe acute respiratory syndrome (SARS) type 1 and 2 (SARS-CoV-2) coronavirus (CoV), Middle East respiratory syndrome (MERS) CoV(Goal et al., 2022; Rathore and Ghosh, 2020), rotavirus (Martella et al., 2010), norovirus (Wilhelm et al., 2015), rabies virus (Kumar et al., 2023) and influenza viruses represent the most significant pathogens reported to date (Kessler et al., 2021).

Zoonotic viruses can be transmitted to humans directly or indirectly from animals. Close interaction between people and infected vertebrate animals (pets, livestock, or wildlife) and indirect contact with their environment are key aspects of zoonotic disease transmission (Rahman et al., 2020).

Direct interaction with an animal infected with zoonotic pathogens may cause infection generally via oronasal route or through skin lesions. Body fluids, secretions, excretions are also potential sources of infection. For instance, saliva during rabies infection could be a source of transmission to humans through bites of rabid carnivores (dog, bat, monkey, skunk, raccoon, or fox) (Kumar et al., 2023). Another example of direct transmission of zoonoses is avian influenza, a viral disease spreading from animals to humans through droplets or fomites (Peacock et al., 2024). Viral pathogens such as yellow fever virus can be also transmitted to human via vectors (i.e. arthropods like mosquitoes and ticks) (Srivastava et al., 2024). Food-borne transmission has been described as one of the modes of transmission for many different viruses, associated with diseases ranging from mild diarrhea to severe neurological symptoms (Olaimat et al., 2024). Norovirus, hepatitis A and E viruses, rotavirus, astrovirus, adenovirus, and sapovirus are the major common viruses associated with water or foodborne illness outbreaks. It is also suspected that many human viruses including Aichi virus, Nipah virus, tick-borne encephalitis virus, H5N1 avian influenza viruses, and coronaviruses (SARS-CoV-1, SARS-CoV-2 and MERS-CoV) also have the potential to be transmitted via food products (Olaimat et al., 2024).

Since the beginning of 1900, viruses have been detected and identified using various techniques. Conventional approaches involve electron microscopy (EM), cell culture inoculation studies and serology. Electron microscopy provides an objective approach for visualizing virus-like particles (VLPs) in clinical samples, cell culture fluids, and tissue sections. However, its sensitivity is limited,

particularly when applied directly to clinical specimens, as effective visualization requires minimum virus concentration of approximately 10^5 to 10^6 particles per milliliter of specimen. Several techniques can enhance the sensitivity of EM screening: increase virus concentration in the specimen by ultracentrifugation, ultrafiltration, and agar diffusion or employing immunoelectron (IE) microscopy. IE microscopy involves incubating clinical samples suspected of containing a virus with convalescent serum from the patient before observation (Kapikian et al., 1972;Feinstone et al., 1973).

Serological detection methods rely on the principle of antigen–specific antibody binding by detecting antibody levels in the host's biological fluids (blood serum or plasma, milk, semen, etc). The main serological detection methods are chemiluminescence immunoassay (CLIA), enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) (Mekonnen et al., 2020;Ejazi et al., 2021). These methods all have their own advantages and disadvantages, based on detection efficiency, system cost, the convenience of operation, and diagnosis restrictions (Kubina and Dziedzic, 2020)

Development of nucleic acid sequence-dependent techniques such as (PCR) (Saiki et al., 1985, Scharf et al., 1986) and more recently microarrays and subtractive hybridization-based methods (Wang, et al., 2002) simplified the diagnosis of viral infections. These methods rely on sensitive detection of specific viral nucleic acids. Due to the prior requirement of sequence information to design primers or hybridization probes and knowledge of closely related viral genomes, these techniques were applied for discovery of new genotypes of known viruses rather than novel, non-sequenced viruses (Pozzetto, 2002).

Metagenomics represent a culture- and sequence-independent approach enabling the detection and identification of microorganisms present in a sample (Wooley and Ye, 2010). Sequence-Independent Amplifications (SIA) techniques such as sequence-independent single-primer amplification (SISPA), rolling circle amplification (RCA) and Degenerate oligonucleotide primed PCR (DOP-PCR) were developed. SISPA represents a random priming method to achieve the non-selective logarithmic amplification of heterogeneous DNA populations (Reyes and Kim, 1991). Genetic sequences are previously converted to double-stranded DNA, followed by non-specific linker ligation and PCR amplification (Allander et al., 2001). SISPA has been used to identify novel viral nucleic acid in various types of samples (Allander et al., 2001, Jones et al., 2005). RCA is an highly effective approach to amplifying circular DNA.. such as plasmids, circular viral genomes, or circularized DNA fragments and relies on the activity of a DNA polymerase enzyme with strand displacement capability

(Ali et al., 2014). The most well-known DNA polymerase enzyme used for RCA is Phi29 DNA polymerase, extracted from bacteriophage phi29 infecting *Bacillus subtilis* (Silander and Saarela, 2008). This technique can be used both for the amplification of known viruses using specific primers and for unknown viruses using non-specific primers (Johne et al., 2009). DOP-PCR was initially developed for genome mapping studies (Telenius et al., 1992), but has emerged as an effective strategy for enriching viral genomic content in whole genome sequencing (WGS) applications (Nanda et al., 2008). This technique is particularly valuable when working with samples containing low concentrations of DNA/RNA viruses where standard library preparation methods may prove insufficient (Gawad et al., 2016). DOP-PCR employs a set of degenerate oligonucleotide primers to non-specifically amplify genetic material across the viral genome. This approach ensures broad coverage and maximizes the likelihood of capturing diverse genomic regions, enhancing downstream sequencing outcomes (Blagodatskikh et al., 2017).

Microbial identification by PCR-based DNA amplification must be confirmed by sequencing of the amplification product. The first sequencing method relied on the Sanger's dideoxy chain termination protocol became instantly popular and it was successfully commercialized into automated DNA sequencing equipment (Sanger et al., 1977). The method was based on DNA labelling with fluorescent chain terminating inhibitors, capillary electrophoresis of the amplicons and detection of fluorescence emission. Sangers'(???) method was used successfully for sequencing templates of up to 1000 base pairs (bp). However, this sequence method is constrained by its low throughput, higher (higher compared to what?) cost, time and labor involved in sequencing larger (larger than what?) genomes. Moreover, the method was completely dependent on specific primers and unable to sequence the genetic material of different microorganisms, thus limiting the use in metagenomic approaches.

Limitations of Sanger sequencing technology in metagenomics prompted research into novel DNA sequencing approaches, known as Next-Generation Sequencing (NGS) (Margulies et al., 2005). NGS techniques displayed a large number of innovations in the amplification technology, sequencing chemistry, microfluidics, imaging technologies and bioinformatics (Buermans and den Dunnen 2014). The development of advanced next generation sequencing (NGS) technologies, like those from Illumina, Ion Torrent, Pacific Biosciences, and Oxford Nanopore, has revolutionized genomics. These technologies enable the sequencing of millions to billions of DNA fragments in a single reaction, making DNA analysis faster and more efficient than ever before (Rhoads and Au, 2015; Vaser et al., 2017) . NGS for molecular diagnosis of infectious diseases could rely on targeted

amplicon and metagenomic sequencing. The former is more efficient and accurate for the identification of a known pathogen (Quick et al., 2017). Metagenomic sequencing can produce the whole genome sequence (WGS) of an emerging, unknown pathogen or that of a mixed population of pathogens with very large depth of sequencing coverage and high throughput. However, the use of the certain method is impaired by high noise to signal ration caused by host or background DNA (Kafetzopoulou et al., 2018; Deng et al., 2020). WGS methods have been developed for many viruses that cause endemic or emerging diseases, including influenza A virus and influenza B viruses (Imai et al., 2018).

To fully benefit from depth of sequencing of viral pathogens in the samples, host DNA/RNA should previously be removed from samples (Temmam et al., 2014). Protocols such as sample filtration nuclease digestion, ultracentrifugation, and random pre-amplification of RNA or DNA have been reported in several metagenomics studies coupled with NGS (Wang et al., 2018). The amount of total nucleic acids isolated from viral particles is often too low for sequencing and may require amplification of viral nucleic acid (Bulcha et al., 2017). Enrichment can be achieved directly through virus isolation or the use of oligonucleotide bait probes targeting the study virus, or indirectly via host nucleic acid depletion. PCR can provide both target enrichment and amplification in a single step, is relatively cheap, and fast compared with other methods.

Nanopore sequencing technology has been extensively employed in research since 2014, when Oxford Nanopore Technologies (ONT) developed MinION sequencer (Jain et al., 2016), To date, nanopore sequencing has found applications in the identification of clinically relevant viruses (Greninger et al., 2015; Davidson et al., 2020), the assembly of new reference genomes (Viehweger et al., 2019), the detection of DNA and RNA variations (Kim et al., 2020; Price et al., 2020), outbreak surveillance (Lu et al., 2020) and on-site metagenomics research (Boykin et al., 2019). Nanopore sequencing technology is capable of sequencing single long strands of DNA or RNA, thereby enhancing the quality of sequencing and throughput (Wang et al., 2021). It offers a number of benefits over traditional methods, including reduced turnaround times and lower equipment cost (Petersen et al., 2019). The technology also provides unique advantages in terms of convenience, real-time genomic surveillance, and data analysis (Ji et al., 2024).

NGS generates large amounts of data that require complex bio-informatics processing. Bioinformatics is the discipline that conceptualizes biology in terms of macromolecules and then applies informatics (applied math, computer science, and statistics) to analyse and organize the

information associated with these molecules, on a large scale (Viehweger et al., 2019). NGS-based bioinformatics analytics are designed to convert raw data into nucleotide bases with associated quality scores, known as "reads". Data are further processed and assessed for quality, trimmed, and filtered based on laboratory established quality thresholds. The sequence reads are either aligned to a reference sequence, or in the absence of a reference, assembled to create a full length sequence using a process referred to as de novo assembly (Chiu et al., 2016). De novo assembly constitutes a cornerstone of novel virology, especially when dealing with viruses for which there is not genetic reference database (Khan et al., 2018; Wee et al., 2019). This technique involves reconstructing complete or near-complete viral genomes using only on the sequence reads without relying on pre-existing genomic information (Cosma et al., 2023) Finally pathogen identification, variant calling, functional annotation, taxonomic classification are able to interpret genetic data obtained (Gargis et al., 2016).

References

Ali MM, Li F, Zhang Z, Zhang K, Kang DK, Ankrum JA, Le XC, Zhao W. Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. Chem Soc Rev. 2014 May 21;43(10):3324-41. doi: 10.1039/c3cs60439j. Epub 2014 Mar 18. PMID: 24643375.

Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11609-14. doi: 10.1073/pnas.211424698. Epub 2001 Sep 18. PMID: 11562506; PMCID: PMC58777.

Blagodatskikh KA, Kramarov VM, Barsova EV, Garkovenko AV, Shcherbo DS, Shelenkov AA, Ustinova VV, Tokarenko MR, Baker SC, Kramarova TV, Ignatov KB. Improved DOP-PCR (iDOP-PCR): A robust and simple WGA method for efficient amplification of low copy number genomic DNA. PLoS One. 2017 Sep 11;12(9):e0184507. doi: 10.1371/journal.pone.0184507. PMID: 28892497; PMCID: PMC5593185.

Boykin LM, Sseruwagi P, Alicai T, Ateka E, Mohammed IU, Stanton JL, Kayuki C, Mark D, Fute T, Erasto J, Bachwenkizi H, Muga B, Mumo N, Mwangi J, Abidrabo P, Okao-Okuja G, Omuut G, Akol J, Apio HB, Osingada F, Kehoe MA, Eccles D, Savill A, Lamb S, Kinene T, Rawle CB, Muralidhar A, Mayall K, Tairo F, Ndunguru J. Tree Lab: Portable genomics for Early Detection of Plant Viruses and Pests in Sub-Saharan Africa. Genes (Basel). 2019 Aug 21;10(9):632. doi: 10.3390/genes10090632. PMID: 31438604; PMCID: PMC6769854.

Buermans, H.P., den Dunnen, J.T., 2014. Next generation sequencing technology: Advances and applications. Biochim Biophys Acta 1842, 1932–1941.

Bulcha, B., Berhane, G., Merera, O., 2017. Review on viral metagenomics and its future perspective in. Zoonotic and arboviral disease surveillance. J Biol Agric Healthcare, 7(21), 35–41.

Cosma BM, Shirali Hossein Zade R, Jordan EN, van Lent P, Peng C, Pillay S, Abeel T. Evaluating long-read de novo assembly tools for eukaryotic genomes: insights and considerations. Gigascience. 2022 Dec 28;12:giad100. doi: 10.1093/gigascience/giad100. Epub 2023 Nov 24. PMID: 38000912; PMCID: PMC10673639.

Davidson AD, Williamson MK, Lewis S, Shoemark D, Carroll MW, Heesom KJ, Zambon M, Ellis J, Lewis PA, Hiscox JA, Matthews DA. Characterisation of the transcriptome and proteome of SARS-CoV-2 reveals a cell passage induced in-frame deletion of the furin-like cleavage site from the spike glycoprotein. Genome Med. 2020 Jul 28;12(1):68.

doi: 10.1186/s13073-020-00763-0. PMID: 32723359; PMCID: PMC7386171.

Deng X, Achari A, Federman S, Yu G, Somasekar S, Bártolo I, Yagi S, Mbala-Kingebeni P, Kapetshi J, Ahuka-Mundeke S, Muyembe-Tamfum JJ, Ahmed AA, Ganesh V, Tamhankar M, Patterson JL, Ndembi N, Mbanya D, Kaptue L, McArthur C, Muñoz-Medina JE, Gonzalez-Bonilla CR, López S, Arias CF, Arevalo S, Miller S, Stone M, Busch M, Hsieh K, Messenger S, Wadford DA, Rodgers M, Cloherty G, Faria NR, Thézé J, Pybus OG, Neto Z, Morais J, Taveira N, Hackett JR Jr, Chiu CY. Author Correction: Metagenomic sequencing with spiked primer enrichment for viral diagnostics and genomic surveillance. Nat Microbiol. 2020 Mar;5(3):525. doi: 10.1038/s41564-020-0671-7. Erratum for: Nat Microbiol. 2020 Mar;5(3):443-454. doi: 10.1038/s41564-019-0637-9. PMID: 31965087; PMCID: PMC7608365.

Eaton BT, Broder CC, Middleton D, Wang LF. Hendra and Nipah viruses: different and dangerous. Nat Rev Microbiol. 2006 Jan;4(1):23-35. doi: 10.1038/nrmicro1323. PMID: 16357858; PMCID: PMC7097447.

Ejazi SA, Ghosh S, Ali N. Antibody detection assays for COVID-19 diagnosis: an early overview. Immunol Cell Biol. 2021 Jan;99(1):21-33. doi: 10.1111/imcb.12397. Epub 2020 Sep 25. PMID: 32864735.

Feinstone SM, Kapikian AZ, Purceli RH. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science. 1973 Dec 7;182(4116):1026-8. doi: 10.1126/science.182.4116.1026. PMID: 4356028.

Gargis AS, Kalman L, Lubin IM. Assuring the Quality of Next-Generation Sequencing in Clinical Microbiology and Public Health Laboratories. J Clin Microbiol. 2016 Dec;54(12):2857-2865. doi: 10.1128/JCM.00949-16. Epub 2016 Aug 10. PMID: 27510831; PMCID: PMC5121372.

Gawad C, Koh W, Quake SR. Single-cell genome sequencing: current state of the science. Nat Rev Genet. 2016 Mar;17(3):175-88. doi: 10.1038/nrg.2015.16. Epub 2016 Jan 25. PMID: 26806412.

Greninger AL, Naccache SN, Federman S, Yu G, Mbala P, Bres V, Stryke D, Bouquet J, Somasekar S, Linnen JM, Dodd R, Mulembakani P, Schneider BS, Muyembe-Tamfum JJ, Stramer SL, Chiu CY. Rapid metagenomic identification of viral pathogens in clinical samples by real-time nanopore sequencing analysis. Genome Med. 2015 Sep 29;7:99. doi: 10.1186/s13073-015-0220-9. PMID: 26416663; PMCID: PMC4587849.

Goyal R, Gautam RK, Chopra H, Dubey AK, Singla RK, Rayan RA, Kamal MA. Comparative highlights on MERS-CoV, SARS-CoV-1, SARS-CoV-2, and NEO-CoV. EXCLI J. 2022 Sep 29; 21:1245-1272. doi: 10.17179/excli2022-5355. PMID: 36483910; PMCID: PMC9727256.

Hussein HA. Brief review on ebola virus disease and one health approach. Heliyon. 2023 Aug 8;9(8):e19036. doi: 10.1016/j.heliyon.2023.e19036. PMID: 37600424; PMCID: PMC10432691.

Imai K, Tamura K, Tanigaki T, Takizawa M, Nakayama E, Taniguchi T, Okamoto M, Nishiyama Y, Tarumoto N, Mitsutake K, Murakami T, Maesaki S, Maeda T. Whole Genome Sequencing of Influenza A and B Viruses With the MinION Sequencer in the Clinical Setting: A Pilot Study. Front Microbiol. 2018 Nov 13;9:2748. doi: 10.3389/fmicb.2018.02748. PMID: 30483243; PMCID: PMC6243006..

Jain M, Olsen HE, Paten B, Akeson M. Erratum to: The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol. 2016 Dec 13;17(1):256. doi: 10.1186/s13059-016-1122-x. Erratum for: Genome Biol. 2016 Nov 25;17(1):239. doi: 10.1186/s13059-016-1103-0. PMID: 27964738; PMCID: PMC5154158.

Ji CM, Feng XY, Huang YW, Chen RA. The Applications of Nanopore Sequencing Technology in Animal and Human Virus Research. Viruses. 2024 May 16;16(5):798. doi: 10.3390/v16050798. PMID: 38793679; PMCID: PMC11125791.

Johne R, Müller H, Rector A, van Ranst M, Stevens H. Rolling-circle amplification of viral DNA genomes using

phi29 polymerase. Trends Microbiol. 2009 May;17(5):205-11. doi: 10.1016/j.tim.2009.02.004. Epub 2009 Apr 15. PMID: 19375325.

Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E. New DNA viruses identified in patients with acute viral infection syndrome. J Virol. 2005 Jul;79(13):8230-6. doi: 10.1128/JVI.79.13.8230-8236.2005. PMID: 15956568; PMCID: PMC1143717.

Kafetzopoulou LE, Efthymiadis K, Lewandowski K, Crook A, Carter D, Osborne J, Aarons E, Hewson R, Hiscox JA, Carroll MW, Vipond R, Pullan ST. Assessment of metagenomic Nanopore and Illumina sequencing for recovering whole genome sequences of chikungunya and dengue viruses directly from clinical samples. Euro Surveill. 2018 Dec;23(50):1800228. doi: 10.2807/1560-7917.ES.2018.23.50.1800228. PMID: 30563591; PMCID: PMC6299504.

Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol. 1972 Nov;10(5):1075-81. doi: 10.1128/JVI.10.5.1075-1081.1972. PMID: 4117963; PMCID: PMC356579.

Kessler S, Harder TC, Schwemmle M, Ciminski K. Influenza A Viruses and Zoonotic Events-Are We Creating Our Own Reservoirs? Viruses. 2021 Nov 9;13(11):2250. doi: 10.3390/v13112250. PMID: 34835056; PMCID: PMC8624301.

Khan AR, Pervez MT, Babar ME, Naveed N, Shoaib M. A Comprehensive Study of De Novo Genome Assemblers: Current Challenges and Future Prospective. Evol Bioinform Online. 2018 Feb 20; 14:1176934318758650. doi: 10.1177/1176934318758650. PMID: 29511353; PMCID: PMC5826002.

Kubina R, Dziedzic A. Molecular and Serological Tests for COVID-19 a Comparative Review of SARS-CoV-2 Coronavirus Laboratory and Point-of-Care Diagnostics. Diagnostics (Basel). 2020 Jun 26;10(6):434. doi: 10.3390/diagnostics10060434. PMID: 32604919; PMCID: PMC7345211.

Kumar A, Bhatt S, Kumar A, Rana T. Canine rabies: An epidemiological significance, pathogenesis, diagnosis, prevention, and public health issues. Comp Immunol Microbiol Infect Dis. 2023 Jun; 97:101992. doi: 10.1016/j.cimid.2023.101992. Epub 2023 May 9. PMID: 37229956.

Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet. 2020 Feb 22;395(10224):565-574. doi: 10.1016/S0140-6736(20)30251-8. Epub 2020 Jan 30. PMID: 32007145; PMCID: PMC7159086.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376–380.

Martella V, Bányai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. Vet Microbiol. 2010 Jan 27;140(3-4):246-55. doi: 10.1016/j.vetmic.2009.08.028. Epub 2009 Aug 28. PMID: 19781872.

Mekonnen D., Mengist H. M., Derbie A., Nibret E., Munshea A., He H., et al. (2020). Diagnostic Accuracy of Serological Tests and Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2 Antibody: A Systematic Review

and Meta-Analysis. Rev. Med. Virol. 31, e2181. 10.1002/rmv.2181

Misu M, Yoshikawa T, Sugimoto S, Takamatsu Y, Kurosu T, Ouji Y, Yoshikawa M, Shimojima M, Ebihara H, Saijo M. Rapid whole genome sequencing methods for RNA viruses. Front Microbiol. 2023 Feb 23; 14:1137086. doi: 10.3389/fmicb.2023.1137086. PMID: 36910229; PMCID: PMC9995502.

Morse SS, Mazet JA, Woolhouse M, Parrish CR, Carroll D, Karesh WB, Zambrana-Torrelio C, Lipkin WI, Daszak P. Prediction and prevention of the next pandemic zoonosis. Lancet. 2012 Dec 1;380(9857):1956-65. doi: 10.1016/S0140-6736(12)61684-5. PMID: 23200504; PMCID: PMC3712877.

Nanda S, Jayan G, Voulgaropoulou F, Sierra-Honigmann AM, Uhlenhaut C, McWatters BJ, Patel A, Krause PR. Universal virus detection by degenerate-oligonucleotide primed polymerase chain reaction of purified viral nucleic acids. J Virol Methods. 2008 Sep;152(1-2):18-24. doi: 10.1016/j.jviromet.2008.06.007. Epub 2008 Jul 16. PMID: 18601953; PMCID: PMC7172433.

Olaimat AN, Taybeh AO, Al-Nabulsi A, Al-Holy M, Hatmal MM, Alzyoud J, Aolymat I, Abughoush MH, Shahbaz H, Alzyoud A, Osaili T, Ayyash M, Coombs KM, Holley R. Common and Potential Emerging Foodborne Viruses: A Comprehensive Review. Life (Basel). 2024 Jan 28;14(2):190. doi: 10.3390/life14020190. PMID: 38398699; PMCID: PMC10890126.

Park WJ, Park BJ, Ahn HS, Lee JB, Park SY, Song CS, Lee SW, Yoo HS, Choi IS. Hepatitis E virus as an emerging zoonotic pathogen. J Vet Sci. 2016 Mar;17(1):1-11. doi: 10.4142/jvs.2016.17.1.1. Epub 2016 Mar 22. PMID: 27051334; PMCID: PMC4808633.

Peacock T, Moncla L, Dudas G, VanInsberghe D, Sukhova K, Lloyd-Smith JO, Worobey M, Lowen AC, Nelson MI. The global H5N1 influenza panzootic in mammals. Nature. 2024 Sep 24. doi: 10.1038/s41586-024-08054-z. Epub ahead of print. PMID: 39317240.

Petersen LM, Martin IW, Moschetti WE, Kershaw CM, Tsongalis GJ. Third-Generation Sequencing in the Clinical Laboratory: Exploring the Advantages and Challenges of Nanopore Sequencing. J Clin Microbiol. 2019 Dec 23;58(1):e01315-19. doi: 10.1128/JCM.01315-19. PMID: 31619531; PMCID: PMC6935936.

Pozzetto, B.,2002. Méthodes de diagnostic en virologie. In Virologie Médicale; Mammette, A., Ed.; Presses Universitaires de Lyon: Lyon, France pp. 187–210.

Price AM, Hayer KE, McIntyre ABR, Gokhale NS, Abebe JS, Della Fera AN, Mason CE, Horner SM, Wilson AC, Depledge DP, Weitzman MD. Direct RNA sequencing reveals m6A modifications on adenovirus RNA are necessary for efficient splicing. Nat Commun. 2020 Nov 26;11(1):6016. doi: 10.1038/s41467-020-19787-6. PMID: 33243990; PMCID: PMC7691994.

Quick J, Grubaugh ND, Pullan ST, Claro IM, Smith AD, Gangavarapu K, Oliveira G, Robles-Sikisaka R, Rogers TF, Beutler NA, Burton DR, Lewis-Ximenez LL, de Jesus JG, Giovanetti M, Hill SC, Black A, Bedford T, Carroll MW, Nunes M, Alcantara LC Jr, Sabino EC, Baylis SA, Faria NR, Loose M, Simpson JT, Pybus OG, Andersen KG, Loman NJ. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat Protoc. 2017 Jun;12(6):1261-1276. doi: 10.1038/nprot.2017.066. Epub 2017 May 24. PMID: 28538739; PMCID: PMC5902022.

Rahman MT, Sobur MA, Islam MS, Ievy S, Hossain MJ, El Zowalaty ME, Rahman AT, Ashour HM. Zoonotic Diseases: Etiology, Impact, and Control. Microorganisms. 2020 Sep 12;8(9):1405. doi: 10.3390/microorganisms8091405. PMID: 32932606; PMCID: PMC7563794.

Rathore JS, Ghosh C. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a newly emerged pathogen: an overview. Pathog Dis. 2020 Aug 1;78(6):ftaa042. doi: 10.1093/femspd/ftaa042. PMID: 32840560; PMCID:

PMC7499575.

Reyes GR, Kim JP. Sequence-independent, single-primer amplification (SISPA) of complex DNA populations. Mol Cell Probes. 1991 Dec;5(6):473-81. doi: 10.1016/s0890-8508(05)80020-9. PMID: 1664049.

Rhoads A, Au KF. PacBio Sequencing and Its Applications. Genomics Proteomics Bioinformatics. 2015 Oct;13(5):278-89. doi: 10.1016/j.gpb.2015.08.002. Epub 2015 Nov 2. PMID: 26542840; PMCID: PMC4678779.

Roy S, Coldren C, Karunamurthy A, Kip NS, Klee EW, Lincoln SE, Leon A, Pullambhatla M, Temple-Smolkin RL, Voelkerding KV, Wang C, Carter AB. Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists. J Mol Diagn. 2018 Jan;20(1):4-27. doi: 10.1016/j.jmoldx.2017.11.003. Epub 2017 Nov 21. PMID: 29154853.

Russotto Y, Micali C, Pellicanò GF, Nunnari G, Venanzi Rullo E. HIV and Mediterranean Zoonoses: A Review of the Literature. Infect Dis Rep. 2022 Sep 16;14(5):694-709. doi: 10.3390/idr14050075. PMID: 36136825; PMCID: PMC9498920.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985 Dec 20;230(4732):1350-4. doi: 10.1126/science.2999980. PMID: 2999980.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA 74, 5463-5467.

Scharf SJ, Horn GT, Erlich HA. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science. 1986 Sep 5;233(4768):1076-8. doi: 10.1126/science.3461561. PMID: 3461561.

Silander K, Saarela J. Whole genome amplification with Phi29 DNA polymerase to enable genetic or genomic analysis of samples of low DNA yield. Methods Mol Biol. 2008; 439:1-18. doi: 10.1007/978-1-59745-188-8_1. PMID: 18370092.

Srivastava S, Dhoundiyal S, Kumar S, Kaur A, Khatib MN, Gaidhane S, Zahiruddin QS, Mohanty A, Henao-Martinez AF, Krsak M, Rodriguez-Morales AJ, Montenegro-Idrogo JJ, Bonilla-Aldana DK, Sah R. Yellow Fever: Global Impact, Epidemiology, Pathogenesis, and Integrated Prevention Approaches. Infez Med. 2024 Dec 1;32(4):434-450. doi: 10.53854/liim-3204-3. PMID: 39660161; PMCID: PMC11627485.

Telenius H, Carter NP, Bebb CE, Nordenskjöld M, Ponder BA, Tunnacliffe A. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics. 1992 Jul;13(3):718-25. doi: 10.1016/0888-7543(92)90147-k. PMID: 1639399.

Temmam S, Davoust B, Berenger JM, Raoult D, Desnues C. Viral metagenomics on animals as a tool for the detection of zoonoses prior to human infection? Int J Mol Sci. 2014 Jun 10;15(6):10377-97. doi: 10.3390/ijms150610377. PMID: 24918293; PMCID: PMC4100157.

Vaser R, Sović I, Nagarajan N, Šikić M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome Res. 2017 May;27(5):737-746. doi: 10.1101/gr.214270.116. Epub 2017 Jan 18. PMID: 28100585; PMCID: PMC5411768.

Viehweger A, Krautwurst S, Lamkiewicz K, Madhugiri R, Ziebuhr J, Hölzer M, Marz M. Direct RNA nanopore sequencing of full-length coronavirus genomes provides novel insights into structural variants and enables modification analysis. Genome Res. 2019 Sep;29(9):1545-1554. doi: 10.1101/gr.247064.118. Epub 2019 Aug 22. PMID: 31439691; PMCID: PMC6724671.

Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Ganem D, DeRisi JL. Microarray-based detection

and genotyping of viral pathogens. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15687-92. doi: 10.1073/pnas.242579699. Epub 2002 Nov 12. PMID: 12429852; PMCID: PMC137777.

Wang C, Zhou S, Xue W, Shen L, Huang W, Zhang Y, Li X, Wang J, Zhang H, Ma X. Comprehensive virome analysis reveals the complexity and diversity of the viral spectrum in pediatric patients diagnosed with severe and mild hand-foot-and-mouth disease. Virology. 2018 May; 518:116-125. doi: 10.1016/j.virol.2018.02.004. Epub 2018 Feb 19. PMID: 29471150.

Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. Nat Biotechnol. 2021 Nov;39(11):1348-1365. doi: 10.1038/s41587-021-01108-x. Epub 2021 Nov 8. PMID: 34750572; PMCID: PMC8988251.

Wee Y, Bhyan SB, Liu Y, Lu J, Li X, Zhao M. The bioinformatics tools for the genome assembly and analysis based on third-generation sequencing. Brief Funct Genomics. 2019 Feb 14;18(1):1-12. doi: 10.1093/bfgp/ely037. PMID: 30462154.

Wilhelm B, Waddell L, Greig J, Rajić A, Houde A, McEwen SA. A scoping review of the evidence for public health risks of three emerging potentially zoonotic viruses: hepatitis E virus, norovirus, and rotavirus. Prev Vet Med. 2015 Apr 1;119(1-2):61-79. doi: 10.1016/j.prevetmed.2015.01.015. Epub 2015 Jan 24. PMID: 25681862.

Wooley JC, Ye Y. Metagenomics: Facts and Artifacts, and Computational Challenges*. J Comput Sci Technol. 2009 Jan;25(1):71-81. doi: 10.1007/s11390-010-9306-4. PMID: 20648230

Chapter 1

Influenza virus at the human-animal interface

Influenza is an acute viral infection affecting different host species worldwide. In humans influenza is associated with respirator clinical signs and high mortality causing pandemics, epidemics, and sporadic outbreaks. Every year almost 10% of the human population is affected by influenza with 500000 patients dying each year. Human influenza viruses (IVs) induce annual epidemics mostly in the winter season. Highly contagious respiratory IV infections lead to fatal outcome in high-risk groups (Liang, 2023). Occasionally, human IV strains jump from an animal host to human populations that lack pre-existing immunity, thus causing pandemics with high mortality and morbidity rates. Four influenza pandemics have been reported in the last century, the 1918–19 "Spanish" flu, the 1957 "Asia" flu, the 1968 "Hong Kong" flu, and the 2009 "Swine flu." (Ma et al., 2007; Munster et al., 2007; Smith et al., 2009a, 2009b; Neumann and Kawaoka, 2011). The 1918–19 "Spanish" flu is regarded as the most severe pandemic in recent times, with ~ 500 million infections and 50–100 million deaths reported worldwide (Taubenberger and Morens, 2006).

IVs belong to the *Orthomyxoviridae* family and comprise four major types A, B, C, and D. Influenza A virus (IAV) is able to infect different avian and mammalian species, i.e. humans, birds, pigs, horses, and dogs (Perez et al., 2011). Influenza B virus (IBV) infects humans and seals, whereas influenza C virus (ICV) infects humans and pigs (Perez et al., 2011). IAV, IBV, and ICV are human pathogens. The influenza D virus (IDV), was first reported in 2011, infects pigs and cattle, with no human infections described (Liu et al., 2020).

IAV can be further divided according to the host species infected by, e.g., human influenza virus (hIAV), swine influenza virus (swIAV), avian influenza virus (AIV), equine influenza virus (EIV), canine influenza virus (CIV), and bat influenza virus (Suarez, 2016). According to the antigenic variations of the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA), IAVs are divided into distinct 18 HA (HA1-HA18) and 11 NA (NA1-NA9) subtypes. hIAVs contain mostly HA1, HA2, HA3 and NA1, NA2 subtypes, AIVs contain HA1 to HA16 and NA1 to NA9 subtypes, while HA17NA10 and HA18NA11 are so far only detected in bats (Abdelwhab and Mettenleiter, 2023).

Swine and poultry represent two rapidly growing livestock industries constitute two key reservoirs of IAVs. As livestock industry farms host several thousand birds or pigs occasionally harboring multiple subtypes of IAVs, they are frequently regarded high-risk areas for emergence of novel IAVs.

Continuous introduction of animals in livestock farming may support IAVs maintenance in livestock. Furthermore, animal professionals and the addition of different animal species could introduce new strains of IAV into flocks or herds (Rajao et al., 2019). These professionals might also unintentionally spread IAVs from infected livestock to humans (Ma et al., 2018).

The segmented nature of the genome favors the reassortment of genetic material during a co-infection with two genetically different type A influenza viruses. The reassortment process, also known as antigenic shift, involving the genome segment encoding HA, result in the generation of viruses displaying surface antigens against which human population may not have pre-existing, protective antibodies. Accumulation of point mutations during virus replication, also mentioned as antigenic drift, potentially result in conservative amino acid replacements. If this process involves genome segment encoding HA this could affect pre-existing immunity, host range, virulence, and other factors (Hale et al., 2010). Antigenic drift and shift resulting in sustained human-to-human virus transmission could develop to a pandemic, (Bouvier and Palese, 2008; Kelly, 2011).

Equine influenza (EI) is a highly contagious respiratory disease affecting horses, with significant economic repercussions on the global equine industry. Clinical symptoms of EI include fever, dry cough, and nasal discharge (Chambers, 2014). EI is primarily transmitted through aerosols from infected horses and can spread quickly within a group of horses and to other susceptible animals nearby (Landolt, 2014). EI can cause loss of horsepower for work and daily tasks, cancellation of equine competitions and leisure activities with high treatment costs and implications in the animal welfare from clinical disease. For instance, an epidemic in a naive horse population in Australia in 2007 was estimated to generate loss corresponding to one billion dollars (Paillot and El-Hage, 2016).

EI virus (EIV) is classified into two subtypes H7N7 and H3N8 based on the HA and NA (Daly et al., 2011). The H3N8 subtype is currently the predominant epidemic type worldwide, including both European and American lineages (Olguin-Perglione and Barrandeguy, 2021). The American lineages are further categorized into South American, Kentucky, and Florida lineages, with the Florida lineages having two additional branches. Florida Clade 1 is primarily found in the Americas and Europe, whereas Florida Clade 2 is primarily found in Europe, Asia, and Africa. Vaccination in horses against EIV is currently considered the primary means of preventing EI, but still needs further confirmation on the efficacy (Newton et al., 2023).

EIV transmission is facilitated by the international transportation of horses, primarily for competition

and breeding purposes (Kleij et al., 2024). In recent years, sporadic outbreaks mainly in unvaccinated horses were reported in several European countries (Xie et al., 2016; Sing et al., 2018; Chambers, 2022).

EIV infection is not limited to equids. Sporadic spillover of EIV to dogs has been reported in the United Kingdom and Australia (Crawford et al., 2005; Daly et al., 2008). During surveillance for influenza viruses in pigs in China two EIV H3N8 strains have been identified (Tu et al., 2009).

Humans are also a potential host for EIV. In experimental infections in antibody-negative human volunteers over 60% seroconversion was observed and virus cultures from throat swabs collected 2–6 days after nasal inoculation tested positive. Most of the human volunteers were found to shed virus up to 5 days post-infection (Alford et al., 1967; Kasel and Couch, 1969). During the 2007 EIV outbreak in Australia epidemiological studies in animal professionals exposed to horses displayed 10% seroprevalence against EIV (Burnell et al., 2014).

References

Abdelwhab EM, Mettenleiter TC. Zoonotic Animal Influenza Virus and Potential Mixing Vessel Hosts. Viruses. 2023 Apr 16;15(4):980. doi: 10.3390/v15040980. PMID: 37112960; PMCID: PMC10145017.

Alford RH, Kasel JA, Lehrich JR, Knight V. Human responses to experimental infection with influenza A/Equi 2 virus. Am J Epidemiol. 1967 Jul;86(1):185-92. doi: 10.1093/oxfordjournals.aje.a120723. PMID: 4951557.

Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008 Sep 12;26 Suppl 4(Suppl 4):D49-53. doi: 10.1016/j.vaccine.2008.07.039. PMID: 19230160; PMCID: PMC3074182.

Burnell FJ, Holmes MA, Roiko AH, Lowe JB, Heil GL, White SK, Gray GC. Little evidence of human infection with equine influenza during the 2007 epizootic, Queensland, Australia. J Clin Virol. 2014 Feb;59(2):100-3. doi: 10.1016/j.jcv.2013.11.011. Epub 2013 Dec 6. PMID: 24360918.

Chambers TM. A brief introduction to equine influenza and equine influenza viruses. Methods Mol Biol. 2014; 1161:365-70. doi: 10.1007/978-1-4939-0758-8_31. PMID: 24899445

Chambers T.M. Equine Influenza. Cold Spring Harb. Perspect Med. 2022;12: a038331. doi: 10.1101/cshperspect.a038331.

Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO. Transmission of equine influenza virus to dogs. Science. 2005 Oct 21;310(5747):482-5. doi: 10.1126/science.1117950. Epub 2005 Sep 26. PMID: 16186182.

Daly JM, MacRae S, Newton JR, Wattrang E, Elton DM. Equine influenza: a review of an unpredictable virus. Vet J. 2011 Jul;189(1):7-14. doi: 10.1016/j.tvjl.2010.06.026. Epub 2010 Aug 3. PMID: 20685140.

Daly JM, Blunden AS, Macrae S, Miller J, Bowman SJ, Kolodziejek J, Nowotny N, Smith KC. Transmission of equine influenza virus to English foxhounds. Emerg Infect Dis. 2008 Mar;14(3):461-4. doi: 10.3201/eid1403.070643. PMID: 18325262; PMCID: PMC2570814.

Hale BG, Albrecht RA, García-Sastre A. Innate immune evasion strategies of influenza viruses. Future Microbiol. 2010 Jan;5(1):23-41. doi: 10.2217/fmb.09.108. PMID: 20020828; PMCID: PMC2820251.

Kasel JA, Couch RB. Experimental infection in man and horses with influenza A viruses. Bull World Health Organ. 1969;41(3):447-52. PMID: 5309454; PMCID: PMC2427734.

Kelly H. The classical definition of a pandemic is not elusive. Bull World Health Organ. 2011 Jul 1;89(7):540-1. doi: 10.2471/BLT.11.088815. PMID: 21734771; PMCID: PMC3127276.

Kleij L, Bruder E, Raoux-Barbot D, Lejal N, Nevers Q, Deloizy C, Da Costa B, Legrand L, Barrey E, Chenal A, Pronost S, Delmas B, Dhorne-Pollet S. Genomic characterization of equine influenza A subtype H3N8 viruses by long read sequencing and functional analyses of the PB1-F2 virulence factor of A/equine/Paris/1/2018. Vet Res. 2024 Mar 22;55(1):36. doi: 10.1186/s13567-024-01289-8. PMID: 38520035; PMCID: PMC10960481.

Landolt GA. Equine influenza virus. Vet Clin North Am Equine Pract. 2014 Dec;30(3):507-22. doi: 10.1016/j.cveq.2014.08.003. Epub 2014 Oct 3. PMID: 25282321.

Liang Y. Pathogenicity and virulence of influenza. Virulence. 2023 Dec;14(1):2223057. doi: 10.1080/21505594.2023.2223057. PMID: 37339323; PMCID: PMC10283447

Liu R, Sheng Z, Huang C, Wang D, Li F. Influenza D virus. Curr Opin Virol. 2020 Oct; 44:154-161. doi: 10.1016/j.coviro.2020.08.004. Epub 2020 Sep 12. PMID: 32932215; PMCID: PMC7755673.

Ma MJ, Wang GL, Anderson BD, Bi ZQ, Lu B, Wang XJ, Wang CX, Chen SH, Qian YH, Song SX, Li M, Lednicky JA, Zhao T, Wu MN, Cao WC, Gray GC. Evidence for Cross-species Influenza A Virus Transmission Within Swine Farms, China: A One Health, Prospective Cohort Study. Clin Infect Dis. 2018 Feb 1;66(4):533-540. doi: 10.1093/cid/cix823. PMID: 29401271; PMCID: PMC5848305.

Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 2007 May 11;3(5):e61. doi: 10.1371/journal.ppat.0030061. PMID: 17500589; PMCID: PMC1876497.

Neumann G, Kawaoka Y. The first influenza pandemic of the new millennium. Influenza Other Respir Viruses. 2011 May;5(3):157-66. doi: 10.1111/j.1750-2659.2011.00231.x. Epub 2011 Feb 28. PMID: 21477134; PMCID: PMC3073629.

Newton JR, Rendle DI, Mountford DR, Marr CM. Equine influenza vaccination catches an autumn cold! But must get over it as soon as it can. Equine Vet J. 2023 Jan;55(1):142-146. doi: 10.1111/evj.13885. Epub 2022 Oct 13. PMID: 36226994.

Olguin-Perglione C, Barrandeguy ME. An Overview of Equine Influenza in South America. Viruses. 2021 May 12;13(5):888. doi: 10.3390/v13050888. PMID: 34065839; PMCID: PMC8151294.

Paillot R, El-Hage CM. The Use of a Recombinant Canarypox-Based Equine Influenza Vaccine during the 2007 Australian Outbreak: A Systematic Review and Summary. Pathogens. 2016 Jun 10;5(2):42. doi: 10.3390/pathogens5020042. PMID: 27294963; PMCID: PMC4931393.

Perez D, Rimstad E, Smith G, et al. Orthomyxoviridae. In: King AMQ, Adams MJ, Carstens EB, et al, eds. Virus Taxonomy. Oxford: Elsevier; 2011:749–762.

Rajao DS, Vincent AL, Perez DR. Adaptation of Human Influenza Viruses to Swine. Front Vet Sci. 2019 Jan 22; 5:347. doi: 10.3389/fvets.2018.00347. PMID: 30723723; PMCID: PMC6349779.

Singh R.K., Dhama K., Karthik K., Khandia R., Munjal A., Khurana S.K., Chakraborty S., Malik Y.S., Virmani N., Singh R., et al. A Comprehensive Review on Equine Influenza Virus: Etiology, Epidemiology,

Pathobiology, Advances in Developing Diagnostics, Vaccines, and Control Strategies. Front. Microbiol. 2018; 9:1941. doi: 10.3389/fmicb.2018.01941.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 2009a Jun 25;459(7250):1122-5. doi: 10.1038/nature08182. PMID: 19516283.

Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS, Guan Y. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A. 2009b Jul 14;106(28):11709-12. doi: 10.1073/pnas.0904991106. Epub 2009 Jul 13. PMID: 19597152; PMCID: PMC2709671.

Suarez DL. Role of the Editor in Scientific Publishing. In: Swayne DE, editor. Veterinary Pathology and Poultry Diseases. 2016 Nov 4; Chapter 1. doi: 10.1002/9781118924341.ch1.

Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis. 2006 Jan;12(1):15-22. doi: 10.3201/eid1201.050979. PMID: 16494711; PMCID: PMC3291398.

Tu J, Zhou H, Jiang T, Li C, Zhang A, Guo X, Zou W, Chen H, Jin M. Isolation and molecular characterization of equine H3N8 influenza viruses from pigs in China. Arch Virol. 2009;154(5):887-90. doi: 10.1007/s00705-009-0381-1. Epub 2009 Apr 26. PMID: 19396578.

Xie T., Anderson B.D., Daramragchaa U., Chuluunbaatar M., Gray G.C. A Review of Evidence that Equine Influenza Viruses Are Zoonotic. Pathogens. 2016; 5:50. doi: 10.3390/pathogens5030050.

A Cold Case of Equine Influenza Disentangled with Nanopore Sequencing

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Abstract

Massive sequencing techniques have allowed us to develop straightforward approaches for the whole genome sequencing of viruses, including influenza viruses, generating information that is useful for improving the levels and dimensions of data analysis, even for archival samples. Using the Nanopore platform, we determined the whole genome sequence of an H3N8 equine influenza virus, identified from a 2005 outbreak in Apulia, Italy, whose origin had remained epidemiologically unexplained. The virus was tightly related (>99% at the nucleotide level) in all the genome segments to viruses identified in Poland in 2005–2008 and it was seemingly introduced locally with horse trading for the meat industry. In the phylogenetic analysis based on the eight genome segments, strain ITA/2005/horse/Bari was found to cluster with sub-lineage Florida 2 in the HA and M genes, whilst in the other genes it clustered with strains of the Eurasian lineage, revealing a multi-reassortant nature.

1. Introduction

Equine influenza A virus (EIAV) infection causes acute disease with variable morbidity and low mortality in horses and odd-toed ungulates. EIAV is a major cause of respiratory disease in horses, able to spread rapidly among susceptible animals (Landolt et al., 2014). The influenza A virus (IAV) genome is composed of eight segments of negative-sense single-strand RNA. The virus is subtyped on the basis of the envelope-associated proteins, the hemagglutinin (HA) and the neuraminidase (NA) (Wright, 2001). To date, 18 HA and 11 NA subtypes have been described in different susceptible hosts (Durães-Carvalho and Salemi, 2018). In particular, two EIAV subtypes have been identified in equines, such as H7N7 and H3N8 (Landolt et al., 2014). The last confirmed report of H7N7 EIAV in horses dates back to 1979 (Webster, 1993). H3N8 EIAVs have been reported worldwide since 1963 (Ito et al., 2008). In the phylogenetic analysis of the HA gene, H3N8 EIAVs seem to have diverged into distinct evolutionary lineages. By the end of the 1980s, two antigenically distinct lineages,

American and Eurasian, have emerged. The American lineage has further evolved into Argentinian, Kentucky, and Florida sub-lineages (Daly et al., 1996; Lai et al., 2001). The evolution of the Florida sub-lineage has led to the emergence of two clades, Florida 1 (FC1) and 2 (FC2), which are currently circulating worldwide (Bryant et al., 2009; Gildea et al., 2012; Laabassi et al., 2015; Murcia et al., 2011; Virmani et al., 2010; Yamanaka et al., 2008). FC1 viruses are endemic in North America and FC2 viruses are predominant in Europe. However, both lineages have originated outbreaks in Europe, South Africa, South America, and Asia (Cullinane and Newton, 2013). More recently, outbreaks of FC1 and FC2 viruses have been also reported on the African continent (Ahmed et al., 2022; Diallo et al., 2021; Shittu et al., 2020).

In December 2005, EIAV infection was established as the cause of clinical respiratory disease in vaccinated horses in Apulia, Italy (Martella et al., 2007). An acute respiratory disease occurred in a horse center managed by the Forest Service Rangers in Martina Franca. A total of 24 out of 33 animals developed acute clinical signs of febrile respiratory disease. The outbreak was seemingly related to a fair/market organized yearly for the promotion of local horse and donkey breeds, although other EIAV outbreaks were not reported in the same time span.

On the genetic characterization of the HA and NA genes, the virus from the outbreak (ITA/2005/horse/Bari) was characterized as H3N8 and was related to American viruses (A/Kentucky/5/02-like). The virus was hypothesized to have been imported into Italy due to the relocation of infected horses from a large outbreak described in 2003 in the United Kingdom (Newton et al., 2006). In Europe, the circulation of Kentucky/5/02-like EIAV strains was first described in the United Kingdom in 2003, where outbreaks of influenza were reported regardless of the immunization status of the animal (Newton et al., 2006). Epidemiological investigation revealed that in May 2003 Kentucky/5/02-like EIAV strains scattered from the United Kingdom as a result of the transport of horses from Newmarket to a racetrack in Rome, Italy, where an outbreak subsequently has risen (Newton et al., 2006). Due to the genetic similarities between the HA and NA genes of strain ITA/2005/horse/Bari and H3N8 EIAV strains from the United Kingdom (A/eq/Stock-on-Trent/410956/04 and A/eq/Aboyne/410355/04), we hypothesized that the local 2005 outbreak could be somehow linked epidemiologically to the 2003 Rome outbreak, although other cases of EIAV had not been reported from May 2003 to December 2005, suggesting that the 2003 outbreak in Rome had been effectively contained. However, the epidemiological link between these two outbreaks remained unknown.

Over the last decade, novel protocols have been developed for whole genome sequencing (WGS) of viruses (Pallen et al., 2010), taking advantage of next-generation sequencing (NGS) chemistries. Oxford Nanopore Technologies (ONT™) is a third-generation NSG platform, capable of producing

long sequences (Lin et al., 2021). The ONT™ platform can be used starting with a negligible investment in terms of hardware and has been quickly adopted by the academic and scientific community.

The shift from single (HA) or dual gene target (HA and NA) sequencing of IAVs to WGS using NGS has allowed us to gather relevant information on IAV evolution (Baillie et al., 2012; Langat et al., 2017; Watson et al., 2015) thus also warranting re-analysis of old IAV isolates in archival collections. This provided the rationale for WGS of strain ITA/2005/horse/Bari (Martella et al., 2007).

2. Materials and Methods

2.1. Virus Cultivation

The virus was cultured in the allantoid cavities of 10-day embryonated hens' eggs with the addition of antibiotics and antimycotics. The eggs were harvested after 3 days, and the allantoid fluid was tested for the presence of the virus by hemagglutination assay with 1% chicken erythrocytes in phosphate-buffered saline. Virus titer was also quantified using a quantitative reverse transcription polymerase chain reaction PCR (qRT-PCR) (Quinlivan et al., 2005).

2.2. Genome Sequencing and Data Analysis

Using a PCR-based enrichment protocol after reverse transcription of the RNA (Wüthrich et al., 2019), the genome of the EIAV isolate ITA/2005/horse/Bari was reconstructed. A total of 1 µg of purified PCR product from each sample was used as input of a library generated with the Ligation sequencing kit 1D SQK-LSK110 (ONT™, Oxford UK). First, DNA was end-repaired (NEBNext Ultra II end repair/dA tailing kit; New England Biolabs, NEB, Ipswich, MA, USA), purified using AMPure XP beads in a ratio of 1:1 volume of beads per sample, and eluted in 30 µL of nuclease-free water. Sequencing adapters (AMx1D; ONT™, Oxford UK) were ligated to the DNA (Blunt/TA ligation master mix; NEB Ipswich, MA, USA) after incubating at room temperature for 10 min. The adapter-ligated DNA library was purified with AMPure XP beads in a ratio of 1:2.5 volume of beads per sample, followed by 2 washes (Adapter bead binding buffer; ONT™) and elution in 15 µL of elution buffer (ONT™). The library was loaded onto a MinION flow cell (MIN106 R9.4; ONT™, Oxford UK) and run via MinKNOW v.18.05.5 for 24 h. MinKNOW-generated FASTQ files containing reads reaching the quality threshold (Q score ≥7) were analyzed using Geneious Prime version 2021.2 (Biomatters Ltd., Auckland, New Zealand). The reads were mapped to a set of reference EIAV genome segments using MiniMap2 (Li, 2018). The Basic Local Alignment Search Tool Nucleotide (BLASTn) tool from NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, accessed on 28 February 2023) was employed to compare EIAV resulting consensus sequences to the GenBank database using default values to search for homologous hits.

2.3. Animal Influxes

The national register for animal trading (NSIS) was consulted to extract archival data of the year 2005 on the trading of horses.

2.4. Sequence and Phylogenetic Analyses

Sequence editing and multiple alignments were performed in Geneious Prime version 2021.2 (Biomatters Ltd., Auckland, New Zealand). The sequences were aligned with cognate EIAV strains retrieved from the GenBank database by Multiple Alignment using Fast Fourier Transform (Katoh et al., 2002). The correct substitution model parameters for the phylogenetic analyses were selected using the "Find the best protein DNA/Protein Models" tool provided by MEGA X version 10.0.5 software (Kumar et al., 2018). The evolutionary history was inferred by using the maximum-likelihood method, the Hasegawa–Kishino–Yano 2-parameter model, a discrete gamma distribution 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.

The curated data sets used for phylogenetic analysis included 101 HA sequences, 98 NA sequences, and 26 complete genome sequences representative of the different lineages and clades.

2.5. GenBank Sequence Submission

The nucleotide sequences of 7 genome segments of strain ITA/2005/horse/Bari were deposited in GenBank under accession numbers OP919615 to OP919621. The HA gene sequence was previously obtained (EF117330) (Martella et al., 2007).

3. Results

The titer of the virus passage used for sequencing was $1{,}00X10^5$ copies (CT mean = 23.95) in qRT-PCR and 1: 256 in HA. A total of 877,687 reads were generated and after MiniMap2 assembling, the sequencing depth ranged from 35,415 to 2,421,160 (mean = 587,597) in the eight genome segments (Table 1).

Table 1. Number of reads obtained and depth of coverage per genome segment for strain A/equine/Bari/2005 by Minimap2

Gene	Segment	Length (nt)	Reads (number)	Coverage Depth	Accession
PB2	1	2341	16,195	35415.1	OP919615
PB1	2	2341	19,245	42029.6	OP919616
PA	3	2233	38,177	63306.4	OP919617
HA	4	1778	14,653	42886.6	EF117330
NP	5	1565	26,191	84524.7	OP919618
NA	6	1413	41,878	153256.3	OP919619
M	7	1027	360,563	1858200.5	OP919620
NS	8	800	409256	2421160.6	OP919621

In this analysis, seven genome segments of the isolate ITA/2005/horse/Bari showed the highest nucleotide (nt) identities (99.7 to 99.9% nt) to an H3N8 EIAV strain from Poland circulating during 2005 (Rozek et al., 2009), whilst the HA-encoding gene shared 99.0% nt identity to a 2008 H3N8 EIAV strain from Poland (Kwasnik et al., 2016) (Table 2).

Table 2. Equine influenza viruses with the highest nucleotide identity to ITA/2005/horse/Bari, determined by Basic Local Alignment Search Tool Nucleotide (BLASTn) (consulted on 28 February 2023).

Gene	Accession No.	Reference strains	Origin	% NtIdentity
PB2	MZ373289	A/equine/Pulawy/1/2005(H3N8)	Poland	99.7
PB1	MZ364002	A/equine/Pulawy/1/2005(H3N8)	Poland	99.8
PA	MZ373291	A/equine/Pulawy/1/2005(H3N8)	Poland	99.9
HA	KT429521	A/equine/Pulawy/1/2008(H3N8)	Poland	99.0
NP	MZ373293	A/equine/Pulawy/1/2005(H3N8)	Poland	99.9
NA	MZ373294	A/equine/Pulawy/1/2005(H3N8)	Poland	99.8
M	MZ373295	A/equine/Pulawy/1/2005(H3N8)	Poland	99.8
NS	MZ373296	A/equine/Pulawy/1/2005(H3N8)	Poland	99.9

Polymerase basic protein 2 (PB2), Polymerase basic protein 1 (PB1), Polymerase acidic (PA), Hemagglutinin (HA), Nucleoprotein (NP), Neuraminidase (NA), Matrix (M), Non-structural protein (NS).

An alignment of the HA protein was generated to inspect amino acid (aa) changes in the antigenic epitopes using reference EIAV strains (Figure S1). Overall, there were six aa changes between the isolate ITA/2005/horse/Bari and the closest relative in the HA gene, strain POL/2008/horse/Pulawy-1, but only two changes were mapped within HA epitope A at position 135 (R to G) and epitope D at position 213 (M to I). The mutation in epitope D was also observed in the reference FC2 strain GBR/2007/horse/Richmond-1, but not the change in epitope A. Three mutations were observed with respect to reference FC1 H3N8 viruses in epitope E (aa 78), B (aa 169), and D (aa 213). Moreover, seven mutations were observed with respect to the Eurasian reference strain GBR/1989/horse/Suffolk-1 in epitopes C (aa 48 and 276), B (aa 163 and 189), and D (aa 207, 213 and 244).

Based on NSIS data, the fluxes of horses (countries of origin) imported into Apulia provinces in 2005 are shown in Figure 1. Overall, 25,392 horses were imported into the Apulia region in 2005, of which 19,868 were destined to slaughter (DPA) and 5524 were non-DPA. The influxes of animals, based on their destinations (DPA and non-DPA) in the Apulia region (year 2005) and the provenience of non-DPA horses in November–December 2005 are also shown in Figure 1.

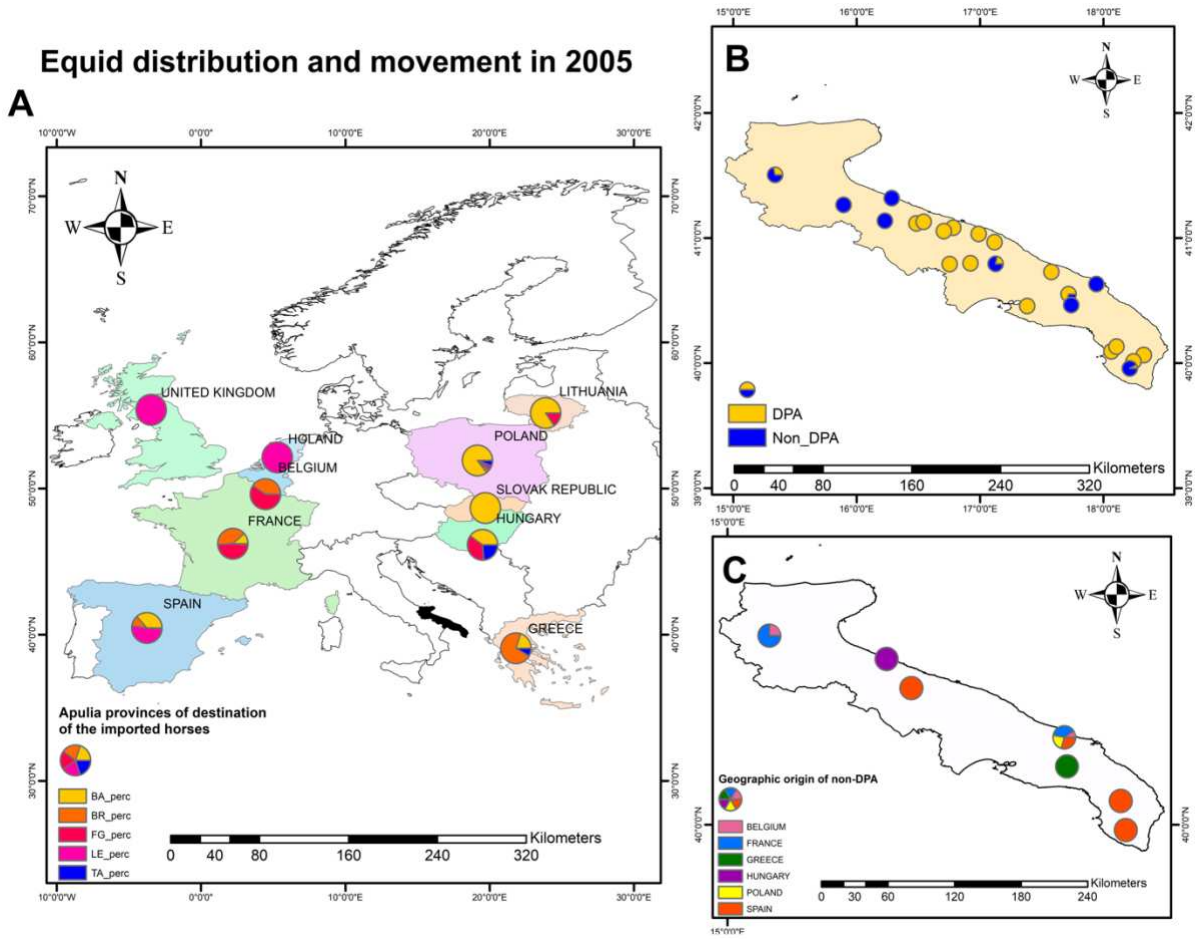

Figure 1. Fluxes of horses (countries of origin) imported in Apulia provinces (BA, Bari; BR, Brindisi; FG, Foggia; LE, Lecce; TA, Taranto) in 2005 (A); influxes of animals, based on their destinations (DPA, animals destined to slaughter; non-DPA, animals with other destination) in Apulia region (B); geographic provenience of non-DPA horse in November-December 2005 (C). Pie charts are not proportionate to the number of animals.

In phylogenetic analysis, based on the complete nucleotide sequences of the eight genome segments, strain ITA/2005/horse/Bari clustered within the Eurasian lineage in all the genome segments (PB2, PB1, PA, NP, NA, NS) but the HA and M (Figure 2 and Figure 3). In the phylogenetic trees based on segments 4 (HA) and 7 (M), the virus clustered with Florida 2 clade EIAV strains, suggesting an inter-lineage multi-reassortant genomic constellation.

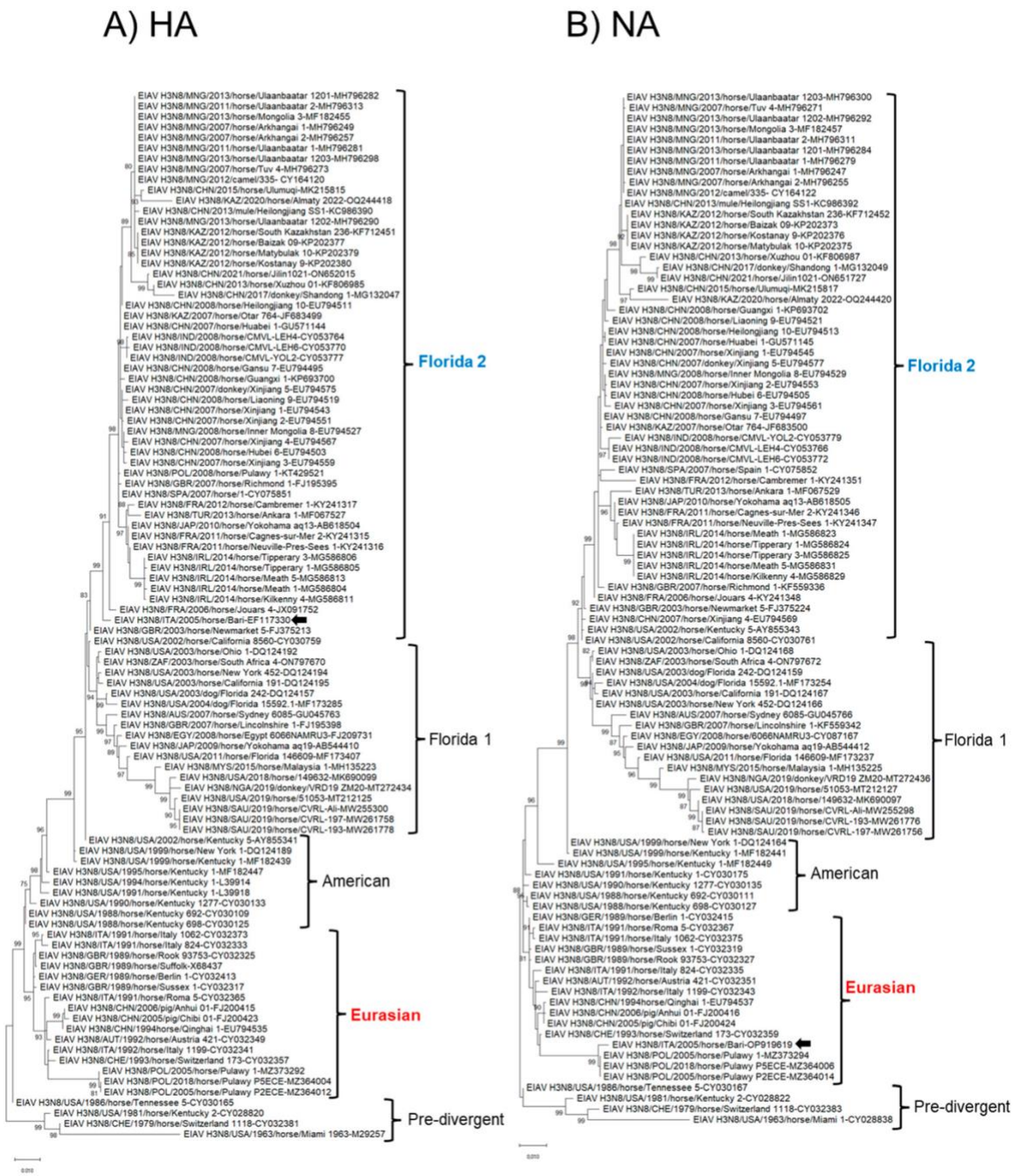

Figure 2. Phylogenetic analysis of haemagglutinin and neuraminidase genes of EIAV subtype H3N8 strain ITA/2005/horse/Bari compared with cognate sequences retrieved from GenBank database. Phylogeny of the hemagglutinin (HA) gene (**A**). Phylogeny of the neuraminidase (NA) (**B**). Phylogenetic trees were performed using the maximum likelihood method and Hasegawa–Kishino–Yano 2-parameter model with a gamma distribution. 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 arrow indicates the strain described in this study. Numbers of nucleotide substitutions are indicated by the scale bar.

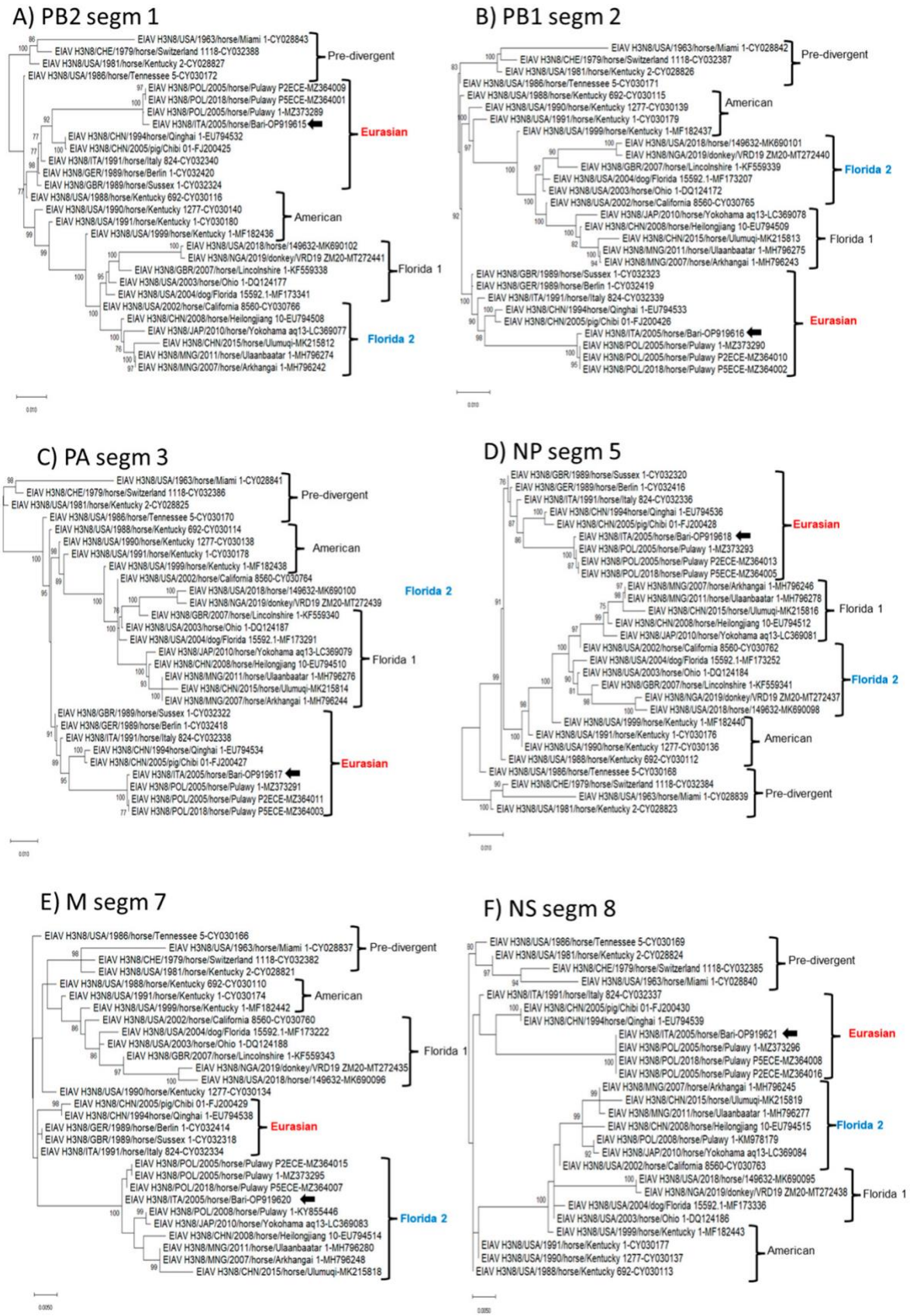

Figure 3. Phylogenetic tree of influenza virus polymerase complex (Polymerase basic protein 2, PB2, Polymerase basic protein 1, PB1, Polymerase acidic, PA), nucleoprotein (NP), matrix protein (M), and non-structural gene (NS) nucleotide sequences of the EIAV subtype H3N8 strain ITA/2005/horse/Bari were compared with cognate sequences

retrieved from GenBank database. Phylogeny of PB2 gene (**A**). Phylogeny of PB1 gene (**B**). Phylogeny of PA gene (**C**). Phylogeny of NP gene (**D**). Phylogeny of M gene (**E**). Phylogeny of NS gene (**F**). Phylogenetic trees were performed using the maximum likelihood method and Hasegawa–Kishino–Yano 2-parameter model with a gamma distribution. 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 arrow indicates the strain described in this study. Numbers of nucleotide substitutions are indicated by the scale bar.

4. Discussion

We applied a WGS protocol based on PCR enrichment of the IAV genome (Wüthrich et al., 2019) and deep sequencing on the ONT™ platform to generate the complete genome of an archival EIAV (Table 1). In the past, several reports have employed Sanger sequencing of single viral RNA segments such as the HA segment (Houghton et al., 2017; Pagani et al., 2015). This approach did not allow us to gather relevant information on other genome segments and to decipher, for instance, the intricated origin of reassortant IAV strains.

In recent years, WGS, coupled with NGS, has become the preferred method for the high-resolution characterization of pathogens, including IAVs. Multiple protocols have been described for WGS-based typing of IAVs (Hoffmann et al., 2001; Meinel et al., 2018; Zhou and Wentworth, 2012) and WGS has been applied to monitor the transmission events of IAVs in various settings (Houghton et al., 2017; McCrone et al., 2018; Pagani et al., 2015; Valley-Omar et al., 2015; Virk et al., 2017) and for retrospective studies on archival samples (Patrono et al., 2022; Xue et al., 2017). This has allowed us to generate an expanding database of WGS data, useful for the analysis of novel IAV strains and also for the re-analysis of archival viruses. Protocols based on NGS allow us to generate WGS sequence data in less than 24 h, dramatically increasing the throughput and decreasing the costs. Moreover, unlike Sanger sequencing, the final sequences are a consensus obtained with a scalable depth coverage, allowing us to retain information on single nucleotide polymorphisms at the quasispecies level. Finally, sequence-independent protocols can be used to generate complete genome sequences of novel uncharacterized organisms, overcoming the limits of Sanger technology. ONT™ is a third-generation NGS platform capable of producing long sequences with no theoretical read length limit (Lin et al., 2021), now declined into a number of applications. Whilst the costs for consumables can vary based on the multiplexing and chemistry among different NGS platforms, the hardware for the ONT™ platform can be acquired with a small investment, thus representing an excellent tool for low-budget research and diagnostics.

In our analysis, the isolate ITA/2005/horse/Bari appeared highly related (99.1 to 99.9% nt) in seven genome segments to an H3N8 EIAV strain from Poland circulating in 2005 (Rozek et al., 2009) (Table 2) and in the HA segment to a 2008 Poland isolate.

On phylogenetic analysis with cognate gene sequences of H3N8 EIAV strains retrieved from the databases, strain ITA/2005/horse/Bari was found to cluster with FC2 viruses in the HA and M genes, whilst in the other genes it clustered with strains of the Eurasian lineage (Figure 2 and Figure 3), intermingled with Poland EIAV strains from 2005 and 2008. This unique pattern is suggestive of intra-typic (inter-lineage) reassortment between H3N8 viruses (Murcia et al., 2011). Interestingly, the closest relative of strain ITA/2005/horse/Bari, strain POL/2005/Horse/Pulawy-1, was also a reassortant with an FC2-like M gene, but, unlike the Italian strain, it retained a HA gene of the Eurasian lineage. It is rather difficult to ascertain the trajectory of the evolution of the 2005 Italian and Poland viruses, i.e., if there were sequential events of reassortment or if they represented a pool of reassortant viruses co-circulating in Europe in 2005. Unfortunately, for the closest relative of strain ITA/2005/horse/Bari in the HA gene, strain POL/2008/Horse/Pulawy-1, the complete genome sequence was not available, although the only sequenced segments (HA, M, and NS) suggest an FC2-like constellation.

Analysis of the complete genome of 82 EIAV H3N8 strains collected in the period 1963 to 2008 from Europe, Asia, and the Americas has shown that all of the trees displayed a similar phylogenetic pattern typical of other mammalian IAVs, in which viruses from consecutive seasons were linked by a main trunk lineage with short side branches stemming from it (Murcia et al., 2011). For all gene segments, the main trunk of the trees was first bifurcated when the American and Eurasian lineages appeared in the late 1980s (Daly et al., 1996). A second bifurcation event occurred in the early 2000s, when the sub-lineages or clades FC1 and FC2 originated, both of which continue to circulate today (*OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition - Videoconference, 16 april 2020*, 2020)whilst the Eurasian lineage seems extinct (OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition - Videoconference, 16 april 2020, 2020). The separation between the two clades FC1 and FC2 is evident for all viruses isolated after 2005 in all phylogenies, although inconsistencies have been observed in the phylogeny of some genome segments, i.e., PB1, PB2, PA, and HA, suggestive of intra-subtype reassortment (Murcia et al., 2011). Intra-subtype reassortment in influenza evolution has been documented for H1N1 and H3N2 human influenza viruses ((Holmes et al., 2005; Nelson et al., 2008). This phenomenon has been suspected to play a role in the onset of viruses with unusual epidemiological and phenotype features, such as variations in antigenic profile and/or virulence (Nelson et al., 2008).

A similar pattern of evolution has been observed in a more recent study focused on the analysis of H3N8 EIAVs in Asia (Miño et al., 2019). This study included 102 complete genomes of viruses from 1963 to 2017 and 202 HA sequences of FC1 and FC2 viruses, collected between 2003 and 2017 worldwide. In these analyses, FC1 and FC2 viruses were shown to split into various sub-clades

seemingly circulating/evolving in restricted areas and occasionally spreading to other geographical areas. Noteworthy, no evidence of reassortment between FC1 and FC2 viruses was identified by the authors (Miño et al., 2019).

On visual inspection of the HA aa alignment, there were six aa changes between the isolate ITA/2005/horse/Bari and the closest relative, in this gene, strain POL/2008/horse/Pulawy-1 classified as clade FC2 but only two changes were mapped within HA epitope A at position 135 (R to G) and epitope D at position 213 (M to I). However, a number of aa changes were present in the antigenic epitopes when compared with reference strains of other lineages and sub-lineages. Three mutations were observed with respect to FC1 H3N8 viruses in epitopes E, B, and D and seven mutations were observed with respect to the Eurasian reference strain GBR/1989/horse/Suffolk-1 in epitopes C, B, and D. Changes in the epitopes of IAVs can drastically affect recognition of vaccine-induced immunity and continual monitoring of circulating viruses is required to update vaccine composition (*OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition - Videoconference, 16 april 2020*, 2020). The HA epitope profile of the 2005 Italian virus was therefore highly conserved since only a single aa change was observed with respect to the reference H3N8 FC2 strain GBR/2007/horse/Richmond-1.

In summary, global surveillance activities have revealed that EIAV outbreaks are reported every year worldwide, and since the early 2000s, they have been caused by strains that belong to either FC1 or FC2 (Nelson et al., 2008), whilst H3N8 viruses of Eurasian lineage are no longer detected and no longer recommended in vaccine composition (*OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition - Videoconference, 16 april 2020*, 2020). However, the results of our analyses indicate that reassortant H3N8 viruses with a wide set of genes of Eurasian lineage were still circulating in Europe in 2005. Since surveillance investigations mostly collected data on the HA gene in those years, it is possible that similar reassortant viruses continued to circulate but they remained undetected. WGS analysis of archival collections could be useful to understand more in depth the extent of this phenomenon.

Since Poland is a major source of horses imported for local horse meat consumption, we interrogated the records of the national register for animal trading (NSIS) and observed that in 2005, 50.4% (12,788/25,392) of horses were imported from Poland, reaching 59.1% (1184/2003) in November and 61,4% (716/1167) in December. Of these, 19,868 (72.2%) were DPA, whilst 5524 (27.8%) were not (non-DPA). Overall, only 8.7% (478/5524) of the non-DPA horses were imported from Poland, suggesting that most animals imported from Poland were for the meat industry. Yet, finding a connection between the horses housed in the center of the Forest Service Rangers and meat horses was difficult considering the different destinations. However, in November and December 2005,

43/1184 (3.6%) and 28/716 (3.9%) non-DPA horses, respectively, were also introduced in Apulia from Poland. All these non-DPA animals were delivered to Latiano (Brindisi prefecture), only 40-km far from Martina Franca. Accordingly, we hypothesize that the 2005 EIAV outbreak in Bari was due to the re-introduction of EIAV with either DPA or non-DPA horses imported from Poland. This evidence, coupled with the genomic data, would re-enforce the hypothesis on the origin of the 2005 Italian EIAV outbreak.

5. Conclusions

Coupling molecular and epidemiological data, a 2005 cold case, i.e., the origin of an outbreak of EIAV, could be finally disentangled and the 2005 H3N8 EIAV strain was found to be a multi-reassortant between Eurasian lineage and FC2 viruses.

WGS is now being applied more routinely to EIAV analysis (Wüthrich et al., 2019), thus helping understand the evolutionary routes of H3N8 EIAV strains on a global scale (Murcia et al., 2011). In addition, since H3N8 EIAVs have also been identified in dogs (Daly et al., 2007), pigs, cats (Su et al., 2014; Tu et al., 2009), and in camels (Yondon et al., 2014), this information is useful to understand promptly and thoroughly inter-species transmission events. Although the evolution of H3N8 EIAV is almost entirely driven by mechanisms of genetic drifts (Bean et al., 1992), WGS has revealed that reassortment may also occur between H3N8 EIAV strains and with IAVs of non-equine origin (Khan et al., 2017; Langat et al., 2017). Genome segments related to influenza A H1N8, H5N1, H7N1, and H9N2 strains have been identified among samples collected from horses, accounting for less than 0.5% of all EIAV cases (Xie et al., 2016). Moreover, a novel H9N2 influenza A virus was detected in 2011, in horses in Guangxi Province, China (He, 2012). Generating a large database of EIAV genome sequences will surely improve the resolution of epidemiological investigations. Understanding the molecular evolution and phylodynamic of EIAV requires a large-scale and comprehensive genome data set. This should also include the analysis of archival EIAV strains.

References

Ahmed BM, Bayoumi MM, Farrag MA, Elgamal MA, Daly JM, Amer HM. Emergence of equine influenza virus H3Nx Florida clade 2 in Arabian racehorses in Egypt. Virol J. 2022 Nov 12;19(1):185. doi: 10.1186/s12985-022-01917-9. PMID: 36371185; PMCID: PMC9652821.

Baillie GJ, Galiano M, Agapow PM, Myers R, Chiam R, Gall A, Palser AL, Watson SJ, Hedge J, Underwood A, Platt S, McLean E, Pebody RG, Rambaut A, Green J, Daniels R, Pybus OG, Kellam P, Zambon M. Evolutionary dynamics of local pandemic H1N1/2009 influenza virus lineages revealed by whole-genome analysis. J Virol. 2012 Jan;86(1):11-8. doi: 10.1128/JVI.05347-11. Epub 2011 Oct 19. PMID: 22013031; PMCID: PMC3255882.

Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, Webster RG. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J Virol. 1992 Feb;66(2):1129-38. doi: 10.1128/JVI.66.2.1129-

1138.1992. PMID: 1731092; PMCID: PMC240817.

Bryant NA, Rash AS, Russell CA, Ross J, Cooke A, Bowman S, MacRae S, Lewis NS, Paillot R, Zanoni R, Meier H, Griffiths LA, Daly JM, Tiwari A, Chambers TM, Newton JR, Elton DM. Antigenic and genetic variations in European and North American equine influenza virus strains (H3N8) isolated from 2006 to 2007. Vet Microbiol. 2009 Jul 2;138(1-2):41-52. doi: 10.1016/j.vetmic.2009.03.004. Epub 2009 Mar 13. PMID: 19346084.

Cullinane A, Newton JR. Equine influenza--a global perspective. Vet Microbiol. 2013 Nov 29;167(1-2):205-14. doi: 10.1016/j.vetmic.2013.03.029. Epub 2013 Apr 9. PMID: 23680107.

Daly JM, Lai AC, Binns MM, Chambers TM, Barrandeguy M, Mumford JA. Antigenic and genetic evolution of equine H3N8 influenza A viruses. J Gen Virol. 1996 Apr;77 (Pt 4):661-71. doi: 10.1099/0022-1317-77-4-661. PMID: 8627254.

Daly JM, Sindle T, Tearle J, Barquero N, Newton JR, Corning S. Equine influenza vaccine containing older H3N8 strains offers protection against A/eq/South Africa/4/03 (H3N8) strain in a short-term vaccine efficacy study. Equine Vet J. 2007 Sep;39(5):446-50. doi: 10.2746/042516407x180327. PMID: 17910270.

Diallo AA, Souley MM, Issa Ibrahim A, Alassane A, Issa R, Gagara H, Yaou B, Issiakou A, Diop M, Ba Diouf RO, Lo FT, Lo MM, Bakhoum T, Sylla M, Seck MT, Meseko C, Shittu I, Cullinane A, Settypalli TBK, Lamien CE, Dundon WG, Cattoli G. Transboundary spread of equine influenza viruses (H3N8) in West and Central Africa: Molecular characterization of identified viruses during outbreaks in Niger and Senegal, in 2019. Transbound Emerg Dis. 2021 May;68(3):1253-1262. doi: 10.1111/tbed.13779. Epub 2020 Aug 17. PMID: 32770642.

Durães-Carvalho R, Salemi M. In-depth phylodynamics, evolutionary analysis and in silico predictions of universal epitopes of Influenza A subtypes and Influenza B viruses. Mol Phylogenet Evol. 2018 Apr;121:174-182. doi: 10.1016/j.ympev.2018.01.008. Epub 2018 Jan 31. PMID: 29355604.

Gildea S, Quinlivan M, Arkins S, Cullinane A. The molecular epidemiology of equine influenza in Ireland from 2007-2010 and its international significance. Equine Vet J. 2012 Jul;44(4):387-92. doi: 10.1111/j.2042-3306.2011.00472.x. Epub 2011 Oct 6. PMID: 21978127.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 2001 Dec;146(12):2275-89. doi: 10.1007/s007050170002. PMID: 11811679.

Holmes EC, Ghedin E, Miller N, Taylor J, Bao Y, St George K, Grenfell BT, Salzberg SL, Fraser CM, Lipman DJ, Taubenberger JK. Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses. PLoS Biol. 2005 Sep;3(9):e300. doi: 10.1371/journal.pbio.0030300. Epub 2005 Jul 26. PMID: 16026181; PMCID: PMC1180517.

Houghton R, Ellis J, Galiano M, Clark TW, Wyllie S. Haemagglutinin and neuraminidase sequencing delineate nosocomial influenza outbreaks with accuracy equivalent to whole genome sequencing. J Infect. 2017 Apr;74(4):377-384. doi: 10.1016/j.jinf.2016.12.015. Epub 2017 Jan 16. PMID: 28104386.

Ito M, Nagai M, Hayakawa Y, Komae H, Murakami N, Yotsuya S, Asakura S, Sakoda Y, Kida H. Genetic Analyses of an H3N8 Influenza Virus Isolate, Causative Strain of the Outbreak of Equine Influenza at the Kanazawa Racecourse in Japan in 2007. J Vet Med Sci. 2008 Sep;70(9):899-906. doi: 10.1292/jvms.70.899. PMID: 18840963.

Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002 Jul 15;30(14):3059-66. doi: 10.1093/nar/gkf436. PMID: 12136088; PMCID: PMC135756.

Khan A, Mushtaq MH, Ahmad MUD, Nazir J, Farooqi SH, Khan A. Molecular Epidemiology of a novel re-assorted epidemic strain of equine influenza virus in Pakistan in 2015-16. Virus Res. 2017 Aug 15;240:56-63. doi:

10.1016/j.virusres.2017.07.022. Epub 2017 Jul 27. PMID: 28757141.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018 Jun 1;35(6):1547-1549. doi: 10.1093/molbev/msy096. PMID: 29722887; PMCID: PMC5967553.

Kwasnik M, Gora IM, Rola J, Zmudzinski JF, Rozek W. NS-gene based phylogenetic analysis of equine influenza viruses isolated in Poland. Vet Microbiol. 2016;182:95-101. doi: 10.1016/j.vetmic.2015.10.028. Epub 2015 Oct 31. PMID: 26711034

Laabassi F, Lecouturier F, Amelot G, Gaudaire D, Mamache B, Laugier C, Legrand L, Zientara S, Hans A. Epidemiology and Genetic Characterization of H3N8 Equine Influenza Virus Responsible for Clinical Disease in Algeria in 2011. Transbound Emerg Dis. 2015 Dec;62(6):623-31. doi: 10.1111/tbed.12209. Epub 2014 Jan 29. PMID: 24472362.

Lai AC, Chambers TM, Holland RE Jr, Morley PS, Haines DM, Townsend HG, Barrandeguy M. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. Arch Virol. 2001;146(6):1063-74. doi: 10.1007/s007050170106. PMID: 11504416.

Landolt, G. A., Townsend, H. G., and Lunn, D. P. (2014). Equine influenza infection. In Equine infectious diseases (pp. 141–151). Elsevier.

Langat P, Raghwani J, Dudas G, Bowden TA, Edwards S, Gall A, Bedford T, Rambaut A, Daniels RS, Russell CA, Pybus OG, McCauley J, Kellam P, Watson SJ. Genome-wide evolutionary dynamics of influenza B viruses on a global scale. PLoS Pathog. 2017 Dec 28;13(12):e1006749. doi: 10.1371/journal.ppat.1006749. PMID: 29284042; PMCID: PMC5790164.

Lin B, Hui J, Mao H. Nanopore Technology and Its Applications in Gene Sequencing. Biosensors (Basel). 2021 Jun 30;11(7):214. doi: 10.3390/bios11070214. PMID: 34208844; PMCID: PMC8301755.

Martella V, Elia G, Decaro N, Di Trani L, Lorusso E, Campolo M, Desario C, Parisi A, Cavaliere N, Buonavoglia C. An outbreak of equine influenza virus in vaccinated horses in Italy is due to an H3N8 strain closely related to recent North American representatives of the Florida sub-lineage. Vet Microbiol. 2007 Mar 31;121(1-2):56-63. doi: 10.1016/j.vetmic.2006.11.017. Epub 2006 Nov 25. PMID: 17197135.

McCrone JT, Woods RJ, Martin ET, Malosh RE, Monto AS, Lauring AS. Stochastic processes constrain the within and between host evolution of influenza virus. Elife. 2018 May 3;7:e35962. doi: 10.7554/eLife.35962. PMID: 29683424; PMCID: PMC5933925.

Meinel DM, Heinzinger S, Eberle U, Ackermann N, Schönberger K, Sing A. Whole genome sequencing identifies influenza A H3N2 transmission and offers superior resolution to classical typing methods. Infection. 2018 Feb;46(1):69-76. doi: 10.1007/s15010-017-1091-3. Epub 2017 Oct 31. PMID: 29086356.

Miño S, Mojsiejczuk L, Guo W, Zhang H, Qi T, Du C, Zhang X, Wang J, Campos R, Wang X. Equine Influenza Virus in Asia: Phylogeographic Pattern and Molecular Features Reveal Circulation of an Autochthonous Lineage. J Virol. 2019 Jun 14;93(13):e00116-19. doi: 10.1128/JVI.00116-19. PMID: 31019053; PMCID: PMC6580976.

Murcia PR, Wood JL, Holmes EC. Genome-scale evolution and phylodynamics of equine H3N8 influenza A virus. J Virol. 2011 Jun;85(11):5312-22. doi: 10.1128/JVI.02619-10. Epub 2011 Mar 23. PMID: 21430049; PMCID: PMC3094979.

Nelson MI, Viboud C, Simonsen L, Bennett RT, Griesemer SB, St George K, Taylor J, Spiro DJ, Sengamalay NA, Ghedin E, Taubenberger JK, Holmes EC. Multiple reassortment events in the evolutionary history of H1N1 influenza A virus since 1918. PLoS Pathog. 2008 Feb 29;4(2):e1000012. doi: 10.1371/journal.ppat.1000012. PMID: 18463694; PMCID: PMC2262849.

Newton JR, Daly JM, Spencer L, Mumford JA. Description of the outbreak of equine influenza (H3N8) in the United Kingdom in 2003, during which recently vaccinated horses in Newmarket developed respiratory disease. Vet Rec. 2006 Feb 11;158(6):185-92. doi: 10.1136/vr.158.6.185. PMID: 16474051.

OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition—Videoconference, 16 april 2020. (2020). https://doi.org/10.20506/bull.2020.1.3134

Pagani L, Thomas Y, Huttner B, Sauvan V, Notaridis G, Kaiser L, Iten A, Pittet D, Harbarth S. Transmission and effect of multiple clusters of seasonal influenza in a Swiss geriatric hospital. J Am Geriatr Soc. 2015 Apr;63(4):739-44. doi: 10.1111/jgs.13339. PMID: 25900487; PMCID: PMC7166655.

Pallen MJ, Loman NJ, Penn CW. High-throughput sequencing and clinical microbiology: progress, opportunities and challenges. Curr Opin Microbiol. 2010 Oct;13(5):625-31. doi: 10.1016/j.mib.2010.08.003. Epub 2010 Sep 16. PMID: 20843733.

Patrono LV, Vrancken B, Budt M, Düx A, Lequime S, Boral S, Gilbert MTP, Gogarten JF, Hoffmann L, Horst D, Merkel K, Morens D, Prepoint B, Schlotterbeck J, Schuenemann VJ, Suchard MA, Taubenberger JK, Tenkhoff L, Urban C, Widulin N, Winter E, Worobey M, Schnalke T, Wolff T, Lemey P, Calvignac-Spencer S. Archival influenza virus genomes from Europe reveal genomic variability during the 1918 pandemic. Nat Commun. 2022 May 10;13(1):2314. doi: 10.1038/s41467-022-29614-9. PMID: 35538057; PMCID: PMC9090925.

Quinlivan M, Dempsey E, Ryan F, Arkins S, Cullinane A. Real-time reverse transcription PCR for detection and quantitative analysis of equine influenza virus. J Clin Microbiol. 2005 Oct;43(10):5055-7. doi: 10.1128/JCM.43.10.5055-5057.2005. PMID: 16207961; PMCID: PMC1248463.

Rozek W, Purzycka M, Polak MP, Gradzki Z, Zmudzinski JF. Genetic typing of equine influenza virus isolated in Poland in 2005 and 2006. Virus Res. 2009 Oct;145(1):121-6. doi: 10.1016/j.virusres.2009.06.017. Epub 2009 Jul 4. PMID: 19580828.

Shittu I, Meseko CA, Sulaiman LP, Inuwa B, Mustapha M, Zakariya PS, Muhammad AA, Muhammad U, Atuman YJ, Barde IJ, Zecchin B, Quaranta EG, Shamaki D, Alabi O, Monne I, Fusaro A, Joannis TM. Fatal multiple outbreaks of equine influenza H3N8 in Nigeria, 2019: The first introduction of Florida clade 1 to West Africa. Vet Microbiol. 2020 Sep;248:108820. doi: 10.1016/j.vetmic.2020.108820. Epub 2020 Aug 13. PMID: 32891950.

Su S, Wang L, Fu X, He S, Hong M, Zhou P, Lai A, Gray G, Li S. Equine influenza A(H3N8) virus infection in cats. Emerg Infect Dis. 2014 Dec;20(12):2096-9. doi: 10.3201/eid2012.140867. PMID: 25417790; PMCID: PMC4257791

Tu J, Zhou H, Jiang T, Li C, Zhang A, Guo X, Zou W, Chen H, Jin M. Isolation and molecular characterization of equine H3N8 influenza viruses from pigs in China. Arch Virol. 2009;154(5):887-90. doi: 10.1007/s00705-009-0381-1. Epub 2009 Apr 26. PMID: 19396578.

Valley-Omar Z, Nindo F, Mudau M, Hsiao M, Martin DP. Phylogenetic Exploration of Nosocomial Transmission Chains of 2009 Influenza A/H1N1 among Children Admitted at Red Cross War Memorial Children's Hospital, Cape Town, South Africa in 2011. PLoS One. 2015 Nov 13;10(11):e0141744. doi: 10.1371/journal.pone.0141744. PMID: 26565994; PMCID: PMC4643913.

Virk RK, Gunalan V, Lee HK, Inoue M, Chua C, Tan BH, Tambyah PA. Molecular Evidence of Transmission of Influenza A/H1N1 2009 on a University Campus. PLoS One. 2017 Jan 6;12(1):e0168596. doi: 10.1371/journal.pone.0168596. PMID: 28060851; PMCID: PMC5218485.

Virmani N, Bera BC, Gulati BR, Karuppusamy S, Singh BK, Kumar Vaid R, Kumar S, Kumar R, Malik P, Khurana SK, Singh J, Manuja A, Dedar R, Gupta AK, Yadav SC, Chugh PK, Narwal PS, Thankur VL, Kaul R, Kanani

A, Rautmare SS, Singh RK. Descriptive epidemiology of equine influenza in India (2008-2009): temporal and spatial trends. Vet Ital. 2010 Oct-Dec;46(4):449-58. PMID: 21120800.

Watson SJ, Langat P, Reid SM, Lam TT, Cotten M, Kelly M, Van Reeth K, Qiu Y, Simon G, Bonin E, Foni E, Chiapponi C, Larsen L, Hjulsager C, Markowska-Daniel I, Urbaniak K, Dürrwald R, Schlegel M, Huovilainen A, Davidson I, Dán Á, Loeffen W, Edwards S, Bublot M, Vila T, Maldonado J, Valls L; ESNIP3 Consortium; Brown IH, Pybus OG, Kellam P. Molecular Epidemiology and Evolution of Influenza Viruses Circulating within European Swine between 2009 and 2013. J Virol. 2015 Oct;89(19):9920-31. doi: 10.1128/JVI.00840-15. Epub 2015 Jul 22. PMID: 26202246; PMCID: PMC4577897.

Webster RG. Are equine 1 influenza viruses still present in horses? Equine Vet J. 1993 Nov;25(6):537-8. doi: 10.1111/j.2042-3306.1993.tb03009.x. PMID: 8276003.

Wright P., Neumann G., Kawaoka Y. Fields Virology. 6th ed. Volume 1. WoltersKluwer-Lippincott Williams and Wilkins; Philadelphia, PA, USA: 2013. Orthomyxoviruses; pp. 1186–1244.

Wüthrich D, Lang D, Müller NF, Neher RA, Stadler T, Egli A. Evaluation of two workflows for whole genome sequencing-based typing of influenza A viruses. J Virol Methods. 2019 Apr;266:30-33. doi: 10.1016/j.jviromet.2019.01.009. Epub 2019 Jan 21. PMID: 30677464.

Xie T, Anderson BD, Daramragchaa U, Chuluunbaatar M, Gray GC. A Review of Evidence that Equine Influenza Viruses Are Zoonotic. Pathogens. 2016 Jul 12;5(3):50. doi: 10.3390/pathogens5030050. PMID: 27420100; PMCID: PMC5039430.

Xue KS, Stevens-Ayers T, Campbell AP, Englund JA, Pergam SA, Boeckh M, Bloom JD. Parallel evolution of influenza across multiple spatiotemporal scales. Elife. 2017 Jun 27;6:e26875. doi: 10.7554/eLife.26875. PMID: 28653624; PMCID: PMC5487208.

Yamanaka T, Niwa H, Tsujimura K, Kondo T, Matsumura T. Epidemic of equine influenza among vaccinated racehorses in Japan in 2007. J Vet Med Sci. 2008 Jun;70(6):623-5. doi: 10.1292/jvms.70.623. PMID: 18628606.

Yondon M, Zayat B, Nelson MI, Heil GL, Anderson BD, Lin X, Halpin RA, McKenzie PP, White SK, Wentworth DE, Gray GC. Equine influenza A(H3N8) virus isolated from Bactrian camel, Mongolia. Emerg Infect Dis. 2014 Dec;20(12):2144-7. doi: 10.3201/eid2012.140435. PMID: 25418532; PMCID: PMC4257804.

Zhou B, Wentworth DE. Influenza A virus molecular virology techniques. Methods Mol Biol. 2012; 865:175-92. doi: 10.1007/978-1-61779-621-0_11. PMID: 22528160.

Chapter 2

Rotaviruses A: etiological agent of enteritis in animals

Rotavirus (RV) is a leading cause of gastrointestinal diarrhea in infants and newborn animals worldwide (Caddy et al., 2021). In the human population, RVs cause yearly 122,322–215,575 deaths in children below five years-age (Steele et al., 2019).

RV was initially identified in the 1950s in rectal swabs collected from monkeys. Subsequently, in the 1960s, RV was observed in intestinal biopsies of mice using electron microscopy (Adams and Kraft, 1963). In 1973, Bishop and colleagues provided the first description of the virus in children who exhibited symptoms of gastroenteritis (Bishop et al., 1973). Later, RV was detected through direct thin-layer electron microscopy and immune electron microscopy in fecal samples from hospitalized children suffering from acute non-bacterial gastroenteritis (Bishop et al., 1974). Under the electron microscope, a viral particle displaying a wheel-like structure, measuring around 70 nanometers in diameter, and was thereof named "rotavirus." The term "rotavirus" is derived from the Latin word "rota," denoting wheel (Flewett et al., 1974). The genus *Rotavirus* belongs to the family *Sedoreoviridae* within the *Reovirales* order (Matthijnssens et al., 2022). The RV is a large, non-enveloped virus that displays three concentric icosahedral capsid structures. Its diameter ranges from 65 to 75 nm. Two capsid proteins, VP4 and VP7, compose the outer layer while VP6 protein is localized in the intermediate layer. VP2 is connected to VP1 (RNA-dependent RNA polymerase) and VP3 (viral capping enzyme) within the internal core. RV genome consists of 11 double-stranded RNA (dsRNA) segments, enclosed within the internal core layer (Jampanil et al., 2023). The rotavirus genome codes six nonstructural proteins (NSP1-NSP6) and six viral structural proteins (VP1-VP4, VP6, and VP7) (Desselberger, 2014).

A total of ten rotavirus species (A − J) and the putative species RVK and RVL are defined, mainly based on genetic and antigenic features of the inner capsid VP6 protein (Johne et al., 2023).

In Africa RVA-associated deaths accounts for about 50% of global disease mortality despite the introduction of RVA vaccines in many ountries (Ndebe et al., 2023). Additionally, rotavirus can infect domesticated and wild animals, resulting in the death of newborn animals (Caddy et al., 2021). RVA is a major cause of rotaviral diarrhea in birds and bats are regarded as RVA reservoir (Islam et al., 2020). While RVB, RVC, RVE, RVH, and RVI have been detected in mammalian hosts, RVD, RVF, and RVG have been detected only in birds and RVI and RVJ were discovered in dogs and bats (Bányai et al., 2017; Mihalov-Kovács et al., 2015). The tentative species rotavirus K (RVK) and L (RVL) have been identified in 2019 in common shrews (*Sorex araneus*) in Germany (Johne et al., 2019). RVL is considered as a separate species ith???? the highest genetic relatedness to RVB-like

clade (Johne et al., 2022). Although 42 G genotypes and 58 P genotypes have been identified globally in both humans and animals (Sadiq and Khan, 2024), only a small number of G and P genotype combinations have been primarily identified in humans. RVA strains G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8], have been shown to account for approximately 90% of global rotavirus transmission in humans (Hakim et al., 2024).

There is a great variety of circulating RVA strains in animals. The G3, G5, G10, G14 and P[12] genotypes are commonly found in equines (Matthijnssens et al., 2012). The RVA that infect bovines are related to G1, G6-G8, G10, G11, G15, G18 and G21, and P[1], P[5], P[11], P[14], P[17], P[21] and P[29] genotypes; in that, G6, G8 and G10 are associated to P[5], P[11] and P[1], which are the most prevalent (Badaracco et al., 2013; Dhama et al., 2009; Martella et al., 2010). G6 is often detected in livestock and G10, in dairy cattle (Varshney et al., 2002). In pigs several genotypes have been described, including G1-G6, G8-G12, P[1], P[5-8], P[11], P[13], P[19], P[21-27] and P[32] (Miyazaki et al., 2011). The RVA strains isolated in lamb belong to the G1, G3, G5, G6, G8, G9 and G10 genotypes, while the G3, G6 and G10 genotypes are often associated to P types belonging to P[1], P[11] and P[14] (Dhama et al., 2009). In goat the most common genotype is G6P[5] (Dhama et al., 2009), but other strains have already been described: G3P[3] in South Korea, G6P[14] in South Africa, and G6P[1] in Italy (Ghosh et al., 2010).

G3 is the most prevalent genotype of RVA and it is usually associated to P[3] and P[5] in dogs, and to P[3] and P[9] in cats (Tsugawa and Hoshino, 2008). G3 is also often detected in rabbits, but associated to P[22] and P[14] (Martella et al., 2005). Information on RVA genotypes infecting birds is limited to date, and some studies reported detection of G7, G23, G22, G6, G10 and P[37] ((Silva et al., 2012; Trojnar et al., 2013).

Rabbits are important livestock, wild, pet and laboratory animals. The rabbit breeding industry has experienced a considerable increase in meat production, despite the vulnerability of this particular species to infectious diseases (Carvalho et al., 2017; Isomursu et al., 2018). Rabbits can suffer from many viral, bacterial, and parasitic diseases; however, viral infections seem to have the highest impact on their health (Kerr and Donnelly, 2013). Enteric diseases can seriously impact rabbit farms because they can generate severe financial losses due to mortality, compromised growth performance and lower conversion rates (Papp et al., 2013; Peeters et al., 1984).

Animal rotavirus vaccines currently available are designed for cattle, swine, and horses, as these

species can suffer significant economic losses due to rotaviral diarrhea. Most of these vaccines are inactivated and can be administered to pregnant animals to stimulate robust maternal (passive) immunity, which helps protect newborns from neonatal diarrhea shortly after birth (Carossino et al., 2024).

References

Adams WR, Kraft LM. Epizootic diarrhea of infant mice: indentification of the etiologic agent. Science. 1963 Jul 26;141(3578):359-60. doi: 10.1126/science.141.3578.359. PMID: 14011026.

Badaracco A, Matthijnssens J, Romero S, Heylen E, Zeller M, Garaicoechea L, Van Ranst M, Parreño V. Discovery and molecular characterization of a group A rotavirus strain detected in an Argentinean vicuña (Vicugna vicugna). Vet Microbiol. 2013 Jan 25;161(3-4):247-54. doi: 10.1016/j.vetmic.2012.07.035. Epub 2012 Jul 27. PMID: 22877519.

Bányai K, Kemenesi G, Budinski I, Földes F, Zana B, Marton S, Varga-Kugler R, Oldal M, Kurucz K, Jakab F. Candidate new rotavirus species in Schreiber's bats, Serbia. Infect Genet Evol. 2017 Mar; 48:19-26. doi: 10.1016/j.meegid.2016.12.002. Epub 2016 Dec 6. PMID: 27932285; PMCID: PMC7106153.

Bishop RF, Davidson GP, Holmes IH, Ruck BJ. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. Lancet. 1974 Feb 2;1(7849):149-51. doi: 10.1016/s0140-6736(74)92440-4. PMID: 4129719.

Bishop RF, Davidson GP, Holmes IH, Ruck BJ. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet. 1973 Dec 8;2(7841):1281-3. doi: 10.1016/s0140-6736(73)92867-5. PMID: 4127639.

Caddy S, Papa G, Borodavka A, Desselberger U. Rotavirus research: 2014-2020. Virus Res. 2021 Oct 15; 304:198499. doi: 10.1016/j.virusres.2021.198499. Epub 2021 Jul 2. PMID: 34224769.

Carossino M, Vissani MA, Barrandeguy ME, Balasuriya UBR, Parreño V. Equine Rotavirus A under the One Health Lens: Potential Impacts on Public Health. Viruses. 2024 Jan 16;16(1):130. doi: 10.3390/v16010130. PMID: 38257830; PMCID: PMC10819593.

Carvalho CL, Silva S, Gouveia P, Costa M, Duarte EL, Henriques AM, Barros SS, Luís T, Ramos F, Fagulha T, Fevereiro M, Duarte MD. Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016-2017). Virus Genes. 2017 Dec;53(6):922-926. doi: 10.1007/s11262-017-1483-6. Epub 2017 Jun 21. PMID: 28639220.

Desselberger U. Rotaviruses. Virus Res. 2014 Sep 22;190:75-96. doi: 10.1016/j.virusres.2014.06.016. Epub 2014 Jul 9. PMID: 25016036.

Dhama K, Chauhan RS, Mahendran M, Malik SV. Rotavirus diarrhea in bovines and other domestic animals. Vet Res Commun. 2009 Jan;33(1):1-23. doi: 10.1007/s11259-008-9070-x. Epub 2008 Jul 12. PMID: 18622713; PMCID: PMC7088678.

Flewett TH, Bryden AS, Davies H, Woode GN, Bridger JC, Derrick JM. Relation between viruses from acute gastroenteritis of children and newborn calves. Lancet. 1974 Jul 13;2(7872):61-3. doi: 10.1016/s0140-6736(74)91631-6. PMID: 4137164.

Ghosh S, Alam MM, Ahmed MU, Talukdar RI, Paul SK, Kobayashi N. Complete genome constellation of a caprine group A rotavirus strain reveals common evolution with ruminant and human rotavirus strains. J Gen Virol. 2010

Sep;91(Pt 9):2367-73. doi: 10.1099/vir.0.022244-0. Epub 2010 May 26. PMID: 20505013.

Hakim MS, Gazali FM, Widyaningsih SA, Parvez MK. Driving forces of continuing evolution of rotaviruses. World J Virol. 2024 Jun 25;13(2):93774. doi: 10.5501/wjv.v13.i2.93774. PMID: 38984077; PMCID: PMC11229848.

Islam A, Hossain ME, Rostal MK, Ferdous J, Islam A, Hasan R, Miah M, Rahman M, Rahman MZ, Daszak P, Epstein JH. Epidemiology and Molecular Characterization of Rotavirus A in Fruit Bats in Bangladesh. Ecohealth. 2020 Sep;17(3):398-405. doi: 10.1007/s10393-020-01488-7. Epub 2020 Sep 2. PMID: 32876756; PMCID: PMC7464061.

Isomursu M, Neimanis A, Karkamo V, Nylund M, Holopainen R, Nokireki T, Gadd T. An Outbreak of Rabbit Hemorrhagic Disease in Finland. J Wildl Dis. 2018 Oct;54(4):838-842. doi: 10.7589/2017-11-286. Epub 2018 Jun 11. PMID: 29889007.

Jampanil N, Kumthip K, Maneekarn N, Khamrin P. Genetic Diversity of Rotaviruses Circulating in Pediatric Patients and Domestic Animals in Thailand. Trop Med Infect Dis. 2023 Jun 29;8(7):347. doi: 10.3390/tropicalmed8070347. PMID: 37505643; PMCID: PMC10383398.

Johne R, Schilling-Loeffler K, Ulrich RG, Tausch SH. Whole Genome Sequence Analysis of a Prototype Strain of the Novel Putative Rotavirus Species L. Viruses. 2022 Feb 24;14(3):462. doi: 10.3390/v14030462. PMID: 35336869; PMCID: PMC8954357.

Johne R, Tausch SH, Grützke J, Falkenhagen A, Patzina-Mehling C, Beer M, Höper D, Ulrich RG. Distantly Related Rotaviruses in Common Shrews, Germany, 2004-2014. Emerg Infect Dis. 2019 Dec;25(12):2310-2314. doi: 10.3201/eid2512.191225. PMID: 31742508; PMCID: PMC6874240.

Johne R, Tausch SH, Ulrich RG, Schilling-Loeffler K. Genome analysis of the novel putative rotavirus species K. Virus Res. 2023 Sep;334:199171. doi: 10.1016/j.virusres.2023.199171. Epub 2023 Jul 11. PMID: 37433351; PMCID: PMC10410577.

Kerr PJ, Donnelly TM. Viral infections of rabbits. Vet Clin North Am Exot Anim Pract. 2013 May;16(2):437-68. doi: 10.1016/j.cvex.2013.02.002. Epub 2013 Mar 17. PMID: 23642871; PMCID: PMC7110462.

Martella V, Bányai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. Vet Microbiol. 2010 Jan 27;140(3-4):246-55. doi: 10.1016/j.vetmic.2009.08.028. Epub 2009 Aug 28. PMID: 19781872.

Martella V, Ciarlet M, Lavazza A, Camarda A, Lorusso E, Terio V, Ricci D, Cariola F, Gentile M, Cavalli A, Camero M, Decaro N, Buonavoglia C. Lapine rotaviruses of the genotype P[22] are widespread in Italian rabbitries. Vet Microbiol. 2005 Nov 30;111(1-2):117-24. doi: 10.1016/j.vetmic.2005.10.005. Epub 2005 Oct 27. PMID: 16257498.

Matthijnssens J, Attoui H, Bányai K, Brussaard CPD, Danthi P, Del Vas M, Dermody TS, Duncan R, Fāng Q, Johne R, Mertens PPC, Mohd Jaafar F, Patton JT, Sasaya T, Suzuki N, Wei T. ICTV Virus Taxonomy Profile: Sedoreoviridae 2022. J Gen Virol. 2022 Oct;103(10). doi: 10.1099/jgv.0.001782. PMID: 36215107.

Matthijnssens J, Miño S, Papp H, Potgieter C, Novo L, Heylen E, Zeller M, Garaicoechea L, Badaracco A, Lengyel G, Kisfali P, Cullinane A, Collins PJ, Ciarlet M, O'Shea H, Parreño V, Bányai K, Barrandeguy M, Van Ranst M. Complete molecular genome analyses of equine rotavirus A strains from different continents reveal several novel genotypes and a largely conserved genotype constellation. J Gen Virol. 2012 Apr;93(Pt 4):866-875. doi: 10.1099/vir.0.039255-0. Epub 2011 Dec 21. PMID: 22190012.

Mihalov-Kovács E, Gellért Á, Marton S, Farkas SL, Fehér E, Oldal M, Jakab F, Martella V, Bányai K. Candidate new rotavirus species in sheltered dogs, Hungary. Emerg Infect Dis. 2015 Apr;21(4):660-3. doi: 10.3201/eid2104.141370. PMID: 25811414; PMCID: PMC4378476.

Miyazaki A, Kuga K, Suzuki T, Kohmoto M, Katsuda K, Tsunemitsu H. Genetic diversity of group A rotaviruses associated with repeated outbreaks of diarrhea in a farrow-to-finish farm: identification of a porcine rotavirus strain

bearing a novel VP7 genotype, G26. Vet Res. 2011 Nov 9;42(1):112. doi: 10.1186/1297-9716-42-112. PMID: 22067072; PMCID: PMC3245447.

Ndebe J, Harima H, Chambaro HM, Sasaki M, Yamagishi J, Kalonda A, Shawa M, Qiu Y, Kajihara M, Takada A, Sawa H, Saasa N, Simulundu E. Prevalence and Genomic Characterization of Rotavirus A from Domestic Pigs in Zambia: Evidence for Possible Porcine-Human Interspecies Transmission. Pathogens. 2023 Sep 27;12(10):1199. doi: 10.3390/pathogens12101199. PMID: 37887715; PMCID: PMC10609906.

Papp H, László B, Jakab F, Ganesh B, De Grazia S, Matthijnssens J, Ciarlet M, Martella V, Bányai K. Review of group A rotavirus strains reported in swine and cattle. Vet Microbiol. 2013 Aug 30;165(3-4):190-9. doi: 10.1016/j.vetmic.2013.03.020. Epub 2013 Apr 4. PMID: 23642647; PMCID: PMC7117210.

Peeters JE, Pohl P, Charlier G. Infectious agents associated with diarrhoea in commercial rabbits: a field study. Ann Rech Vet. 1984;15(3):335-40. PMID: 6097152

Sadiq A, Khan J. Rotavirus in developing countries: molecular diversity, epidemiological insights, and strategies for effective vaccination. Front Microbiol. 2024 Jan 5; 14:1297269. doi: 10.3389/fmicb.2023.1297269. PMID: 38249482; PMCID: PMC10797100.

Silva LC, Sanches AA, Gregori F, Brandão PE, Alfieri AA, Headley SA, Jerez JA. First description of group A rotavirus from fecal samples of ostriches (Struthio camelus). Res Vet Sci. 2012 Oct;93(2):1066-9. doi: 10.1016/j.rvsc.2011.12.007. Epub 2011 Dec 30. PMID: 22209018.

Steele AD, Victor JC, Carey ME, Tate JE, Atherly DE, Pecenka C, Diaz Z, Parashar UD, Kirkwood CD. Experiences with rotavirus vaccines: can we improve rotavirus vaccine impact in developing countries? Hum Vaccin Immunother. 2019;15(6):1215-1227. doi: 10.1080/21645515.2018.1553593. Epub 2019 Feb 8. PMID: 30735087; PMCID: PMC6663148.

Trojnar E, Sachsenröder J, Twardziok S, Reetz J, Otto PH, Johne R. Identification of an avian group A rotavirus containing a novel VP4 gene with a close relationship to those of mammalian rotaviruses. J Gen Virol. 2013 Jan;94(Pt 1):136-142. doi: 10.1099/vir.0.047381-0. Epub 2012 Oct 10. PMID: 23052396.

Tsugawa T, Hoshino Y. Whole genome sequence and phylogenetic analyses reveal human rotavirus G3P[3] strains Ro1845 and HCR3A are examples of direct virion transmission of canine/feline rotaviruses to humans. Virology.2008;380(2):344–353.

Varshney B, Jagannath MR, Vethanayagam RR, Kodhandharaman S, Jagannath HV, Gowda K, Singh DK, Rao CD. Prevalence of, and antigenic variation in, serotype G10 rotaviruses and detection of serotype G3 strains in diarrheic calves: implications for the origin of G10P11 or P11 type reassortant asymptomatic strains in newborn children in India. Arch Virol. 2002;147(1):143-65. doi: 10.1007/s705-002-8308-z. PMID: 11855628.

Complete genome sequencing of a G3P[14] rabbit rotavirus

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Abstract

Group A rotaviruses (RVAs) are a major cause of acute dehydrating diarrhea in infants and young animals worldwide. In rabbits, RVAs are associated with enteric disease, likely in combination with other pathogens. We report the identification and characterization of a lapine RVA strain in an Italian rabbit breeding farm. Increased mortality rates associated with enteric symptoms were reported in the facility in post-weaning rabbits around 40 days of age. By quantitative RT-PCR, a RVA strain was identified in the intestinal contents of deceased rabbits. A PCR-based enrichment protocol coupled with Nanopore sequencing allowed the reconstruction of the near complete genome of a rabbit RVA strain, Rabbit-wt/ITA/36-9/2022/G3P[14], with a genotype constellation (G3-P[14]-I2-R2-C2-M3-A9-N2-T6-E5-H3) conserved among lapine RVAs. Each of the 11 gene segments displayed high nucleotide identity and phylogenetic clustering with lapine rotavirus strains, as well as two Belgian human G3P[14] strains, which had been shown to have a zoonotic (lapine) origin. However, the NSP2 gene of strain 39-6 clustered closer with a group of rare human G3P[9] strains, suggesting a common path during their evolution. Gathering sequence data on animal RVA is pivotal to reconstructing the history of homologous and heterologous RVAs in various mammals, including humans.

Keywords: rotavirus, enteritis; genome reconstruction; G3P[14]; rabbit

Introduction

Viruses belonging to the species *Rotavirus alphagastroenteritidis* formerly known as Group A or species A rotaviruses (RVAs) are enteric pathogens and a leading cause of severe diarrhea in human infants, as well as in young animals worldwide (Bishop, 2009). In humans, gastroenteritis may lead to death, mostly in undeveloped countries. Globally, RVA infection was the leading cause of diarrheal deaths, accounting for nearly 20% of infant deaths from diarrhea in 2019 (Du et al., 2022). RVA caused a high death burden in African, Oceanian, and South Asian Countries in the past three decades (Du *et al*., 2022). The development of four live-attenuated oral RVA vaccines (Rotarix®, Rotavac®, Rotasiil®, and RotaTeq®) with WHO prequalification and recommendations resulted in their inclusion in national immunization programs in over 120 countries (*Chen et al.,* 2022). However, RVA remains the major cause of severe viral diarrhea in low-income countries (Buttery &Kirkwood, 2021). RVA infection in animals is also responsible for relevant financial losses due to decreased productivity of livestock animals (Tate *et al.,* 2012).

The RVA genome consists of 11 segments of double-stranded RNA encoding six structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) (Pesavento et al., 2006). The two outer capsid proteins (VP7 and VP4) induce the production of neutralizing antibodies and form the basis for the G (Glycoprotein) and P (Protease-sensitive) dual (G/P) genotyping system for RVA strains (Kim et al., 2012). This dual genotyping system is often used for RVA classification, and is updated continuously by the Rotavirus Classification Working Group (Matthijnssens et al., 2011). According to this classification system, 42 G- and 58 P genotypes have been described globally (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg).

A limited number of studies have investigated the molecular characteristics of RVA strains of rabbits, with only a few lapine RVA strains isolated and characterized. Lapine rotavirus strains typically share the G3 genotype in combination with P[22] or P[14] (Martella *et al.,* 2005). Thus far, the complete genome sequence (CGS) of five rabbit RVA strains has been determined, including G3P[14] and G3P[22] strains from Italy, China and South Korea (Matthijnssens et al., 2006; Guo et al., 2012; Oem et al., 2019) . Heterologous RVA infections in rabbits have been also reported. One lapine strain, RVA/Rabbit-tc/NLD/K1130027/2011/G6P[11], was shown to possess the typical bovine-like RVA genome constellation (Schoondermark-van de Ven *et al.,* 2013). A human-derived G3P[8] strain, C-3/15, has been identified from a Mexican commercial rabbitry (Reynoso-Utrera et al., 2024).

Also, a few examples exist of lapine RVA infecting humans. In 2000, the first reported case of a

lapine-like G3P[14] RVA strain (RVA/Human-wt/BEL/B4106/2000/G3P[14]) infecting a child was reported in Belgium (De Leener *et al.,* 2004). Subsequently, the strain was demonstrated to be entirely of lapine origin, but still able to cause severe gastroenteritis in a child (Matthijnssens *et al.,* 2006). A second report of human infection by a lapine-like RVA was reported in Belgium during the 2012-2013 rotavirus season (Bonica *et al.,* 2015) with a third zoonotic infection identified in Australia in 2012 (RVA/Human-wt/AUS/RCH272/2012/G3P[14]) in a 12-year-old child hospitalized with acute gastroenteritis (Donato *et al.,* 2014).

Due to the segmented nature of the viral genome, RVA genetic diversity can be generated through reassortment events, involving one or multiple gene segments (Watanabe et al., 2001) eventually coupled with interspecies transmission among animal species and from animals to humans (Martella et al., 2010).

In this study, we report the detection and characterization of a rabbit RVA strain identified in a large breeding farm with recurring problems of enteric disease in young rabbits.

2. Materials and Methods

2.1 Collection of samples

The study was carried out in the genetic center of the Italian Rabbit Breeders Association (ANCI), in Volturara Appula, FG, Southern Italy, authorized by the Ministry of Agricultural, Food and Forestry Policies (MIPAF) for the maintenance of rabbit breeding farms. Pure Italian White, Silver, and Spotted breeds were selected and reared in the facility. A total of 30,000 rabbits were housed in 10 sheds in the farm, with about 4000 does. A closed-cycle production system was used without introducing animals from external farms. However, does were frequently moved from one cage to another or from one shed to another due to routine husbandry procedures.

Increased mortality rates due to the enteric syndrome were reported cyclically in the facility in post-weaning rabbits (about 40 days old). In January 2022, two groups (A and B) of rabbits with fatal severe enteric signs were sampled. At necropsy, a total of 22 samples (intestinal content) were collected, 11 for each group, respectively. The gross lesions included catarrhal enteritis and, in some animals, stasis of either the cecal tract or the stomach.

2.2 Sample preparation and nucleic acid extraction

Stool samples were diluted with sterile PBS, homogenized at 10% by Qiagen TissueLyser (QiagenTM, Hilden, Germany) and centrifuged at 16,000× g for 3 min, as previously described (Pellegrini et al., 2024). Nucleic acid extraction was carried out using the Indispin Pathogen DNA/RNA Mini Kit (Indical®, Leipzig, Germany) from 200 µL of the supernatants, following the

manufacturer's instructions. Nucleic acid samples were eluted (100 µL) in laboratory grade water and stored at −80 °C until later use.

2.3 Quantitative reverse transcription PCR (qRT-PCR) specific for RVA

Samples from group A (n=11) and B (n=11) were pooled and tested for RVA by quantitative RT-PCR designed on the VP2 gene (Gutierrez-Aguirre *et al.,* 2008). RNA denaturation with dimethylsulphoxide (DMSO) at 97°C for 10 min was carried out. Reverse transcription was performed on nucleic acid extracts in a total reaction volume of 20 µL containing 2.5 U of random hexamers, as previously reported (Ndiana et al., 2023). Cycling conditions consisted in 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min. Ten µL of sample cDNA were combined with the 15-µL reaction master mix (IQ Supermix; Bio-Rad Laboratories SRL, Segrate, Italy) comprising multiple forward and reverse primers (0.6 µmol/L of each) and 0.2 µmol/L of degenerate MGB TaqMan probe (Table 1). Thermal cycling was set as follows: activation of iTaq DNA polymerase at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 15 s and annealing/ extension at 60 °C for 30 s.

2.4 Screening for Other Pathogens

Based on the gross lesions, differential diagnosis was oriented to colibacillosis or coccidiosis. Bacteriological and parasitological investigations were carried out using standard diagnostic procedures. PCR assays with primers targeting the fimbrial genes *afr2* and *eae,* encoding the intimin protein of *E. coli* (Wieler, 1997; Dow *et al*., 2005) (table 1) were also performed. Nucleic acids were also tested by a PCR assay targeting 18srRNA of Eimeria spp. (Jinneman *et al*. 1999).

2.5 Amplification of rotavirus A genome

The complete genome sequence of RVA strains was amplified using a previously described protocol (Faizuloev *et al*., 2021, Froussard *et al.,* 1993). Four µL of the nucleic acid samples were combined with 2.8 µL of DMSO, and subjected to RNA denaturation at 97°C for10 min and cooling on ice for 1 min. The RT reaction was performed in a total volume of 20 µL using SuperScript™ III First-Strand Synthesis System (Thermo Fisher-Scientific) kit. Briefly, 6 µL of denatured RNA was combined with 1 µL of annealing buffer and 1 µL of primer mix containing 3 pmol/µl final concentration of each primer unRAf1, unRAf2, unRAf3, unRAr1, unRAr2, unRAr3 (Table 1) complementary to the highly conserved regions of the 5'- and 3'-ends of the RVA gene segments (Faizuloev et al., 2021). This reaction mixture also mentioned as Mix 1 was incubated at 65 °C for 5 min, and cooled for 1 min on ice. Mix 1 was combined with 10 µL of 2x First Strand reaction and 2 µL of SS-RNase out and incubated at 50°C for 50 min and 85°C for 5 min. All primers for RT were extended at the 5'-end with a 20-nt sequence for binding to the universal Up primer (Froussard, 1993), together used as forward and reverse primer for PCR (Table 1).

Amplification of all RVA gene segments was performed using the "universal" primer Up (table 1) (Faizuloev *et al.,* 2021; Froussard, 1993) (Table 1). The reaction was carried out in a total volume of 50 µL containing cDNA, TaKaRa LA Taq DNA polymerase (2.5 units), LA PCR Buffer II with Mg^{2+} (final concentration at 3.5 mM), dNTPs (final concentration of 1 mM each), universal primer Up at 1uM. Initial denaturation was performed at 95°C for 2 min, followed by 1 cycle of denaturation at 94°C for 30s, annealing at 42°C for 30s and extension at 68°C for 20 min. Subsequently, further 10 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 68°C for 10 min were carried out. The last step of the thermal file comprised an additional 30 cycles of denaturation at 94°C for 30s, annealing at 65°C for 30s and annealing at 68°C for 30s, and a final extension at 68°C for 10 min.

Table 1: Oligonucleotides used in this study

Pathogen	Target gene	Assay	Primer/Probes	Sequence 5'-3'	Reference
RVA	VP2	qRT-PCR	Vp2f1	TCTGCAGACAGTTGAACCTATTAA	Gutierrez-aguirre et al., 2008
			Vp2f2	CAGACACGGTTGAACCCATTAA	
			Vp2f3	TCGGCTTGATACAGTAGAACCTATAAATG	
			Vp2f4	TGTCAGCTGATACAGTAGAACCTATAAATG	
			Vp2f5	TCAGCTGAC ACAGTAGAACCTATAAATG	
			Vp2R1	GTTGGCGTTTACAGTTCGTTCAT	
			Vp2R2	GTTGGCGTCTACAATTCGTTCAT	
			VP2-probe	FAM- ATGCGCATRTTRTCAAAHGCAA-MGB-NFQ	
RVA	Complete genome	RT	unRAf1	GCCGGAGCTCTGCAGAATTCGGCTWTWAAA	Faizuloev et al., 2021
			unRAf2	GCCGGAGCTCTGCAGAATTCGGCTTTTTTT	
			unRAf3	GCCGGAGCTCTGCAGAATTCGGCTTTTAAT	
			unRAr1	GCCGGAGCTCTGCAGAATTCGGTCAYATC	
			unRAr2	GCCGGAGCTCTGCAGAATTCGGTCACAWA	
			unRAr3	GCCGGAGCTCTGCAGAATTCAGCCACATG	
	Universal	PCR	Up	GCCGGAGCTCTGCAGAATTC	Froussard, 1993
E. Coli	eae	PCR	ECW1 s	TGCGGCACAACAGGCGGCGA	Wieler, 1997
			ECW2 as	CGGTCGCCGCACCAGGATTC	
	afr2	PCR	AF/R2-F	AAGTTAGGGGACGCCATTAC	Dow et al., 2005
			AF/R2-R	CCAGGACTTATTCTGACCAG	
Eimeria spp.	18srRNA	PCR	1FE	TACCCAATGAAAACAGTTT	Jinneman et al. 1999
			4RB	CGTCTTCAAACCCCCTACTG	

RVA: rotavirus A; qRT-PCR: quantitative reverse transcription PCR; E. coli: *Escherichia coli*
afr2: fimbrial gene of E. Coli.; eae: intimin gene of E. Coli; 18srRNA: 18S ribosomal RNA

2.6 Oxford Nanopore Technologies (ONT) sequencing

Amplicons were evaluated by A2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with a High Sensitivity DNA chip, to assess the length distribution of the obtained fragments. DNA concentration was evaluated in a Qubit 4.0 Fluorometer using a Qubit dsDNA HS kit (Invitrogen,

Life Technologies, Milan, Italy). The Ligation Sequencing Kit V14 (SQK-LSK114) (Oxford Nanopore Technologies, ONT™, Oxford, UK) was used to prepare libraries, which were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter™). The libraries were pooled and sequenced for 24 h using MinION Flongle Flow Cell (R10.4.1) FLO-FLG001 on the MinION- Mk1C device (ONT™, UK) provided with Flongle adapter.

2.7 Sequence and phylogenetic analyses

Sequencing reads produced by the Nanopore platform were evaluated using the online bioinformatics server Genome Detective Virus Tool, v 2.48 (Vilsker *et al.,* 2019) with the research bioinformatic pipeline for quality control, adaptor trimming, and de novo assembly of reads using SPAdes was previously reported (Beikpour *et al.,* 2023). In parallel, FastQ MinION files were also subjected to quality control, trimming and reference assembly by Minimap2 implemented in Geneious Prime software, v2024.0. Open reading frame predictions and annotations were performed in Geneious Prime software, v 2024.0. The online tool Blast Nucleotide (https://blast.ncbi.nlm.nih.gov, accessed on 24th January 2025) was employed using the default values to find homologous hits.

The obtained sequences were aligned with cognate rotavirus sequences recovered from the National Center for Biotechnology Information (NCBI) database using MAFFT plugin implemented in Geneious Prime software, v 2024.0. The most reliable substitution model for the phylogeny was estimated by using "Find the best protein DNA/Protein Models" comprised in MEGA X, v 10.0.5 software (Kumar *et al*., 2018). Maximum-likelihood method, Tamura 3-parameter model, a discrete gamma distribution and invariant sites to model evolutionary rate differences among sites (6 categories) were selected. One thousand replicates were used for statistical support.

2.8 GenBank Sequence Submission

The nt sequences of the 11 genome segments of the RVA strain Rabbit-wt/ITA/36-9/2022/G3P[14] were deposited in GenBank under accession numbers PQ822044 to PQ822054.

Results

RVA was detected at a Ct equal to 42.66 in pool A at a Ct equal to 25.88 in pool B. Samples of pool B were also tested individually, yielding 9/11 (81.8%) RVA-positive animals with CT ranging between 15.33 and 31.34. Microbiological and parasitological investigations also revealed the presence of different biotypes (18, 28, and 31) of *E. coli* with virulence-associated genes (*eae* and *afr2*) and the presence of *Eimeria* spp.

The RVA-positive sample 36/22-9 with the highest Ct (15.33) was subjected to an amplification protocol to recover the complete genome of the RVA strain (Faizuloev *et al.,* 2021; Froussard, 1993). Nanopore sequencing allowed the reconstruction of 11 genome segments of strain RVA/Rabbit-wt/ITA/36-9/2022/G3P[14]. The nearly complete genome was assembled, using a total of 1,224,360 reads, with a 67,318 mean depth coverage (range 8050-244,403) (Table 2). Results obtained using the research bioinformatic pipeline of the online software Genome Detective Virus Tool and in the Minimap2 implemented in the Geneious Prime software were comparable.

Table 2: Number of reads obtained and depth and genome coverage per genome segment for strain RVA/Rabbit-wt/ITA/36-9/2022/G3[P14]

Gene	Segment	Length (nt)	Reads (Nr)	Coverage Depth	Accession
VP1	1	3302	61226	37211	PQ822044
VP2	2	2687	113982	85274	PQ822045
VP3	3	2591	9989	8050	PQ822046
VP4	4	2361	14713	13158	PQ822047
NSP1	5	1597	25116	35791	PQ822048
VP6	6	1356	37152	55260	PQ822049
NSP3	7	1072	129942	244403	PQ822050
NSP2	8	1059	132699	25339	PQ822051
VP7	9	1062	64508	129080	PQ822052
NSP4	10	751	13765	37195	PQ822053
NSP5	11	1035	621268	69740	PQ822054

RNA-dependent RNA polymerase (VP1); capsid protein (VP2); capping enzyme (VP3); spike protein (VP4); nonstructural RNA-binding protein (NSP1); major inner capsid protein (VP6); translational regulator (NSP3); multifunctional RNA chaperone (NSP2); outer capsid protein (VP7); viral enterotoxin (NSP4); viral phosphoprotein (NSP5)

Gene segments 1, 2 and 10 encoding VP1, VP2 and NSP4 genes, respectively, of the RVA strain Rabbit-wt/ITA/36-9/2022/G3P[14] detected in this study displayed the highest nt identity in NCBI database to the Belgian RVA strain Human-wt/BEL/B4106/2000/G3P[14]. Gene segments 3 to 7, and 9 and 11 encoding VP3, VP4, NSP1, VP6, NSP3, VP7, and NSP5 genes, respectively, were closely related to the Belgian RVA strain Human-wt/BEL/BE5028/2012/G3P[14]. Gene segment 8, coding the NSP2, was highly related to the RVA strain Human-wt/RUS/Omsk08-442/2008/G3P[9] isolated in Russia (Table 3).

Table 3 RVA strains with the highest nt identity to Rabbit-wt/ITA/36-9/2022/G3P[14], determined by Basic Local Alignment Search Tool Nucleotide (BLASTn) (consulted on 24th January 2025).

Gene	Segment	Genotype	Reference strain	Accession No	% nt identity
VP1	1	R2	RVA/Human wt/BEL/B4106/2000/G3P[14]	AY740741	93.7%
VP2	2	C2	RVA/Human-wt/BEL/B4106/2000/G3P[14]	AY740740	97.4%
VP3	3	M3	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258400	98.9%
VP4	4	P[14]	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258401	98.1%
NSP1	5	A9	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258404	98.3%
VP6	6	I2	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258402	97.9%
NSP3	7	T6	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258406	98.3%
NSP2	8	N2	RVA/Human-wt/RUS/Omsk08-442/2008/G3P[9]	KC822934	96.2%
VP7	9	G3	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258403	96.7%
NSP4	10	E5	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258407	98.1%
NSP5	11	H3	RVA/Human-wt/BEL/BE5028/2012/G3P[14]	KP258408	98.5%

The novel RVA strain Rabbit-wt/ITA/36-9/2022/G3P[14] displayed the same genotype constellation G3-P[14]-I2-R2-C2-M3-A9-N2-T6-E5-H3 also described in human RVA strains BE5028 and B4106 from Belgium and in a rabbit RVA strain 30-96 from Italy (Table 4).

Table 4: Comparison of the geotype constellation of the rotavirus A strain detected in this study (in bold) with other human and lapine rotavirus A strains. The dark grey shade indicates the highest nt identity on BLAST-based interrogation (accessed on 24th January 2025) of GenBank database. Light gray shade is used to indicate the same genotype.

RVA strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5/6
Human-tc/USA/Wa/1974/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Human-tc/USA/DS-1/1976/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
Human-wt/BEL/BE5028/2012/G3P[14]	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3
Human-wt/BEL/B4106/2000/G3P[14]	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3
Human-wt/AUS/RCH272/2012/G3P[14]	G3	P[14]	I2	R3	C3	M3	A9	N2	T6	E2	H3
Human-wt/RUS/Omsk08-442/2008/G3P[9]	G3	P[9]	I2	ND	ND	ND	ND	N2	ND	ND	ND
Human-wt/HUN/ERN5162/2012/G3P[9]	G3	P[9]	I2	R2	C2	M2	A3	N2	T3	E3	H3
Human-wt/ITA/PA158/1996/G3P[9]	G3	P[9]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Human-wt/USA/0537/2002/G3P[9]	G3	P[9]	I2	R2	C2	M2	A3	N2	T1	E2	H3
Rabbit-wt/ITA/36-9/2022/G3P[14]	**G3**	**P[14]**	**I2**	**R2**	**C2**	**M3**	**A9**	**N2**	**T6**	**E5**	**H3**
Rabbit-tc/ITA/30-96/1996/G3P[14]	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3
Rabbit-tc/CHN/N5/1992/G3P[14]	G3	P[14]	I17	R3	C3	M3	A9	N1	T1	E3	H2
Rabbit-tc/NLD/K1130027/2011/G6P[11]	G6	P[11]	I2	R2	C2	M2	A13	N2	T6	E2	H3
Rabbit-tc/CHN/Z3171/2020/G3P[22]	G3	P[22]	I2	R3	C3	M3	A9	N2	T1	E3	H3
Rabbit-wt/SKR/Rab1404/2014/G3P[22]	G3	P[22]	I2	R3	C3	M3	A9	N2	T3	E3	H3
Rabbit-wt/MEX/C-3-15/2015/G3P[8]	G3	P[8]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cat-wt/ITA/BA222/2005/G3P[9]	G3	P[9]	I2	R2	C2	M2	A3	N1	T3	E2	H3

A phylogenetic tree was generated using a subset of RVA NSP2 genes, selected based on the results of database interrogation with BLAST Nucleotide (interrogation on 24th January 2025) and including

NSP2 sequences of N2 genotype (Figure 1). In this analysis, the RVA strain Rabbit-wt/ITA/36-9/2022/G3P[14] segregated with a group encompassing human and rabbit G3P[14] and G3P[22] RVAs (93.4 to 94.3% nt identity) and a group formed by G3P[9] RVAs detected in USA, Europe, and Russia (94.7 to 96.2% nt identity).

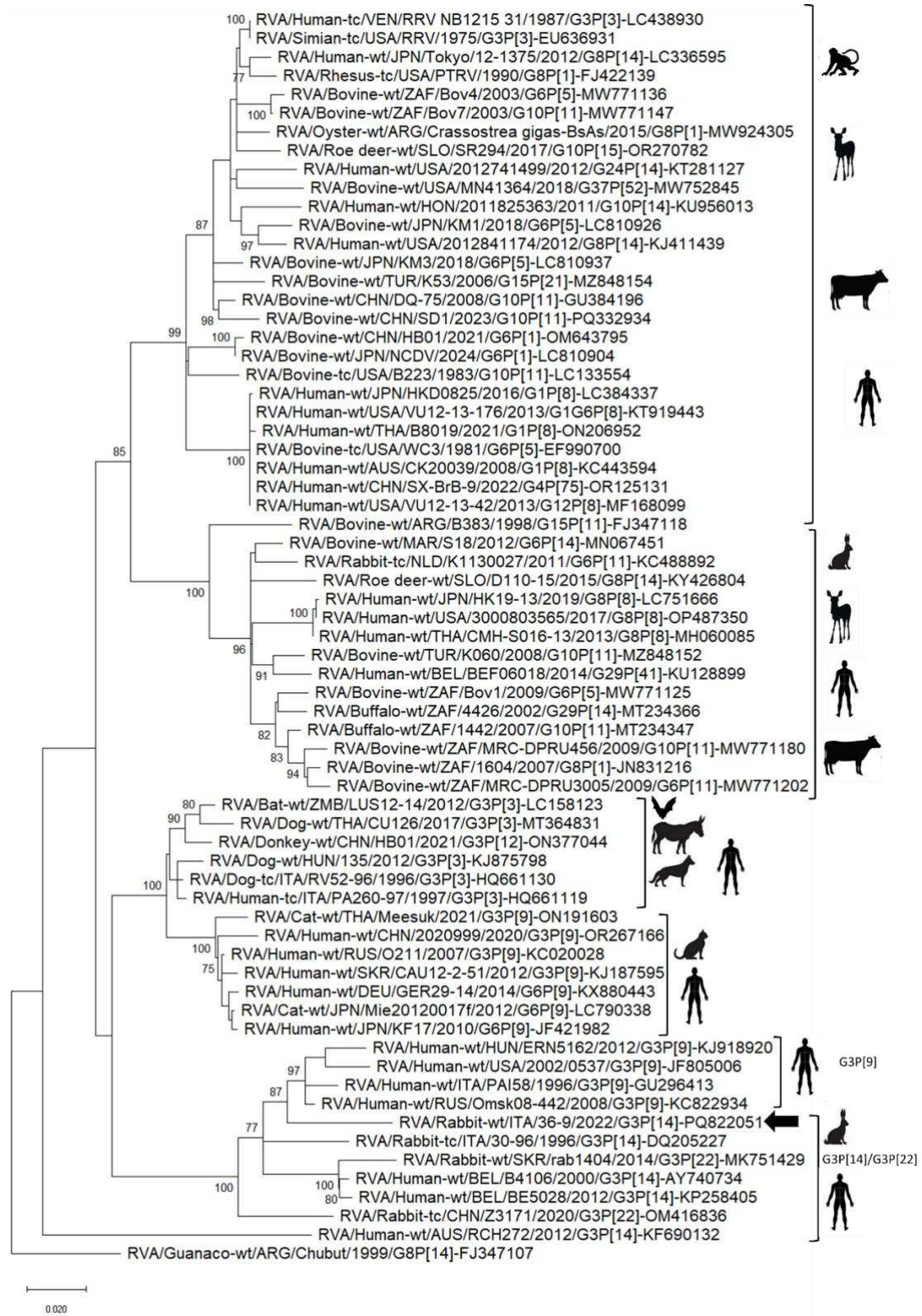

Figure 1: Partial NSP2 gene-based phylogenetic tree of the rotavirus A (RVA) strain identified in this study and cognate sequences of RVA strains of genotype N2 retrieved from the NCBI database. Maximum likelihood method and Tamura model (three parameters) with a gamma distribution and invariable sites 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. The black arrow indicates the lapine RVA strain detected in this study. The scale bar represents the number of nt substitutions per site.

Discussion

RVA infection is endemic in rabbitries and antibodies to RVA are common in rabbits after 4 months of age (Ciarlet *et al.,* 1998a, Ciarlet *et al.,* 1998b, Conner *et al.,* 1988, Petric *et al.,* 1978, Sato *et al.,* 1982, Thouless *et al.,* 1988). Since passively transferred antibodies protect young rabbits up to 2 months of age, RVA-associated disease is usually described after weaning in 1- to 3-month-old animals (Ciarlet *et al.,* 1998b; Conner *et al*., 1988, Thouless *et al.,* 1988). In experimental infection of rabbits with RVA, the enteric disease seems age-restricted to the neonatal period (≤2 week) (*Ciarlet et al.,* 1998b), although strain-related variations in terms of virulence or tropism could result in different patterns of infection/disease (Hall et al., 1993). Overall, RVA could play a role in the etiology of enteric disease by either exerting direct pathogenic activity or in synergism with other pathogens (i.e., *Clostridium difficile*, *C. spiroforme*, or *E. coli*) (Ciarlet *et al.,* 1998b). In this study, RVA was identified in young rabbits deceased with enteric disease. In the facility, increased mortality rates due to enteric syndrome were reported cyclically in post-weaning rabbits (about 40 days old). Consequently, laboratory investigations were requested to assess the possible causes of mortality, which also revealed the presence of pathogenic *E. coli* strains with the virulence-associated genes (*eae* and *afr2*) (Camguilhem& Milon, 1989) and Eimeria spp. (Hughes, 2024), thus confirming a multi-factorial etiology.

Genotyping of VP4 and VP7 antigens of rabbit RVA strains has revealed the typical circulation of G3 strains in combination with either [P14] or P[22] VP4 antigens (Ciarlet *et al.,* 1997; Martella et al., 2003). In a previous large-scale epidemiological study on 350 stool samples from 1998 to 2004 in Italy, the vast majority of strains were typed as G3P[22], whilst only one RVA strain was G3P[14], and two samples contained a mixed (G3P[14]+[22]) RVA infection (Martella *et al.,* 2005). This suggests that these G3P[14] strains are not common or that there may be geographical/temporal variations.

Since information on the CGS of rabbit RVAs is still limited, we generated the CGS of a rabbit RVA identified in a rabbitry located in Southern Italy in 2022. Ten out of eleven segments of strain Rabbit-

wt/ITA/36-9/2022/G3P[14] detected in this study were highly similar to lapine-like RVA strains identified in Belgium in human patients (Matthijnssens *et al*., 2006; Bonica *et al.,* 2015) and to other lapine RVAs. However, the NSP2 genome segment displayed the highest nt identity to the RVA strain Human-wt/RUS/Omsk08-442/2008/G3P[9] identified from a human patient in Russia in 2008 (Zhirakovskaia *et al.,* 2012) (Table 3). A phylogenetic tree was generated using a subset of RVA NSP2 genes, selected based on the results of database interrogation with BLAST. In this analysis, strain Rabbit-wt/ITA/36-9/2022/G3P[14] appeared intermingled between rabbit RVAs and a group of human G3P[9] RVA strains, suggesting that the NSP2 gene of these human G3P[9] viruses likely shared an evolutionary pathway with rabbit RVAs (Figure 1). Interestingly, for some of these G3P[9] human RVAs, CGS data have been generated, revealing an unusual pattern of reassortment in the genome, shared with RVAs of ruminants and cats (De Grazia *et al*., 2010, Grant *et al.,* 2011) and markedly different from AU-1-like G3P[9] human RVAs (Table 4).

Heterologous RVAs can infect rabbits under experimental conditions, although the infection is usually not transmitted horizontally (Ciarlet *et al.,* 1998 a, 1998b). In 2013, the isolation of a bovine-like RVA strain (RVA/Rabbit-tc/NLD/K1130027/2011/G6P[11]) was reported in the Netherlands in rabbits of a laboratory animal facility. Upon CGS analysis, the virus was shown to be related in all genes to either RVAs of ruminants or to bovine-like G6 human RVAs (Schoondermark-van de Ven *et al.,* 2013). Also, a human-like G3P[8] strain was identified from a rabbit production farm, with enteric disease in 40 to 60-day-old animals (Reynoso-Utrera *et al.,* 2024). These findings reinforce the notion that the evolution of human RVAs is tightly intermingled with that of animal RVAs, with a 2-way flow exchange of genetic material, implying forward and backward transmission events among different animal species. Of note, it is known that rabbits can host other viruses with zoonotic potential, as approximately 0.5% (5/919) of human cases of hepatitis E in France in 2015-2016 were caused by rabbit hepatitis E viruses (Abravanel *et al*. 2017).

Surveillance and studies of animal viruses is not only relevant to improving animal health and understanding their diversity and evolution, but it is also relevant to pursuing the principles of the One Health paradigm to decipher the origin of viruses with zoonotic potential.

References

Abravanel F, Lhomme S, El Costa H, Schvartz B, Peron JM, Kamar N, Izopet J. Rabbit Hepatitis E Virus Infections in Humans, France. Emerg Infect Dis. 2017 Jul;23(7):1191-1193. doi: 10.3201/eid2307.170318. PMID: 28628452; PMCID: PMC5512490.

Beikpour F, Pellegrini F, Lanave G, Camero M, Catella C, Di Martino B, Di Profio F, Masotti C,

Battistini R, Serracca L, La Rosa G, Martella V, Suffredini E. Exploring the Astrovirome of Shellfish Matrices Using Nanopore Sequencing. Vet Sci. 2023 Feb 21;10(3):175. doi: 10.3390/vetsci10030175. PMID: 36977214; PMCID: PMC10055021.

Bishop R. Discovery of rotavirus: Implications for child health. J Gastroenterol Hepatol. 2009 Oct;24 Suppl 3:S81-5. doi: 10.1111/j.1440-1746.2009.06076.x. PMID: 19799704.

Bonica, M. B., Zeller, M., Van Ranst, M., Matthijnssens, J., & Heylen, E. (2015). Complete genome analysis of a rabbit rotavirus causing gastroenteritis in a human infant. Viruses, 7(2), 844–856.

Buttery JP, Kirkwood C. Rotavirus vaccine implementation: evidence to fill the gap? Lancet Glob Health. 2021 Jul;9(7):e885-e886. doi: 10.1016/S2214-109X(21)00265-5.

Camguilhem R, Milon A. Biotypes and O serogroups of Escherichia coli involved in intestinal infections of weaned rabbits: clues to diagnosis of pathogenic strains. J Clin Microbiol. 1989 Apr;27(4):743-7. doi: 10.1128/jcm.27.4.743-747.1989. PMID: 2656746; PMCID: PMC267409.

Chen J, Grow S, Iturriza-Gómara M, Hausdorff WP, Fix A, Kirkwood CD. The Challenges and Opportunities of Next-Generation Rotavirus Vaccines: Summary of an Expert Meeting with Vaccine Developers. Viruses. 2022 Nov 19;14(11):2565. doi: 10.3390/v14112565. PMID: 36423174; PMCID: PMC9699535.

Ciarlet M, Estes MK, Conner ME. Comparative amino acid sequence analysis of the outer capsid protein VP4 from four lapine rotavirus strains reveals identity with genotype P[14] human rotaviruses. Arch Virol. 1997;142(5):1059-69. doi: 10.1007/s007050050142. PMID: 9191871.

Ciarlet M, Estes MK, Barone C, Ramig RF, Conner ME. Analysis of host range restriction determinants in the rabbit model: comparison of homologous and heterologous rotavirus infections. J Virol. 1998a Mar;72(3):2341-51. doi: 10.1128/JVI.72.3.2341-2351.1998. PMID: 9499095; PMCID: PMC109534.

Ciarlet M, Gilger MA, Barone C, McArthur M, Estes MK, Conner ME. Rotavirus disease, but not infection and development of intestinal histopathological lesions, is age restricted in rabbits. Virology. 1998b Nov 25;251(2):343-60. doi: 10.1006/viro.1998.9406. PMID: 9837799.

Conner ME, Estes MK, Graham DY. Rabbit model of rotavirus infection. J Virol. 1988 May;62(5):1625-33. doi: 10.1128/JVI.62.5.1625-1633.1988. PMID: 2833612; PMCID: PMC253190.

De Grazia S, Giammanco GM, Potgieter CA, Matthijnssens J, Banyai K, Platia MA, Colomba C, Martella V. Unusual assortment of segments in 2 rare human rotavirus genomes. Emerg Infect Dis. 2010 May;16(5):859-62. doi: 10.3201/eid1605.091826. PMID: 20409385; PMCID: PMC2954010.

De Leener K, Rahman M, Matthijnssens J, Van Hoovels L, Goegebuer T, van der Donck I, Van Ranst M. Human infection with a P[14], G3 lapine rotavirus. Virology. 2004 Jul 20;325(1):11-7. doi: 10.1016/j.virol.2004.04.020. PMID: 15231381.

Donato CM, Manuelpillai NM, Cowley D, Roczo-Farkas S, Buttery JP, Crawford NW, Kirkwood CD. Genetic characterization of a novel G3P[14] rotavirus strain causing gastroenteritis in 12 year old Australian child. Infect Genet Evol. 2014 Jul; 25:97-109. doi: 10.1016/j.meegid.2014.04.009. Epub 2014 Apr 26. PMID: 24780429.

Dow MA, Tóth I, Alexa P, Davies M, Malik A, Oswald E, Nagy B. Predominance of afr2 and ral fimbrial genes related to those encoding the K88 and CS31A fimbrial adhesins in enteropathogenic Escherichia coli isolates from rabbits with postweaning diarrhea in Central Europe. J Clin Microbiol. 2005 Mar;43(3):1366-71. doi: 10.1128/JCM.43.3.1366-1371.2005. PMID: 15750109; PMCID: PMC1081305.

Du Y, Chen C, Zhang X, Yan D, Jiang D, Liu X, Yang M, Ding C, Lan L, Hecht R, Zhu C, Yang S. Global burden and trends of rotavirus infection-associated deaths from 1990 to 2019: an observational trend study. Virol J. 2022 Oct 20;19(1):166. doi: 10.1186/s12985-022-01898-9. PMID: 36266651; PMCID: PMC9585833.

Faizuloev E, Mintaev R, Petrusha O, Marova A, Smirnova D, Ammour Y, Meskina E, Sergeev O, Zhavoronok S, Karaulov A, Svitich O, Zverev V. New approach of genetic characterization of group A rotaviruses by the nanopore sequencing method. J Virol Methods. 2021 Jun; 292:114114. doi: 10.1016/j.jviromet.2021.114114. Epub 2021 Mar 1. PMID: 33662411.

Froussard P. rPCR: a powerful tool for random amplification of whole RNA sequences. PCR Methods Appl. 1993 Feb;2(3):185-90. doi: 10.1101/gr.2.3.185. PMID: 7680262.

Grant L, Esona M, Gentsch J, Watt J, Reid R, Weatherholtz R, Santosham M, Parashar U, O'Brien K. Detection of G3P[3] and G3P[9] rotavirus strains in American Indian children with evidence of gene reassortment between human and animal rotaviruses. J Med Virol. 2011 Jul;83(7):1288-99. doi: 10.1002/jmv.22076. PMID: 21567432.

Guo D, Liu J, Lu Y, Sun Y, Yuan D, Jiang Q, Lin H, Li C, Si C, Qu L. Full genomic analysis of rabbit rotavirus G3P[14] strain N5 in China: identification of a novel VP6 genotype. Infect Genet Evol. 2012 Oct;12(7):1567-76. doi: 10.1016/j.meegid.2012.06.010. Epub 2012 Jun 28. PMID: 22750552.

Gutiérrez-Aguirre I, Steyer A, Boben J, Gruden K, Poljsak-Prijatelj M, Ravnikar M. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. J Clin Microbiol. 2008 Aug;46(8):2547-54. doi: 10.1128/JCM.02428-07. Epub 2008 Jun 4. PMID: 18524966; PMCID: PMC2519481.

Hall GA, Bridger JC, Parsons KR, Cook R. Variation in rotavirus virulence: a comparison of pathogenesis in calves between two rotaviruses of different virulence. Vet Pathol. 1993 May;30(3):223-33. doi: 10.1177/030098589303000302. PMID: 8392764.

Hughes K. Endoparasites of rabbits and hares. J Vet Diagn Invest. 2024 Sep;36(5):599-616. doi: 10.1177/10406387241261991. Epub 2024 Aug 6. PMID: 39108102; PMCID: PMC11459662.

Jinneman KC, Wetherington JH, Hill WE, Omiescinski CJ, Adams AM, Johnson JM, Tenge BJ, Dang NL, Wekell MM. An oligonucleotide-ligation assay for the differentiation between Cyclospora and Eimeria spp. polymerase chain reaction amplification products. J Food Prot. 1999 Jun;62(6):682-5. doi: 10.4315/0362-028x-62.6.682. PMID: 10382662.

Kim HH, Matthijnssens J, Kim HJ, Kwon HJ, Park JG, Son KY, Ryu EH, Kim DS, Lee WS, Kang MI, Yang DK, Hyun BH, Park SI, Park SJ, Cho KO. Full-length genomic analysis of porcine G9P[23] and G9P[7] rotavirus strains isolated from pigs with diarrhea in South Korea. Infect Genet Evol. 2012

Oct;12(7):1427-35. doi: 10.1016/j.meegid.2012.04.028. Epub 2012 May 14. PMID: 22613801.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018 Jun 1;35(6):1547-1549. doi: 10.1093/molbev/msy096. PMID: 29722887; PMCID: PMC5967553.

Martella V, Bányai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. Vet Microbiol. 2010 Jan 27;140(3-4):246-55. doi: 10.1016/j.vetmic.2009.08.028. Epub 2009 Aug 28. PMID: 19781872.

Martella V, Ciarlet M, Camarda A, Pratelli A, Tempesta M, Greco G, Cavalli A, Elia G, Decaro N, Terio V, Bozzo G, Camero M, Buonavoglia C. Molecular characterization of the VP4, VP6, VP7, and NSP4 genes of lapine rotaviruses identified in italy: emergence of a novel VP4 genotype. Virology. 2003 Sep 15;314(1):358-70. doi: 10.1016/s0042-6822(03)00418-5. PMID: 14517088.

Martella V, Ciarlet M, Lavazza A, Camarda A, Lorusso E, Terio V, Ricci D, Cariola F, Gentile M, Cavalli A, Camero M, Decaro N, Buonavoglia C. Lapine rotaviruses of the genotype P[22] are widespread in Italian rabbitries. Vet Microbiol. 2005 Nov 30;111(1-2):117-24. doi: 10.1016/j.vetmic.2005.10.005. Epub 2005 Oct 27. PMID: 16257498.

Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, Brister JR, Buesa J, Esona MD, Estes MK, Gentsch JR, Iturriza-Gómara M, Johne R, Kirkwood CD, Martella V, Mertens PP, Nakagomi O, Parreño V, Rahman M, Ruggeri FM, Saif LJ, Santos N, Steyer A, Taniguchi K, Patton JT, Desselberger U, Van Ranst M. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol. 2011 Aug;156(8):1397-413. doi: 10.1007/s00705-011-1006-z. Epub 2011 May 20. PMID: 21597953; PMCID: PMC3398998.

Matthijnssens J, Rahman M, Martella V, Xuelei Y, De Vos S, De Leener K, Ciarlet M, Buonavoglia C, Van Ranst M. Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. J Virol. 2006 Apr;80(8):3801-10. doi: 10.1128/JVI.80.8.3801-3810.2006. PMID: 16571797; PMCID: PMC1440464.

Ndiana LA, Lanave G, Desario C, Odigie AE, Madubuike KG, Lucente MS, Ezeifeka CA, Patruno G, Lorusso E, Elia G, Buonavoglia C, Decaro N. Detection of Selected Canine Viruses in Nigerian Free-Ranging Dogs Traded for Meat Consumption. Animals (Basel). 2023 Mar 22;13(6):1119. doi: 10.3390/ani13061119. PMID: 36978659; PMCID: PMC10044693.

Oem JK, Lee SY, Kim YS, Na EJ, Choi KS. Genetic characteristics and analysis of a novel rotavirus G3P[22] identified in diarrheic feces of Korean rabbit. Infect Genet Evol. 2019 Sep;73:368-377. doi: 10.1016/j.meegid.2019.06.003. Epub 2019 Jun 4. PMID: 31173932; PMCID: PMC7106088.

Pellegrini F, Lanave G, Caringella F, Diakoudi G, Salvaggiulo A, Cavalli A, Papaleo A, Di Martino B, Camero M, Bányai K, Matthijnssens J, Martella V. Identification of Recombinant Aichivirus D in Cattle, Italy. Animals (Basel). 2024 Nov 18;14(22):3315. doi: 10.3390/ani14223315. PMID: 39595367; PMCID: PMC11591108.

Pesavento JB, Crawford SE, Estes MK, Prasad BV. Rotavirus proteins: structure and assembly. Curr

Top Microbiol Immunol. 2006; 309:189-219. doi: 10.1007/3-540-30773-7_7. PMID: 16913048.

Petric M, Middleton PJ, Grant C, Tam JS, Hewitt CM. Lapine rotavirus: preliminary studies on epizoology and transmission. Can J Comp Med. 1978 Jan;42(1):143-7. PMID: 206325; PMCID: PMC1277806.

Reynoso-Utrera E, Bautista-Gómez LG, Fonseca-Coronado S, Pérez-de la Rosa JD, Rodríguez-Villavicencio VJ, Romero-Núñez C, Flores-Ortega A, Hernández-García PA, Martínez-Castañeda JS. New Genotype G3 P[8] of Rotavirus Identified in a Mexican Gastroenteric Rabbit. Viruses. 2024 Nov 2;16(11):1729. doi: 10.3390/v16111729. PMID: 39599844; PMCID: PMC11599003.

Sato K, Inaba Y, Miura Y, Tokuhisa S, Matumoto M. Isolation of lapine rotavirus in cell cultures. Brief report. Arch Virol. 1982;71(3):267-71. doi: 10.1007/BF01314878. PMID: 6285861.

Schoondermark-van de Ven E, Van Ranst M, de Bruin W, van den Hurk P, Zeller M, Matthijnssens J, Heylen E. Rabbit colony infected with a bovine-like G6P[11] rotavirus strain. Vet Microbiol. 2013 Sep 27;166(1-2):154-64. doi: 10.1016/j.vetmic.2013.05.028. Epub 2013 Jun 20. PMID: 23830050.

Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD; WHO-coordinated Global Rotavirus Surveillance Network. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. Lancet Infect Dis. 2012 Feb;12(2):136-41. doi: 10.1016/S1473-3099(11)70253-5. Epub 2011 Oct 24. PMID: 22030330.

Thouless ME, DiGiacomo RF, Deeb BJ, Howard H. Pathogenicity of rotavirus in rabbits. J Clin Microbiol. 1988 May;26(5):943-7. doi: 10.1128/jcm.26.5.943-947.1988. PMID: 2838507; PMCID: PMC266491.

Vilsker M., Moosa Y., Nooij S., Fonseca V., Ghysens Y., Dumon K., Pauwels R., Alcantara L.C., vanden Eynden E., Vandamme A.-M., et al. Genome Detective: An Automated System for Virus Identification from High-Throughput Sequencing Data. Bioinformatics. 2019; 35:871–873. doi: 10.1093/bioinformatics/bty695.

Watanabe M, Nakagomi T, Koshimura Y, Nakagomi O. Direct evidence for genome segment reassortment between concurrently-circulating human rotavirus strains. Arch Virol. 2001;146(3):557-70. doi: 10.1007/s007050170162. PMID: 11338390.

Wieler, L. H.: Bestimmung von Virulenzfaktoren bovine Shiga-Toxin-bildender Escherichia coli (STEC-) Stämme als Bewertungsgrundlage ihrer klinischen Bedeutung für Rind und Mensch, p. 194. Habilitation thesis, University of Giessen 1997.

Zhirakovskaia EV, Aksanova RKh, Gorbunova MG, Tikunov AIu, Kuril'shchikov AM, Sokolov SN, Netesov SV, Tikunova NV. [Genetic diversity of group A rotavirus isolates found in Western Siberia in 2007-2011]. Mol Gen Mikrobiol Virusol. 2012;(4):33-41. Russian. PMID: 23248851.

Chapter 3

Domestic cat hepadnavirus: human hepatitis B-like virus discovered in cats

The family *Hepadnaviridae* contains five genera: hepatitis viruses specific for human and other mammals (i.e., cats, chipmunks, primates, woodchucks, ground-squirrels) grouped in the genus *Orthohepadnavirus*, hepadnaviruses infecting birds (i.e., ducks, herons, snow geese) classified into the genus *Avihepadnavirus*, hepatitis viruses infecting amphibians and reptiles named as *Herpetohepadnavirus*, hepadnaviruses which infect fish belonging to the genera *Metahepadnavirus* and *Parahepadnavirus* (Magnius, 2020). The name hepadnavirus is an acronym for hepatitis DNA viruses. The prototype virus of the family *Hepadnaviridae* is hepatitis B virus (HBV), a hepatotropic DNA virus, with a genome composed of partially double-stranded circular DNA. HBV is the causative agent of both acute and chronic viral hepatitis in humans. Chronic hepatitis B infection accounts for approximately 3.5% prevalence and below 300 million cases with positive hepatitis B surface antigen (HBsAg) (World Health Organization, 2017; World Health Organization, 2021). Chronic hepatitis can develop into liver cirrhosis, and primary hepatocellular carcinoma (HCC).
Similarly, woodchucks either naturally or experimentally infected with woodchuck hepatitis virus (WHV) display serologically evident chronic infection and may develop acute hepatitis, occasionally becoming chronic virus carriers (Michalak, 2020). Adult infection is usually self-limiting, as described in humans. Chronically WHV-infected woodchucks may develop hepatocellular carcinoma without any evidence of cirrhosis. Ground squirrels infected with ground squirrel hepatitis virus develop mild hepatitis, and hepatocellular carcinoma can occur in chronically infected animals, although later in life than in woodchuck hepatitis virus-infected woodchucks (Seeger et al., 1991).
Domestic cat hepadnavirus (DCH) was first detected in 2018 in a domestic cat in Australia as part of a metagenomic investigation (Aghazadeh et al., 2018). The identification of DCH was subsequently confirmed in European, Asian and American countries (Lanave et al., 2019; Pesavento et al., 2019; Piewbang et al., 2022). The prevalence of DCH across countries, ranges from 0.2% to 18% (Shofa et al., 2022; Stone et al., 2022; Adıgüzel et al., 2023), with a higher prevalence in cats co-infected with feline retroviruses (Lanave et al., 2019; Piewbang et al., 2020) .

A case control study performed on 71 formalin-fixed, paraffin-embedded liver biopsies from cats showed a high detection rate of DCH in cats with chronic hepatitis (43%) and HCC (28%). Conversely, histologically normal biliary tract and liver-associated diseases were DCH-negative in PCR tests (Pesavento et al., 2019). In addition, in situ hybridization assay evidenced DCH in tissue biopsies of animals displaying liver inflammation and neoplasia (Pesavento et al., 2019). These findings suggest a possible association between DCH infection and both chronic hepatitis and HCC

in cats.

In a subsequent study the presence of DCH was also identified in cats in several organs (i.e., heart, lungs, intestines, kidneys, and spleen) by qPCR, and confirmed also by immunohistochemistry (IHC) (Piewbang et al., 2020). Systemic inflammatory diseases in various organs, such as hepatitis, hepatic fibrosis, glomerulonephritis, lymphoplasmacytic enteritis, and histiocytic lymphadenitis were also observed b IHC (Piewbang et al., 2020). Moreover, DCH particles were localized in cells from different tissue transmission electron microscopy, suggesting a broader cell tropism for DCH.

In a 2023 study, DCH DNA was reported aside from liver tissues, in serum and in inflammatory and non-inflammatory peritoneal effusion samples of cats infected with DCH (Ratti et al., 2023). Moreover, DCH viremia has been described in cats with normal or mildly elevated liver enzymes (Scavone et al., 2022).

Recent studies in Hong Kong confirmed the presence of DCH in housed cats and demonstrated a circulation of DCH among community cats (Capozza et al., 2023a; Beatty et al., 2024). A molecular confirmation for DCH detection is necessary, though negative qPCR does not rule out infection. This could be explained since not all infected cats are expected to be viraemic. Prevalence of DCH is possibly underestimated, since anti-DCH core antibodies have been reported in almost 25% of the tested animals (Fruci et al., 2022). In humans, circulating HBV DNA is a proxy for viraemia and is detected in both acute and chronic infections (Tang et al., 2018). However, many HBV-infected humans can restrain viraemia, either through immune control (inactive HBeAg-negative phase) or functionally clearing the infection (measured by HBsAg loss). In either case, covalently closed circular DNA (cccDNA) is not totally removed from the liver but HBV remains persistently able to infect.

Progress has been made towards developing a panel of markers that will help to clarify the threat that DCH poses to feline health, as well as its natural history and global epidemiology (Capozza et al., 2023b).

References

Adıgüzel E, Erdem-Şahinkesen E, Koç BT, Demirden C, Oğuzoğlu TÇ. The detection and full genomic characterization of domestic cat Orthohepadnaviruses from Türkiye. Vet Med Sci. 2023 Sep;9(5):1965-1972. doi: 10.1002/vms3.1217. Epub 2023 Jul 20. PMID: 37471581; PMCID: PMC10508501.

Aghazadeh M, Shi M, Barrs VR, McLuckie AJ, Lindsay SA, Jameson B, Hampson B, Holmes EC, Beatty JA. A Novel Hepadnavirus Identified in an Immunocompromised Domestic Cat in Australia. Viruses. 2018 May 17;10(5):269. doi: 10.3390/v10050269. PMID: 29772771; PMCID: PMC5977262.

Beatty JA, Choi YR, Nekouei O, Woodhouse FM, Gray JJ, Hofmann-Lehmann R, Barrs VR. Epidemiology of Pathogenic Retroviruses and Domestic Cat Hepadnavirus in Community and Client-Owned Cats in Hong Kong. Viruses. 2024 Jan 23;16(2):167. doi: 10.3390/v16020167. PMID: 38399943; PMCID: PMC10893229.

Capozza P., Carrai M., Choi Y.R., Tu T., Nekouei O., Lanave G., Martella V., Beatty J.A., Barrs V.R. Domestic Cat Hepadnavirus: Molecular Epidemiology and Phylogeny in Cats in Hong Kong. Viruses. 2023a; 15:150. doi: 10.3390/v15010150.

Capozza P., Pellegrini F., Camero M., Diakoudi G., Omar A.H., Salvaggiulo A., Decaro N., Elia G., Catucci L., Di Martino B., et al. Hepadnavirus Infection in a Cat with Chronic Liver Disease: A Multi-Disciplinary Diagnostic Approach. Vet. Sci. 2023b; 10:668. doi: 10.3390/vetsci10120668.

Fruci P., Di Profio F., Palombieri A., Massirio I., Lanave G., Diakoudi G., Pellegrini F., Marsilio F., Martella V., Di Martino B. Detection of antibodies against domestic cat hepadnavirus using baculovirus-expressed core protein. Transbound. Emerg. Dis. 2022; 69:2980–2986. doi: 10.1111/tbed.14461.

Lanave G, Capozza P, Diakoudi G, Catella C, Catucci L, Ghergo P, Stasi F, Barrs V, Beatty J, Decaro N, Buonavoglia C, Martella V, Camero M. Identification of hepadnavirus in the sera of cats. Sci Rep. 2019 Jul 23;9(1):10668. doi: 10.1038/s41598-019-47175-8. PMID: 31337847; PMCID: PMC6650429.

Magnius L, Mason WS, Taylor J, Kann M, Glebe D, Dény P, Sureau C, Norder H, Ictv Report Consortium. ICTV Virus Taxonomy Profile: Hepadnaviridae. J Gen Virol. 2020 Jun;101(6):571-572. doi: 10.1099/jgv.0.001415. PMID: 32416744; PMCID: PMC7414443.

Michalak TI. Diverse Virus and Host-Dependent Mechanisms Influence the Systemic and Intrahepatic Immune Responses in the Woodchuck Model of Hepatitis B. Front Immunol. 2020 May 27; 11:853. doi: 10.3389/fimmu.2020.00853. PMID: 32536912; PMCID: PMC7267019.

Pesavento PA, Jackson K, Hampson TSTTB, Munday JS, Barrs VR, Beatty JA. A Novel Hepadnavirus is Associated with Chronic Hepatitis and Hepatocellular Carcinoma in Cats. Viruses. 2019 Oct 21;11(10):969. doi: 10.3390/v11100969. PMID: 31640283; PMCID: PMC6832243.

Piewbang C, Dankaona W, Poonsin P, Yostawonkul J, Lacharoje S, Sirivisoot S, Kasantikul T, Tummaruk P, Techangamsuwan S. Domestic cat hepadnavirus associated with hepatopathy in cats: A retrospective study. J Vet Intern Med. 2022 Sep;36(5):1648-1659. doi: 10.1111/jvim.16525. Epub 2022 Aug 24. PMID: 36054642; PMCID: PMC9511090.

Piewbang C, Wardhani SW, Chaiyasak S, Yostawonkul J, Chai-In P, Boonrungsiman S, Kasantikul T, Techangamsuwan S. Insights into the genetic diversity, recombination, and systemic infections with evidence of intracellular maturation of hepadnavirus in cats. PLoS One. 2020 Oct 23;15(10):e0241212. doi: 10.1371/journal.pone.0241212. PMID: 33095800; PMCID: PMC7584178.

Ratti G., Stranieri A., Scavone D., Cafiso A., Meazzi S., Luzzago C., Dall'Ara P., Tagliasacchi F., Cavicchioli L., Ferrari F., et al. Detection and genetic characterization of domestic cat hepadnavirus in cats with cavitary effusions. Vet. Microbiol. 2023; 284:109828. doi: 10.1016/j.vetmic.2023.109828.

Scavone D., Lauzi S., Stranieri A., Tramontano G., Ratti G., Paltrinieri S. Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. Vet. Rec. 2022;191:e1626. doi: 10.1002/vetr.1626.

Seeger C, Baldwin B, Hornbuckle WE, Yeager AE, Tennant BC, Cote P, Ferrell L, Ganem D, Varmus HE. Woodchuck hepatitis virus is a more efficient oncogenic agent than ground squirrel hepatitis virus in a common host. J Virol. 1991 Apr;65(4):1673-9. doi: 10.1128/JVI.65.4.1673-1679.1991. PMID: 2002538; PMCID: PMC239970.

Shofa M, Kaneko Y, Takahashi K, Okabayashi T, Saito A. Global Prevalence of Domestic Cat Hepadnavirus: An Emerging Threat to Cats' Health? Front Microbiol. 2022 Jun 24; 13:938154. doi: 10.3389/fmicb.2022.938154. PMID: 35814669; PMCID: PMC9263588.

Stone C, Petch R, Gagne RB, Nehring M, Tu T, Beatty JA, VandeWoude S. Prevalence and Genomic Sequence Analysis of Domestic Cat Hepadnavirus in the United States. Viruses. 2022 Sep 21;14(10):2091. doi: 10.3390/v14102091. PMID: 36298647; PMCID: PMC9607532.

Tang L.S.Y., Covert E., Wilson E., Kottilil S. Chronic Hepatitis B Infection. JAMA. 2018; 319:1802. doi: 10.1001/jama.2018.3795.

World Health Organization. Global hepatitis report, 2017. https://www.who.int/publications/i/item/9789241565455

World Health Organization. Global progress report on HIV, viral hepatitis and sexually transmitted infections, 2021. https://www.who.int/publications/i/item/9789240027077

Hepadnavirus Infection in a Cat with Chronic Liver Disease: A Multi-Disciplinary Diagnostic Approach

Adapted from:

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Simple Summary

Domestic cat hepadnavirus (DCH) is a newly identified virus, similar to the hepatitis B virus (HBV). The pathogenicity of DCH in its host needs further investigation. In this report, a case of DCH infection was described in a cat with clinical signs and serum chemistry markers indicative of chronic hepatic disease using a multi-disciplinary diagnostic approach, combining clinical, pathological, virological, and serological information, mirroring the diagnostic approach used for HBV in human patients. Improving the intra-vitam diagnostics for DCH will be useful in developing specific treatment and control strategies.

Abstract

A 3-year-old female stray, shorthair cat, with clinical signs and serum chemistry markers indicative of hepatic disease, was diagnosed with domestic cat hepadnavirus (DCH) infection. Coupling molecular and serological data, the infection was seemingly contextualized into a chronic phase, since IgM anti-core antibodies, a marker of early-stage Hepatitis B Virus (HBV) infection, were not detected. However, the cat possessed IgG anti-core, a common indicator of chronic HBV infection in human patients and did not show seroconversion to the anti-DCH surface antigen, considered protective during HBV infection and associated with long-term protective immunity. On genome sequencing, the DCH strain showed 98.3% nucleotide identity to strains previously identified in Italy.

Keywords:

cat; domestic cat hepadnavirus; serum; liver; hepatitis

1. Introduction

Domestic cat hepadnavirus (DCH), discovered in 2018, is genetically similar to human hepatitis B virus (HBV). The DCH genome consists of approximately 3200 bases of circular, partially double-stranded DNA (Aghazadeh et al., 2018). In molecular investigations from countries in Asia, Australia, the USA, and Europe, DCH has been identified in 0.2 to 18% of cats (Capozza, Decaro, et al., 2021; Capozza et al., 2023; Ratti et al., 2023; Sakamoto et al., 2023; Shofa et al., 2022). This is likely an underrated estimation of the prevalence of DCH, since anti-DCH core antibodies (anti-DCHc) have been revealed in about 25% of the tested animals (Fruci et al., 2022). The pathobiology of this novel hepadnavirus is still largely unknown, but a possible etiopathogenetic role has been hypothesized in the development of feline liver disease. Hepatic histological alterations suggestive of inflammation and neoplasia, as documented for HBV, have been described in DCH-positive cats with chronic hepatitis or hepatocellular carcinoma (HCC) (Pesavento et al., 2019). Furthermore, a positive correlation has been observed between the hepatic markers and the presence of DCH DNA (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Capozza, Decaro, et al., 2021; Capozza, Lanave, et al., 2021; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020, 2022; Sakamoto et al., 2023; Shofa et al., 2022, 2022; Stone et al., 2022). More recently, DCH DNA was detected in serum and in inflammatory and non-inflammatory peritoneal effusion samples but not in liver biopsy from cats infected with DCH with hematological and biochemical alterations (Ratti et al., 2023). In addition, viremia from DCH has also been identified in cats with normal or mildly elevated liver enzymes (Scavone et al., 2022). Coinfection with feline retroviruses and age > 2 years are additional risk factors for DCH viremia (Capozza, Lanave, et al., 2021, 2021; Fruci et al., 2022; Lanave et al., 2019; Shofa et al., 2022). The disease spectrum and the course of HBV infection vary in terms of severity, progression, and prognosis, ranging from acute inapparent infections to progressive chronic hepatitis (Mason and Seeger, 2012). Antigens, antibodies, and viral DNA may be determined and combined with the haematological parameters to identify HBV and predict the stage of infection in human patients (Lee and Kim, 2021; Mason and Seeger, 2012). Accordingly, DCH diagnosis could benefit from a combined virological/serological approach, following the outline of the HBV diagnostic algorithm in human patients. We, herein, describe a case of DCH infection in a cat with evidence of hepatic illness, for whom intra-vitam diagnosis was obtained with direct and indirect diagnostics. The full-genome sequence of the virus infecting the cat was also generated.

2. Case Description

A 3-year-old female stray, shorthair (DSH) cat was referred to a private veterinary clinic in March 2021 for lethargy, anorexia, and weakness. On physical examination, the cat showed a temperature in the physiological range (38 °C), pale mucous membranes, jaundice, and weight loss. After the initial physical examination, a baseline assessment was carried out, consisting of a complete blood count, serum biochemistry, urinalysis, and retroviral screening, using a point-of-care (POC) immunochromatographic test (SNAP FIV/FeLV Combo Test, IDEXX). Abnormalities in serum biochemistry included elevations of alkaline phosphatase (ALP; 160 International Unit (IU)/L; Reference Interval (RI) 14–62), gamma-glutamyl transferase (GGT; 6.2 IU/L; RI

0.0–1.0), total bilirubin (4.42 mg/dL; RI 0.14–0.25), globulins (5.5 g/dL, RI 2.9–4.3), γ-globulin (33.6%; RI 15.0–28.0), and amylase (1755 IU/L; RI 648–1262). Cholesterol (91 mg/dL; RI 112–194), total iron (24 µg/dL; RI 55–152), and saturation (6.8%; RI 20–53) were decreased. A complete blood count revealed leucocytosis (increased white blood cells (WBCs) 46.7×10^3/µL, $5.5–12 \times 10^3$/µL) associated with anaemia (3.51×10^6/µL; RI $6.0–9.5 \times 10^6$/µL). Using the POC assay, the cat tested negative for retroviral infections. Haematological parameters were monitored repeatedly over four additional time points (April 2021, October 2021, November 2021, and February 2022), evidencing a persistent elevation in ALP, GGT, total bilirubin, and WBC values, along with anaemia and leucocytosis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values were out of range at two- and three-time monitoring points, respectively (Table 1).

Table 1. Blood parameters of the patient. Parameters that exceed the normal ranges are in bold.

Sampling Date	RBC 6.0–9.5 × 10^6/µL	WBC 5.5–12 × 10^3/µL	PLT 130–400 × 10^3/µL	CPK 52–295 IU/L	AST 16–46 IU/L	ALT 33–70 IU/L	ALP 14–62 IU/L	GGT 0–1 IU/L	Total Bilirubin 0.14–0.25 mg/dL	DCH DNA	Notes
18 March 2021	**3.51**	**46.7**	382	146	26	30	**160**	**6.2**	**4.42**	NT	
9 April 2021	**5.11**	**60.5**	397	62	30	**101**	**94**	**5.0**	**4.16**	NT	
14 October 2021	**6.33**	**27.7**	**482**	68	**167**	**162**	**63**	**2.8**	**1.87**	NT	
25 November 2021	**5.78**	**23.8**	256	76	20	38	**76**	**4.0**	**1.49**	Pos.	Liver biopsy positive for hepadnavirus (2.9×10^1 DNA copies/mL)
11 February 2022	**3.68**	**25.7**	348	148	**183**	**1285**	**93**	**4.1**	**0.31**	Neg.	

Abbreviations: RBC, red blood cells; WBC, white blood cells; PLT, platelets; CPK, creatine phosphokinase; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; IU, International Unit; L, Liter; mg, milligrams; µL, microlitres; mL, millilitres; DCH, domestic cat hepadnavirus, Neg., negative; Pos., positive; NT, not tested.

November 2021 (Figure 1) showed chronic liver disease and chronic gall-bladder abnormalities, with mild cholestasis.

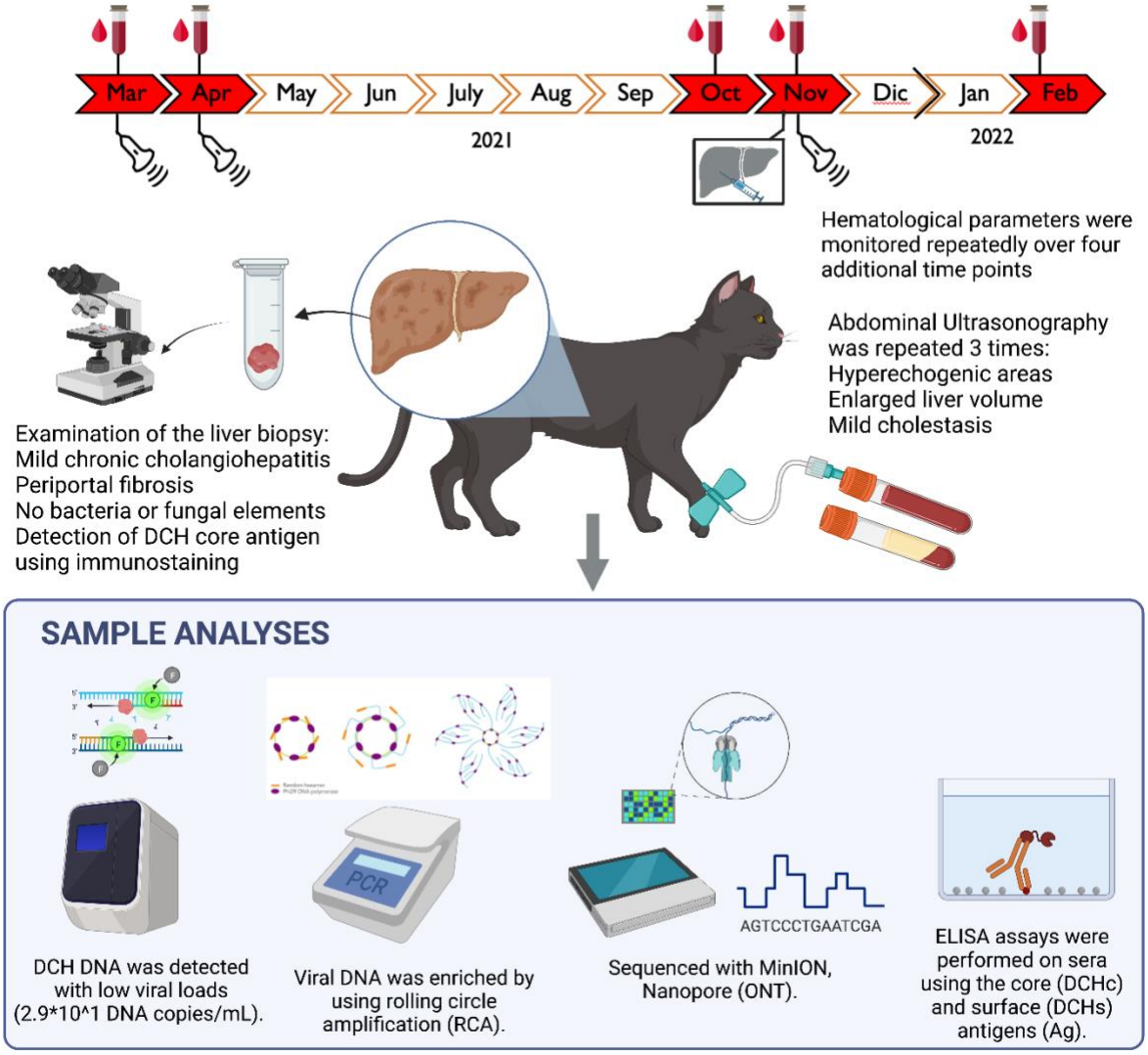

Figure 1. Phases of the investigation.

A liver biopsy specimen (collection date November 2021) was fixed in 10% buffered formalin, processed for paraffin sectioning, and cell staining was performed using haematoxylin and eosin. On histological examination, almost all the portal tracts were characterized by moderate fibrosis with mild lymphoplasmacytic infiltrate and ductular hyperplasia, leading to a diagnosis of lymphoplasmacytic diffuse mild chronic periportal hepatitis (Figure 2A). Bacterial or fungal elements were not evident. Upon virological investigations performed on the liver biopsy sample and on two serum specimens collected in November 2021 and February 2022, DCH DNA was identified using quantitative PCR (qPCR) (Lanave et al., 2019) only from the liver sample, with a viral load of 2.9×10^1 DNA copies/mL of extracted DNA template.

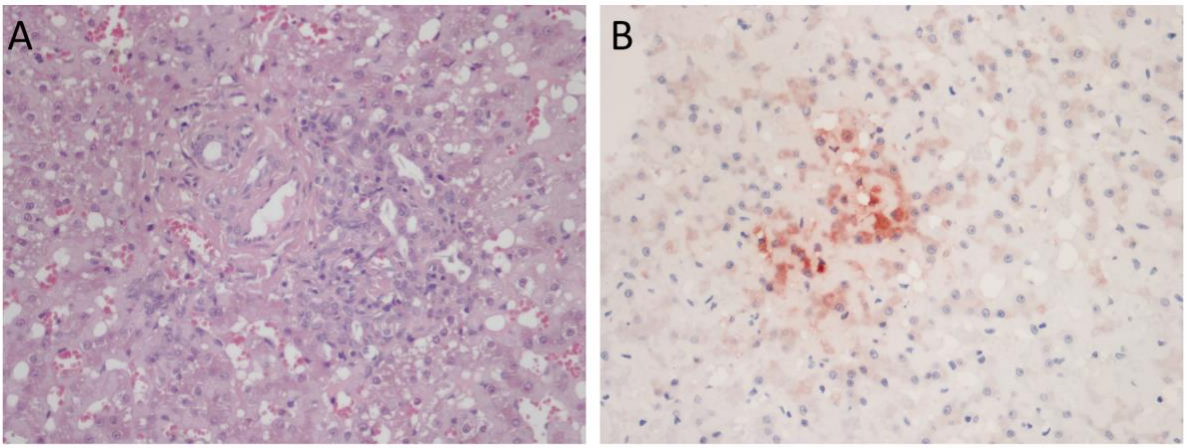

Figure 2. (**A**) Lymphoplasmacytic mild chronic periportal hepatitis with ductular hyperplasia. HandE, 400× magnification; (**B**) multifocal cytoplasmic immunostaining. Domestic cat hepadnavirus immunohistochemistry, 400× magnification.

When retesting all the three specimens with a qualitative PCR protocol (Capozza et al., 2023), both liver and serum samples, collected in November 2021, were positive, whilst the serum collected in February 2022, 3 months after the initial diagnosis, tested negative for DCH. All the specimens were negative for feline calicivirus, feline herpesvirus type 1, feline panleukopenia parvovirus, feline coronavirus, and feline retroviruses, thus ruling out co-infections (Decaro et al., 2008; Gut et al., 1999; Marsilio et al., 2005; Quackenbush et al., 1996; Stiles et al., 1999; Vogtlin et al., 2002). The immunohistochemistry (IHC) assay, performed on the liver biopsy by using a rabbit polyclonal anti-HBV core antigen (HBcAg) (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (Piewbang et al., 2020, 2022), revealed cytoplasmic immunoreactivity with focal strong intensity (Figure 2B), confirming the presence of DCHcAg.

To characterize the DCH strain, the viral DNA detected in the liver sample was enriched using a rolling circle amplification (RCA) with a TempliPhi and RCA primer mix. Subsequently, a total of 1 µg of a dilution 1:100 of the purified RCA product was used as a template for a library prepared with the Ligation sequencing kit 1D SQK-LSK110 (ONT™, Oxford, UK) for MinION sequencing (Oxford Nanopore Technology™). The genome was assembled using Geneious Prime version 2021.2 (Biomatters Ltd., Auckland, New Zealand). The DCH sequence was aligned with other DCH sequences selected from the GenBank (NCBI) database (examined in May 2023) using the Geneious Alignment plugin. The substitution model parameters for phylogenetic inference and assessment of selection pressure were selected using MEGA X version 10.0.5 (Kumar et al., 2018). The phylogenetic reconstruction was carried out using the Maximum Likelihood method and Tamura–Nei model (four parameters) (Hasegawa et al., 1985), a discrete gamma distribution, and invariable sites (six categories), with bootstrapping over 1000 replicates.

In order to investigate the serological response against DCH, the sera samples collected in November 2021 and in February 2022 were tested using two in-house ELISA assays based on a recombinant DCHcAg and DCH surface antigen (DCHsAg) (Fruci et al., 2022, 2023). Only IgG anti-DCHcAg was detected, whilst both sera resulted as negative for IgM anti-DCHcAg and IgG anti-DCHsAg. Sera obtained before November 2021 were not available for virological and serological examinations. The cat was found dead without traumatic injuries in May 2022, and post-mortem examination could not be made.

3. Discussion and Conclusions

Since the identification of DCH in cats in 2018, numerous global studies have been conducted to assess this virus's epidemiology and potential role in feline liver disease (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Capozza et al., 2023; Fruci et al., 2022; Jeanes et al., 2022; Lanave et al., 2019; Pesavento et al., 2019; Piewbang et al., 2020, 2022; Sakamoto et al., 2023; Scavone et al., 2022; Shofa et al., 2022; Stone et al., 2022). An association has been identified between DCH infection and clinical signs or markers related to hepatic damage (Anpuanandam et al., 2021; Capozza et al., 2023; Capozza, Lanave, et al., 2021, 2021; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020, 2022; Sakamoto et al., 2023; Shofa et al., 2022; Stone et al., 2022; Takahashi et al., 2022). DCH-infected cats are likely to have elevated ALT levels (Anpuanandam et al., 2021; Lanave et al., 2019). Cats with haematochemical profiles indicative of hepatic damage are about three-times more likely to test positive for DCH than animals with normal haematochemical parameters (Anpuanandam et al., 2021). These findings suggest the potential contribution of DCH in the development of hepatic disease in these animals. Based on the clinical signs, history, instrumental diagnostic analysis, laboratory results, and viral investigation, the case described in this report was framed as a chronic periportal hepatitis associated with DCH infection. Lymphocytic cholangio-hepatitis was suspected due to the increase in the serum concentration in ALT, ALP, GGT, bile acids, and total bilirubin, associated with the alteration to the leukogram (Jaffey, 2022). Subsequently, the histological examination performed on liver biopsy revealed moderate fibrosis with mild lymphoplasmacytic infiltrate and ductular hyperplasia in almost all portal areas, a picture compatible with periportal hepatitis (Fuentealba and Aburto, 2003; Weiss et al., 1997). In a retrospective investigation, out of 71 cat liver biopsies, a high prevalence of DCH was described in cats with chronic hepatitis (43%) and hepatocellular carcinoma (28%), compared with liver tissues without histological alterations (Pesavento et al., 2019). Animals with chronic hepatitis positive for DCH in PCR showed lymphocytic periportal inflammation, with some hepatic areas presenting inflammation at the portal–lobular interface. Additionally, lymphocytes and plasma cells were dispersed within

sinusoidal spaces where they occasionally clustered. Neutrophils were uncommon and, when present, were close to single-cell regions or to necrotic areas. Fibrosis irregularly spanned the portal regions and affected regional sinusoids (Pesavento et al., 2019). In a more recent investigation, Thai researchers established a correlation between DCH and liver histological parenchymal disease (HPD). Specifically, they revealed a higher proportion of DCH-positive HPD cases, with portal areas surrounded by thick bands of fibrosis connecting the portal structures in some tracts and showing infiltrates of numerous lymphocytes and plasma cells (Piewbang et al., 2020, 2022) .

In our analysis, IHC staining revealed cytoplasmic reactivity for DCH that appeared as multifocal and diffuse staining in the hepatocytes and bile duct epithelial cells adjacent to the areas of inflammation and fibrosis. These findings are comparable to the results of a previous Thai investigation (Piewbang et al., 2020), in which the presence of DCH was confirmed via IHC performed using a polyclonal antibody to the HbcAg antigen, with a positive signal being observed in the hepatocytes and biliary epithelial cells. The IHC assay against both HBsAg and HBcAg is well described in human medicine, and this pattern is correlated with viral replication activity and virus titre. In feline species, a correlation between the immunohistochemical pattern and the above-mentioned features is not yet possible. Therefore, the results obtained should be interpreted considering the margin of variability that has remained untested.

Interestingly, DCH DNA was detected in the serum sample and in liver biopsy, collected in November 2021, using a PCR with primers specific for DCH but not in a follow-up serum sample collected in February 2022. During HBV infection, some patients may exhibit a status of chronic inactive carrier or a form of HBeAg-negative chronic hepatitis, related to mutations in the HBV genome, with the hepadnavirus DNA being detectable in a negligible titre or being undetectable in the blood ((Coffin et al., 2019; Mason and Seeger, 2012). The cat possessed IgG anti-DCHcAg, a common indicator of chronic infection in HBV-infected human patients, and did not show seroconversion to anti-DCHsAg, considered protective during HBV infection and associated with the recovery from infection (Coffin et al., 2019; Mason and Seeger, 2012). In addition, both sera were negative for IgM anti-core, a serological marker indicative of a very early stage of HBV infection. Accordingly, coupling molecular and serological data, the DCH infection was seemingly contextualized into a chronic phase.

The full-length genome sequence of 3185 bp was generated for the DCH strain (GenBank accession nr. OR389995) (Figure 3).

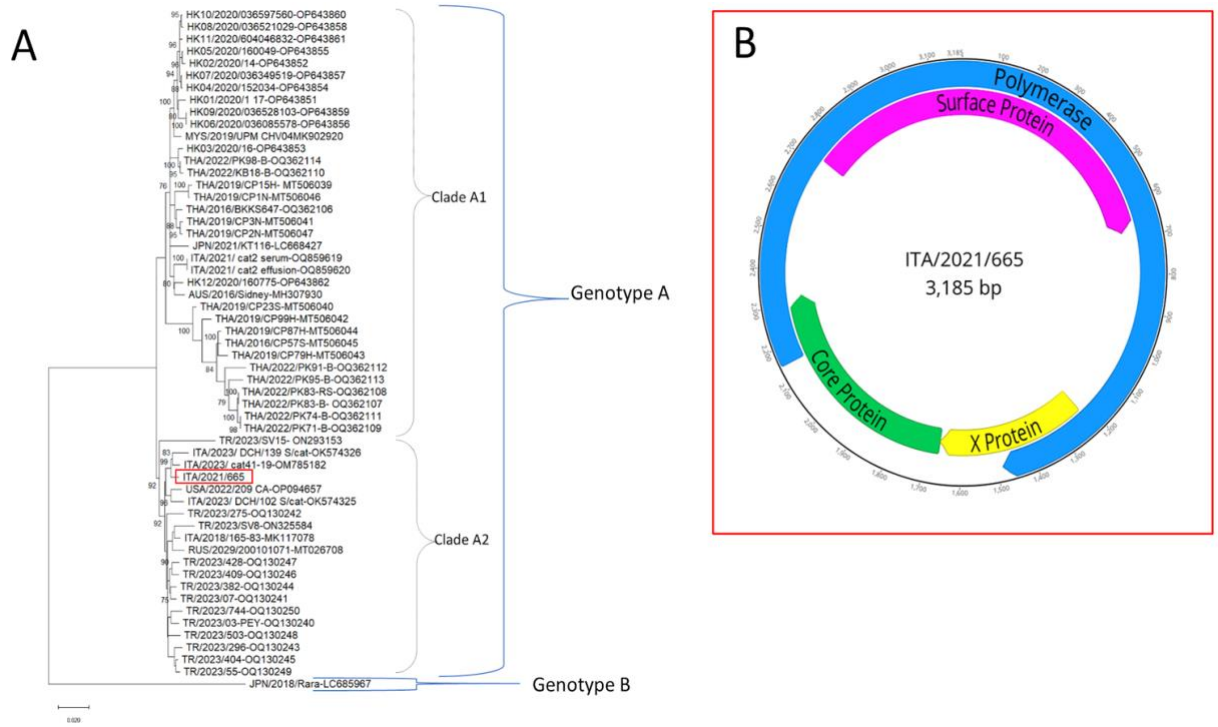

Figure 3. (**A**) Phylogenetic reconstruction based on the complete genomes of domestic cat hepadnavirus obtained in this study (red square) and sequences obtained from the databases. Statistical support was determined using 1000 bootstrap replicates. Bootstrap values greater than 75% are shown. Genotypes A and B, and clades A1 and A2 within genotype A are indicated. The scale bar represents the number of nucleotide substitutions per site. (**B**) Structure of the DCH genome.

In genome-wise sequence analysis, the DCH strain showed nucleotide identity between 97 and 98% with other Italian strains (Fruci et al., 2022; Lanave et al., 2019; Scavone et al., 2022). On phylogenetic analysis (Figure 3) using a whole-genome sequence, the DCH strain ITA/2021/665 was segregated with Malaysian, Thai, Australian, European, and American DCH viruses in genotype A. Strain ITA/2021/665 fell into a well-defined sub-clade, proposed as clade A2, along with Italian, Russian, Turkish, and American strains (Figure 3), distinguishable from sub-clade A1 that contained DCH viruses from Malaysia, Thailand, and Australia, and from the Japanese DCH strain Rara, genotype.

In conclusion, this study provides information on the clinical case of a cat with clinical signs and serum chemistry markers indicative of hepatic disease related to DCH chronic infection. This hypothesis was supported by abdominal ultrasonographic examinations, histological and immuno-histological analysis of liver biopsy, the persistent alteration of the markers of hepatocellular functionality, and the positivity to IgG anti-DCHcAg. Gathering information on DCH is pivotal to understanding its pathobiological role and developing specific treatment and control strategies. Since chronic HBV infection in human patients can evolve across different stages with marked fluctuations in virus activity and even with virus-undetectable phases, combining clinical, bio-chemical,

virological, and serological data must be considered as an elective approach for DCH diagnosis.

Author Contributions

Conceptualization, G.E. and M.C.; methodology, M.C. and G.L.; software, F.P. and G.L.; validation, M.C.; formal analysis, P.C.; investigation, P.C., F.P., G.D., A.H.O., A.S., P.F. and E.L.; resources, L.C., L.T. and E.L.; data curation, P.C.; writing—original draft preparation, P.C. and V.M.; writing—review and editing, V.M. and B.D.M.; visualization, G.D.; supervision, N.D., B.D.M. and V.M.; project administration, G.L.; funding acquisition, N.D. and V.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, were adhered to. Ethical approval was not required, and an ethical statement is not applicable as all the samples were collected for diagnostic purposes.

Informed Consent Statement

The owner of the animals involved in this study signed informed consent. All the samples were collected upon request of the attending veterinary surgeon. No additional requests were made by the researchers who performed the laboratory analyses for the purposes of drafting this study.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

Leonardo Catucci was employed by ACV Triggiano S.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Aghazadeh M, Shi M, Barrs VR, McLuckie AJ, Lindsay SA, Jameson B, Hampson B, Holmes EC, Beatty JA. A Novel Hepadnavirus Identified in an Immunocompromised Domestic Cat in Australia. Viruses. 2018 May 17;10(5):269. doi: 10.3390/v10050269. PMID: 29772771; PMCID: PMC5977262.

Anpuanandam K, Selvarajah GT, Choy MMK, Ng SW, Kumar K, Ali RM, Rajendran SK, Ho KL, Tan WS. Molecular detection and characterisation of Domestic Cat Hepadnavirus (DCH) from blood and liver tissues of cats in Malaysia. BMC Vet Res. 2021 Jan 6;17(1):9. doi: 10.1186/s12917-020-02700-0. PMID: 33407487; PMCID: PMC7788742.

Capozza P, Carrai M, Choi YR, Tu T, Nekouei O, Lanave G, Martella V, Beatty JA, Barrs VR. Domestic Cat Hepadnavirus: Molecular Epidemiology and Phylogeny in Cats in Hong Kong. Viruses. 2023 Jan 3;15(1):150. doi: 10.3390/v15010150. PMID: 36680190; PMCID: PMC9865086.

Capozza P, Decaro N, Beikpour F, Buonavoglia C, Martella V. Emerging Hepatotropic Viruses in Cats: A Brief Review. Viruses. 2021 Jun 17;13(6):1162. doi: 10.3390/v13061162. PMID: 34204394; PMCID: PMC8233973.

Capozza P, Lanave G, Diakoudi G, Stasi F, Ghergo P, Ricci D, Santo G, Arena G, Grillo I, Delle Donne E, Di Lisio F, Zini E, Callegari C, Valente L, Camero M, Di Martino B, Beatty J, Barrs VR, Buonavoglia C, Martella V. A longitudinal observational study in two cats naturally-infected with hepadnavirus. Vet Microbiol. 2021 Mar;254:108999.

doi: 10.1016/j.vetmic.2021.108999. Epub 2021 Jan 22. PMID: 33524809.

Coffin CS, Zhou K, Terrault NA. New and Old Biomarkers for Diagnosis and Management of Chronic Hepatitis B Virus Infection. Gastroenterology. 2019 Jan;156(2):355-368.e3. doi: 10.1053/j.gastro.2018.11.037. Epub 2018 Nov 22. PMID: 30472225; PMCID: PMC6433165.

Decaro N, Desario C, Lucente MS, Amorisco F, Campolo M, Elia G, Cavalli A, Martella V, Buonavoglia C. Specific identification of feline panleukopenia virus and its rapid differentiation from canine parvoviruses using minor groove binder probes. J Virol Methods. 2008 Jan;147(1):67-71. doi: 10.1016/j.jviromet.2007.08.006. Epub 2007 Sep 11. PMID: 17850892.

Fruci P, Di Profio F, Palombieri A, Massirio I, Lanave G, Diakoudi G, Pellegrini F, Marsilio F, Martella V, Di Martino B. Detection of antibodies against domestic cat hepadnavirus using baculovirus-expressed core protein. Transbound Emerg Dis. 2022 Sep;69(5):2980-2986. doi: 10.1111/tbed.14461. Epub 2022 Feb 1. PMID: 35077025.

Fruci P, Palombieri A, Sarchese V, Aste G, Friedrich KG, Martella V, Di Martino B, Di Profio F. Serological and Molecular Survey on Domestic Dog Hepadnavirus in Household Dogs, Italy. Animals (Basel). 2023 Feb 17;13(4):729. doi: 10.3390/ani13040729. PMID: 36830516; PMCID: PMC9951874.

Fuentealba IC, Aburto EM. Animal models of copper-associated liver disease. Comp Hepatol. 2003 Apr 3;2(1):5. doi: 10.1186/1476-5926-2-5. PMID: 12769823; PMCID: PMC156612.

Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H. One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. J Virol Methods. 1999 Jan;77(1):37-46. doi: 10.1016/s0166-0934(98)00129-3. PMID: 10029323; PMCID: PMC7185542.

Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol. 1985;22(2):160-74. doi: 10.1007/BF02101694. PMID: 3934395.

Jaffey JA. Feline cholangitis/cholangiohepatitis complex. J Small Anim Pract. 2022 Aug;63(8):573-589. doi: 10.1111/jsap.13508. Epub 2022 May 6. PMID: 35522164.

Jeanes EC, Wegg ML, Mitchell JA, Priestnall SL, Fleming L, Dawson C. Comparison of the prevalence of Domestic Cat Hepadnavirus in a population of cats with uveitis and in a healthy blood donor cat population in the United Kingdom. Vet Ophthalmol. 2022 Mar;25(2):165-172. doi: 10.1111/vop.12956. Epub 2021 Nov 22. PMID: 34806802.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018 Jun 1;35(6):1547-1549. doi: 10.1093/molbev/msy096. PMID: 29722887; PMCID: PMC5967553.

Lanave G, Capozza P, Diakoudi G, Catella C, Catucci L, Ghergo P, Stasi F, Barrs V, Beatty J, Decaro N, Buonavoglia C, Martella V, Camero M. Identification of hepadnavirus in the sera of cats. Sci Rep. 2019 Jul 23;9(1):10668. doi: 10.1038/s41598-019-47175-8. PMID: 31337847; PMCID: PMC6650429.

Lee JH, Kim HS. Current laboratory tests for diagnosis of hepatitis B virus infection. Int J Clin Pract. 2021 Dec;75(12):e14812. doi: 10.1111/ijcp.14812. Epub 2021 Sep 23. PMID: 34487586.

Marsilio F, Di Martino B, Decaro N, Buonavoglia C. A novel nested PCR for the diagnosis of calicivirus infections in the cat. Vet Microbiol. 2005 Jan 5;105(1):1-7. doi: 10.1016/j.vetmic.2004.09.017. Epub 2004 Nov 19. PMID: 15607078.

Mason, W. S., and Seeger, C. (2012). Hepadnaviruses: Molecular biology and pathogenesis (Vol. 168). Springer Science and Business Media.

Pesavento PA, Jackson K, Hampson TSTTB, Munday JS, Barrs VR, Beatty JA. A Novel Hepadnavirus is Associated with Chronic Hepatitis and Hepatocellular Carcinoma in Cats. Viruses. 2019 Oct 21;11(10):969. doi:

10.3390/v11100969. PMID: 31640283; PMCID: PMC6832243.

Piewbang C, Dankaona W, Poonsin P, Yostawonkul J, Lacharoje S, Sirivisoot S, Kasantikul T, Tummaruk P, Techangamsuwan S. Domestic cat hepadnavirus associated with hepatopathy in cats: A retrospective study. J Vet Intern Med. 2022 Sep;36(5):1648-1659. doi: 10.1111/jvim.16525. Epub 2022 Aug 24. PMID: 36054642; PMCID: PMC9511090.

Piewbang C, Wardhani SW, Chaiyasak S, Yostawonkul J, Chai-In P, Boonrungsiman S, Kasantikul T, Techangamsuwan S. Insights into the genetic diversity, recombination, and systemic infections with evidence of intracellular maturation of hepadnavirus in cats. PLoS One. 2020 Oct 23;15(10):e0241212. doi: 10.1371/journal.pone.0241212. PMID: 33095800; PMCID: PMC7584178.

Quackenbush SL, Dean GA, Mullins JI, Hoover EA. Analysis of FeLV-FAIDS provirus burden and productive infection in lymphocyte subsets in vivo. Virology. 1996 Sep 1;223(1):1-9. doi: 10.1006/viro.1996.0449. PMID: 8806534.

Ratti G, Stranieri A, Scavone D, Cafiso A, Meazzi S, Luzzago C, Dall'Ara P, Tagliasacchi F, Cavicchioli L, Ferrari F, Giordano A, Paltrinieri S, Lauzi S. Detection and genetic characterization of domestic cat hepadnavirus in cats with cavitary effusions. Vet Microbiol. 2023 Sep; 284:109828. doi: 10.1016/j.vetmic.2023.109828. Epub 2023 Jun 29. PMID: 37406408.

Sakamoto H, Ito G, Goto-Koshino Y, Sakamoto M, Nishimura R, Momoi Y. Detection of domestic cat hepadnavirus by next-generation sequencing and epidemiological survey in Japan. J Vet Med Sci. 2023 Jun 13;85(6):642-646. doi: 10.1292/jvms.22-0439. Epub 2023 May 15. PMID: 37183016; PMCID: PMC10315552.

Scavone D, Lauzi S, Stranieri A, Tramontano G, Ratti G, Paltrinieri S. Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. Vet Rec. 2022 Aug;191(4):e1626. doi: 10.1002/vetr.1626. Epub 2022 Apr 8. PMID: 35393638.

Shofa M, Kaneko Y, Takahashi K, Okabayashi T, Saito A. Global Prevalence of Domestic Cat Hepadnavirus: An Emerging Threat to Cats' Health? Front Microbiol. 2022 Jun 24;13:938154. doi: 10.3389/fmicb.2022.938154. PMID: 35814669; PMCID: PMC9263588.

Stiles J, Bienzle D, Render JA, Buyukmihci NC, Johnson EC. Use of nested polymerase chain reaction (PCR) for detection of retroviruses from formalin-fixed, paraffin-embedded uveal melanomas in cats. Vet Ophthalmol. 1999;2(2):113-116. doi: 10.1046/j.1463-5224.1999.00066.x. PMID: 11397251.

Stone C, Petch R, Gagne RB, Nehring M, Tu T, Beatty JA, VandeWoude S. Prevalence and Genomic Sequence Analysis of Domestic Cat Hepadnavirus in the United States. Viruses. 2022 Sep 21;14(10):2091. doi: 10.3390/v14102091. PMID: 36298647; PMCID: PMC9607532.

Takahashi K, Kaneko Y, Shibanai A, Yamamoto S, Katagiri A, Osuga T, Inoue Y, Kuroda K, Tanabe M, Okabayashi T, Naganobu K, Minobe I, Saito A. Identification of domestic cat hepadnavirus from a cat blood sample in Japan. J Vet Med Sci. 2022 May 1;84(5):648-652. doi: 10.1292/jvms.22-0010. Epub 2022 Mar 24. PMID: 35321970; PMCID: PMC9177394.

Vögtlin A, Fraefel C, Albini S, Leutenegger CM, Schraner E, Spiess B, Lutz H, Ackermann M. Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR. J Clin Microbiol. 2002 Feb;40(2):519-23. doi: 10.1128/JCM.40.2.519-523.2002. PMID: 11825966; PMCID: PMC153372.

Weiss, D. J., Armstrong, P., and Gagne, J. (1997). Inflammatory liver disease. 12(1), 22–27.

Chapter 4

Equine papillomavirus: the potential as oncogenic agent

Papillomaviruses (PVs) are small, non-enveloped viruses that possess a circular, double-stranded DNA genome. The term “papilloma” derives from the Latin words *papilla* (plural: *papillae*) and oma denoting benign tumour (Ennaji et al., 2019). Before papillomas were identified as pathological, Hippocrates (460–377 BC) used the Greek term *condyloma* to describe single genital warts (Karamanou et al., 2010) and, more generally, to refer to abnormal skin growths like lumps or warts. In the 1800s, genital warts were formally named *condyloma acuminatum* (von Krogh, 1991; Syrjänen and Syrjänen, 2008), maintaining the same meaning as in ancient times. Today, the term "papilloma" is used in pathology to describe small, nipple-like, benign tumors found on mucous membranes (Lowy and Douglas, 2006; Pennycook and McCready, 2019; Frias-De-Diego et al., 2019).

PVs belong to the family *Papillomaviridae* and are known to infect the epithelia of vertebrae (Bernard et al., 2010). To date, PVs have been identified in different animal species, including mammals, birds and reptiles (Bernard et al., 2010). More than 400 types of human papillomaviruses (HPV) have been identified with over 220 types fully classified (Mlynarczyk-Bonikowska and Rudnicka, 2024).

Most papillomaviruses are highly species-specific, but Bovine Papillomavirus (BPV) is an exception, as it can naturally infect multiple species of domestic animals.. BPVs have been associated with the development of sarcoids in horses, donkeys, zebras, and buffaloes (Chambers et al., 2003; Olson Jr and Cook, 1951). In addition, there is strong evidence that BPV-1 can also infects cats, with development of sarcoid-like lesions (Carney et al., 1990). In cats, BPV cross-infection produce characteristic proliferation of neoplastic fibroblasts, often with picket-fence arrangement (Schulman et al., 2001). In the first reported cross specie infection induced by BPV proliferative papillomatous lesions characterized by variable cytopathic effect and hypergranulosis of the stratum spinosum were observed in two Persian cats (Carney et al., 1990). Due to similarities between equine and feline BPV-induced tumors, the lesion in cats has been named “feline sarcoid”. In 2003, BPV-1 and -2 DNA has been detected from mesenchymal cells of feline sarcoids, with no evidence of viral detection in the overlying epidermis (Teifke et al., 2003). More recently, BPV-14 infection was identified from oral and mucosal sarcoids in cats (Greenwood et al., 2019; Kiefer et al., 2017).

PVs can infect equids with various manifestations including cutaneous papillomas, aural plaques, and genital papillomas. Skin warts are very common in horses, and they are localized on the distal limbs

and face of young horses, usually due to trauma which allows infection in immunologically naïve animals (Baker and Leyland, 1975; Sundberg et al., 1977; Junge et al., 1984). Horses often develop aural plaques on the inner surface of the pinnae (Torres et al., 2010). Equine aural plaques may be contagious, with the causative PV types hypothesized to be spread between horses by an insect vector (Fairley et al., 2014). Aural plaques do not spontaneously resolve and there is a single report of progression to squamous cell carcinoma (SCC) (Peters-Kennedy et al., 2020). It is noteworthy that genital papillomas in horses have been linked to the development of penile and preputial squamous cell carcinomas (MacLachlan and Dubovi, 2017). Genital warts appear as single or multiple (up to many hundreds) raised plaques or exophytic cauliflower-like masses (Torres and Koch, 2013). Warts can be covered by a thick layer of keratin resulting in a hard gray-brown rough surface. Warts typically involve the vulval lips, vestibular walls and clitoris of mares (Torres and Koch, 2013). Penile papillomatosis describes a condition in which the free part and glans of the penis are covered by innumerable coalescing warts (Knight et al., 2011).

Horses are susceptible to infection by *Equus caballus* papillomavirus (EcPV), of which 10 types (EcPV-1–10) have been to date identified (Lange et al., 2013; Nasir and Brandt, 2013; Linder et al., 2018; Turco et al., 2023). EcPV-1, commonly identified in younger horses, is associated with cutaneous papillomatosis and is highly contagious and typically self-limiting (Knottenbelt, 2009). EcPV-2 has been associated with anogenital lesions and neoplastic transformation to SCCs (Bogaert et al., 2012; C. Lange et al., 2013). EcPV-3 is responsible for aural plaques that typically afflict mature horses and are persistent but they are not considered pre-neoplastic (Knottenbelt, 2009). EcPV-4, -5, and -6 have been identified in aural plaques, often as part of mixed infections (Gorino et al., 2013). Additionally, EcPV-4 has been also identified in inguinal and vulvar plaques (Lange et al., 2013). EcPV-7 has been associated with penile masses (Lange et al., 2013) while EcPV-8 has been identified in horses with generalized papillomatosis (Linder et al., 2018) as well as in horse with inguinal plaques, papillomas, and SCC (Peters-Kennedy et al., 2019). EcPV-9 has been identified in the semen of a horse with a penile lesion (Li et al., 2019) while EcPV-10 has been isolated from a vaginal cytobrush of an infertile mare (Turco et al., 2023).

Classic equine viral papillomatosis, caused by EcPV-1, typically affects young horses under the age of three years. It commonly appears on the muzzle and lips and usually resolves on its own within 2–3 months (Maggi et al., 2023). Aural plaque, associated with EcPV3–6, is a form of cutaneous papillomatosis. This condition is characterized by well-defined, bright red or depigmented areas on the inner surface of one or both ears and may appear as single lesions, multiple spots, or larger,

connected patches (Mira et al., 2018). The development of aural plaques …do not show any specific breed or sex predisposition and can be found in horses of any age (Scott and Miller, 2010). Aural plaques may cause reduction in the commercial value of a horse, which may also be barred from participation in certain exhibitions, competitions, and auctions (Mira et al., 2018).

Transmission of papillomaviruses (PVs) often seems to occur through direct contact or by fomites, as indicated by the location of the lesions. For instance, EcPV1 (equine papillomavirus type 1) frequently causes lesions on horses' faces, while cattle papillomas or fibropapillomas commonly develop on the head and udder. Due to the lack of viral envelope, PVs display high resistance to freezing and drying thus allowing their survival in the environment for extended periods (Roden et al., 1997). Infection typically happens when virions enter a new host through skin abrasions, as they cannot penetrate unbroken epithelium (Jones, 2022). It is thought, although not yet proven, that EcPVs implicated in aural plaques are transmitted by flying insects, such as black flies (*Simulium* spp.), which are often seen in horses' ears (Fairley et al., 2014). The principle of flying insects acting as mechanical vectors for PVs has been experimentally proven in rabbits (Dalmat, 1958).

Equine papillomavirus type 2 (EcPV2) was first identified from cases of equine genital carcinoma in situ (CIS) and penile SCCs in 2008, and its genome was fully characterized (Scase et al., 2010). Since then, substantial evidence has indicated that EcPV-2 infection is aetiologically associated to the development of equine genital SCCs (Sykora and Brandt, 2017). Considering all the published studies, EcPV-2 appears to be associated with approximately 85% of penile squamous cell carcinomas (SCCs) (Knight et al., 2011; Scase et al., 2010; Van den Top et al., 2015) nearly 100% of vulvar SCCs (Bogaert et al., 2012; Scase et al., 2010) and around 20% of internal head and neck SCCs (Knight et al., 2013; Sykora et al., 2017). The exact mechanism of EcPV2 transmission is still unknown (Bogaert et al., 2012) and vertical transmission or infection from the mare could occur during or shortly after birth (Greenwood et al., 2020). Sexual transmission of EcPV2 is also possible, but lesions have been found in geldings with no sexual contact history (Bogaert et al., 2012). Other possible route of transmission could include non-sexual direct contact between horses (muzzle to genital) or mechanical transmission by biting flies or human cleaning of the penis and prepuce (Sykora and Brandt, 2017).

References

Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010 May 25;401(1):70-9. doi: 10.1016/j.virol.2010.02.002. Epub 2010 Mar 5. PMID: 20206957; PMCID: PMC3400342.

Baker, J., Leyland, A., 1975. Histological survey of tumours of the horse, with particular reference to those of the skin. The Veterinary Record 96, 419–422.

Bogaert L, Willemsen A, Vanderstraeten E, Bracho MA, De Baere C, Bravo IG, Martens A. EcPV2 DNA in equine genital squamous cell carcinomas and normal genital mucosa. Vet Microbiol. 2012 Jul 6;158(1-2):33-41. doi: 10.1016/j.vetmic.2012.02.005. Epub 2012 Feb 11. PMID: 22397936.

Carney HC, England JJ, Hodgin EC, Whiteley HE, Adkison DL, Sundberg JP. Papillomavirus infection of aged Persian cats. J Vet Diagn Invest. 1990 Oct;2(4):294-9. doi: 10.1177/104063879000200407. PMID: 1965634

Chambers G, Ellsmore VA, O'Brien PM, Reid SW, Love S, Campo MS, Nasir L. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. Virus Res. 2003 Oct;96(1-2):141-5. doi: 10.1016/s0168-1702(03)00175-8. PMID: 12951274.

Ennaji MM. Emergence of oncogenic high-risk human papillomavirus types and cervical cancer. In: Tadlaoui KA, Hassou N, Bennani B, eds. Emerging and Reemerging Viral Pathogens: Volume 1: Fundamental and Basic Virology Aspects of Human, Animal and Plant Pathogens. Cambridge, MA: Academic Press; 2019:539-570.

Fairley RA, Morley CM, Williams SD, Senior DA, Neill MA. Aural plaques in two imported horses in New Zealand. N Z Vet J. 2014 Jul;62(4):232-3. doi: 10.1080/00480169.2013.865570. Epub 2014 Mar 31. PMID: 24245824.

Frias-De-Diego A, Jara M, Escobar LE. Papillomavirus in wildlife. Front Ecol Evol. 2019; 7:1–11. https://dx.doi.org/10.3389/fevo.2019.00406.

Gorino AC, Oliveira-Filho JP, Taniwaki SA, Basso RM, Zakia LS, Araujo JP Jr, Borges AS. Use of PCR to estimate the prevalence of Equus caballus papillomavirus in aural plaques in horses. Vet J. 2013 Sep;197(3):903-4. doi: 10.1016/j.tvjl.2013.05.014. Epub 2013 Jun 15. PMID: 23773810.

Greenwood S, Campbell O, Movasseghi AR. Oral sarcoid in a cat. Can Vet J. 2019 May;60(5):485-489. PMID: 31080260; PMCID: PMC6463769.

Greenwood S, Chow-Lockerbie B, Ramsauer S, Wachoski-Dark G, Knight C, Wobeser B. Prevalence of Equus caballus Papillomavirus Type-2 Infection and Seropositivity in Asymptomatic Western Canadian Horses. Vet Pathol. 2020 Sep;57(5):632-641. doi: 10.1177/0300985820941270. Epub 2020 Aug 19. PMID: 32812517.

Jones, S. (2022). Papillomaviruses in equids: A decade of discovery and more to come? Equine Veterinary Education, 34(5), 236–240.

Junge, R., Sundberg, J., Lancaster, W., 1984. Papillomas and squamous cell carcinomas of horses. Journal of the American Veterinary Medical Association 185, 656–659

Karamanou M, Agapitos E, Kousoulis A, Androutsos G. From the humble wart to HPV: a fascinating story throughout centuries. Oncol Rev. 2010; 4:133-135.

Kiefer C, Tobler K, Ramsauer A, Biegel U, Kuehn N, Ruetten M. Felines Sarcoid bei einer 1-jährigen europäischen Hauskatze ausgelöst durch bovines Papillomavirus Typ 14 in der Schweiz [Feline sarcoid in a 1-year-old domestic short-haired cat caused by bovine papillomavirus type 14 in Switzerland]. Schweiz Arch Tierheilkd. 2017 Sep;159(9):487-491. French. doi: 10.17236/sat00127. PMID: 28952958.

Knight, C.G., Munday, J.S., Rosa, B.V., Kiupel, M., 2011. Persistent, widespread papilloma formation on the penis of a horse: a novel presentation of equine papillomavirus type 2 infection. Veterinary Dermatology 22, 570–574.

Knight CG, Dunowska M, Munday JS, Peters-Kennedy J, Rosa BV. Comparison of the levels of Equus caballus papillomavirus type 2 (EcPV-2) DNA in equine squamous cell carcinomas and non-cancerous tissues using quantitative PCR. Vet Microbiol. 2013 Sep 27;166(1-2):257-62. doi: 10.1016/j.vetmic.2013.06.004. Epub 2013 Jun 20. PMID: 23845733.

Knight CG, Munday JS, Peters J, Dunowska M. Equine penile squamous cell carcinomas are associated with the presence of equine papillomavirus type 2 DNA sequences. Vet Pathol. 2011 Nov;48(6):1190-4. doi: 10.1177/0300985810396516. Epub 2011 Jan 31. PMID: 21282669.

Knottenbelt, D. C. (2009). Pascoe's principles and practice of equine dermatology E-book: Pascoe's principles and practice of equine dermatology E-book. Elsevier Health Sciences.

Lange CE, Vetsch E, Ackermann M, Favrot C, Tobler K. Four novel papillomavirus sequences support a broad diversity among equine papillomaviruses. J Gen Virol. 2013 Jun;94(Pt 6):1365-1372. doi: 10.1099/vir.0.052092-0. Epub 2013 Mar 13. PMID: 23486670.

Lange CE, Tobler K, Lehner A, Grest P, Welle MM, Schwarzwald CC, Favrot C. EcPV2 DNA in equine papillomas and in situ and invasive squamous cell carcinomas supports papillomavirus etiology. Vet Pathol. 2013 Jul;50(4):686-92. doi: 10.1177/0300985812463403. Epub 2012 Oct 11. PMID: 23064881.

Li CX, Chang WS, Mitsakos K, Rodger J, Holmes EC, Hudson BJ. Identification of a Novel Equine Papillomavirus in Semen from a Thoroughbred Stallion with a Penile Lesion. Viruses. 2019 Aug 4;11(8):713. doi: 10.3390/v11080713. PMID: 31382657; PMCID: PMC6723834.

Linder KE, Bizikova P, Luff J, Zhou D, Yuan H, Breuhaus B, Nelson E, Mackay R. Generalized papillomatosis in three horses associated with a novel equine papillomavirus (EcPV8). Vet Dermatol. 2018 Feb;29(1):72-e30. doi: 10.1111/vde.12481. Epub 2017 Aug 22. PMID: 28833761.

Lowy R, Douglas MS. Papillomavirus research: from natural history to vaccines and beyond. In: Campo MS, ed. Papillomavirus Research: From Natural History to Vaccines and Beyond. Hethersett, England: Caister Academic Press; 2006:13-18.

MacLachlan, N. J., & Dubovi, E. J. (Eds.). (2017). Chapter 11—Papillomaviridae and Polyomaviridae. In Fenner's Veterinary Virology (Fifth Edition) (pp. 229–243). Academic Press. https://doi.org/10.1016/B978-0-12-800946-8.00011-8

Maggi R, De Paolis L, De Santis D, Vellone VG, De Ciucis CG, Fruscione F, Mazzocco K, Ghelardi A, Marruchella G, Razzuoli E. Bovine Papillomavirus Type 1 Infection in an Equine Congenital Papilloma. Pathogens. 2023 Aug 18;12(8):1059. doi: 10.3390/pathogens12081059. PMID: 37624019; PMCID: PMC10458069.

Mira J, Herman M, Zakia LS, Olivo G, Araújo JP Jr, Borges AS, Oliveira-Filho JP. Frequency of Equus caballus papillomavirus in equine aural plaques. J Vet Diagn Invest. 2018 Jul;30(4):565-568. doi: 10.1177/1040638717753495. Epub 2018 Mar 30. PMID: 29601778; PMCID: PMC6505913.

Mlynarczyk-Bonikowska B, Rudnicka L. HPV Infections-Classification, Pathogenesis, and Potential New Therapies. Int J Mol Sci. 2024 Jul 11;25(14):7616. doi: 10.3390/ijms25147616. PMID: 39062859; PMCID: PMC11277246.

Olson C Jr, Cook RH. Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. Proc Soc Exp Biol Med. 1951 Jun;77(2):281-4. doi: 10.3181/00379727-77-18750. PMID: 14854020.

Pennycook KB, McCready TA. Condyloma Acuminata. StatPearls. Treasure Island, FL: StatPearls Publishing; 2019.

Peters-Kennedy J, Lange CE, Rine SL, Hackett RP. Equus caballus papillomavirus 8 (EcPV8) associated with multiple viral plaques, viral papillomas, and squamous cell carcinoma in a horse. Equine Vet J. 2019 Jul;51(4):470-474. doi: 10.1111/evj.13046. Epub 2018 Dec 17. PMID: 30456819.

Roden RB, Lowy DR, Schiller JT. Papillomavirus is resistant to desiccation. J Infect Dis. 1997 Oct;176(4):1076-9. doi: 10.1086/516515. PMID: 9333171.

Scase T, Brandt S, Kainzbauer C, Sykora S, Bijmholt S, Hughes K, Sharpe S, Foote A. Equus caballus papillomavirus-2 (EcPV-2): an infectious cause for equine genital cancer? Equine Vet J. 2010 Nov;42(8):738-45. doi: 10.1111/j.2042-3306.2010.00311.x. PMID: 21039805.

Schulman FY, Krafft AE, Janczewski T. Feline cutaneous fibropapillomas: clinicopathologic findings and association with papillomavirus infection. Vet Pathol. 2001 May;38(3):291-6. doi: 10.1354/vp.38-3-291. PMID: 11355659.

Sykora S, Brandt S. Papillomavirus infection and squamous cell carcinoma in horses. Vet J. 2017 May; 223:48-54. doi: 10.1016/j.tvjl.2017.05.007. Epub 2017 May 31. PMID: 28671071.

Sykora S, Jindra C, Hofer M, Steinborn R, Brandt S. Equine papillomavirus type 2: An equine equivalent to human papillomavirus 16? Vet J. 2017 Jul; 225:3-8. doi: 10.1016/j.tvjl.2017.04.014. Epub 2017 May 3. PMID: 28720295.

Syrjänen S, Syrjänen K. The history of papillomavirus research. Cent Eur J Public Health. 2008;16(Suppl):S7-S13.

Sundberg, J., Burnstein, T., Page, E., Kirkham, W., Robinson, F., 1977. Neoplasms of equidae. Journal of the American Veterinary Medical Association 170, 150–152.

Teifke JP, Kidney BA, Löhr CV, Yager JA. Detection of papillomavirus-DNA in mesenchymal tumour cells and not in the hyperplastic epithelium of feline sarcoids. Vet Dermatol. 2003 Feb;14(1):47-56. doi: 10.1046/j.1365-3164.2003.00324.x. PMID: 12603685.

Torres SM, Malone ED, White SD, Koch SN, Watson JL, 2010. The efficacy of imiquimod 5% cream (Aldara®) in the treatment of aural plaque in horses: a pilot open-label clinical trial. Veterinary Dermatology 21, 503–509.

Torres, S.M., Koch, S.N., 2013. Papillomavirus-associated diseases. Veterinary Clinics of North America: Equine Practice 29, 643–655.

Turco S, Gabbianelli F, Mavian CN, Pietrucci D, De Paolis L, Gialletti R, Mechelli L, De Ciucis CG, Cappelli K, Dell'Anno F, Mecocci S, Donato GG, Nervo T, Fruscione F, Crescio MI, Ghelardi A, Chillemi G, Razzuoli E. Genetic Characterization of a Novel Equus caballus Papillomavirus Isolated from a Thoroughbred Mare. Viruses. 2023 Feb 28;15(3):650. doi: 10.3390/v15030650. PMID: 36992359; PMCID: PMC10059215.

van den Top JG, Harkema L, Lange C, Ensink JM, van de Lest CH, Barneveld A, van Weeren PR, Gröne A, Martens A. Expression of p53, Ki67, EcPV2- and EcPV3 DNA, and viral genes in relation to metastasis and outcome in equine penile and preputial squamous cell carcinoma. Equine Vet J. 2015 Mar;47(2):188-95. doi: 10.1111/evj.12245. Epub 2014 May 4. PMID: 24898479.

von Krogh G. Genitoanal papillomavirus infection: diagnostic and therapeutic objectives in the light of current epidemiological observations. Int J STD AIDS. 1991; 2:391-404.

Exploring the genetic diversity of horse papillomaviruses

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Abstract:

Equine aural plaques (EAPs), also known as papillary acanthoma or ear papilloma, are caused by Equus caballus papillomavirus (EcPV). To date, at least ten EcPV types have been described, although only EcPV-1, -3, -4, -5, and -6 have been reported in association with EAPs. Based on the current classification criteria, PV genera display less than 60% sequence identity to types of other genera based on multiple or pairwise sequence alignments of the L1 genes. In the present study, we report the identification of EcPVs in horses affected by EAPs.

A total of 25 EAP samples were collected from the internal pinnae of 22 horses displaying unilateral (nr=19) and bilateral (nr=3) lesions. The DNA extracts were screened using three pan-papillomavirus PCR protocols targeting the L1 and E1 genes of PVs. The obtained PCR amplicons were subjected to Sanger sequencing. A selection of samples was subjected to enrichment of the circular DNA template using rolling circle amplification (RCA) and subsequently sequenced by Oxford Nanopore Technologies (ONTTM) platform. All samples were re-screened using quantitative real-time PCR (qPCR) assays designed on the L1 gene sequences of the detected EcPVs.

Overall, 72% (18/25) of the samples tested positive by pan-papillomavirus PCRs. Interrogation of the NCBI database by Basic Local Alignment Search Tool (BLAST) nucleotide revealed the highest identity of the samples to distinct EcPVs. EcPV-6 (8/18; 44.4%), EcPV-4 (5/18; 27.8%), and EcPV-3 (5/18; 27.8%) were the most common types identified in the study. The complete genome sequences (7.5 kb) of 16 EcPV strains were reconstructed. Nine strains displayed the highest identity (up to 99.9%) to EcPV3 (NC_020500), EcPV4 (NC_017862), and EcPV6 (NC_020085), while seven strains were assigned to five novels putative EcPV species.

Based on the specific qPCR results, the infection rate was 92% (23/25) and co-infections were

observed in 91.3% (21/23) of the EcPV-positive samples. To date, 15 species of PVs have been reported to infect equines, including three bovine PVs (BPV1, BPV2, and BPV13), two donkey PVs (EaPV1 and EaPV2), and ten EcPVs. Our findings extend by about one-third the number of PV species identified in horses, thus far. Epidemiological investigations are required to assess the epidemiological and clinical relevance of these novel EcPVs.

Keywords: equine papillomavirus, aural plaque, next generation sequencing (NGS).

1. Introduction

Papillomatosis is a non-malignant cutaneous tumor often seen in equines (Munday et al., 2022; Sousa et al., 2008). It is associate to Equus caballus papillomavirus (EcPV), a single-stranded DNA virus belonging to the *Papillomaviridae* family, and are small, non-enveloped viruses that have circular, double-stranded DNA genome (Bernard et al., 2010; Jones, 2022). EcPV has a genomic size of roughly 8 kilobases in size (de Villiers et al., 2004; Hainisch et al., 2023; Jones, 2022), and encodes for at least five genes, divided into early non-structural proteins (E1-7) and late structural (L1 and L2 capsid proteins) according to their expression during the infection cycle, and an upstream regulatory region (URR) located between the L1 and E6 open reading frames, contains the origin of replication and binding sites for viral and cellular transcription factors (De Paolis et al., 2022; Hainisch et al., 2023; Jones, 2022).

E1 is a helicase involved in replication, while E2 is a transcription factor that enhances E1 and E2 expression, both expressed during the early stage of the infection (García-Vallvé et al., 2005). E4 is contained within E2 Open Reading Frame (ORF), and it is actively spliced to finalize virus amplification and synthesis (Doorbar, 2013). L1 and L2, instead, encode for the icosahedral capsid protein and for a structural protein that helps the packing into viral particles, both expressed during the late infection. Three additional genes (E5, E6, E7), involved in cell growth and immune response, are considered accessory genes acquired during the PV evolution, and their presence vary among the different phylogenetic groups (Van Doorslaer, 2013). Among the PV genes, L1 major capsid protein nucleotide sequence has been used in the classification of papillomaviruses (PV) because it is the most conserved gene in the genome (Bernard et al., 2010; De Paolis et al., 2022; de Villiers et al., 2004; Hainisch et al., 2023).

The current classification of Papillomaviruses is mainly based on the nucleotide sequence identity of the ORF. A novel papillomavirus type is described as a cloned full-lenght papilloma viral genome, whose L1 nucleotide sequence is at least 10% not identical with that of any other Papillomavirus type. New genera in the family *Papillomaviridae* are proposed when the nucleotide

similarities are lower than 60%. Open reading frame L1 differences between 2% and 10% ascertain a new subtype and a variant is defined if the difference is minus 2% (Bernard et al., 2010; Van Doorslaer et al., 2018; Van Doorslaer et al., 2013).

Therefore, based on their clinical history and current conditions, horses visited by practitioners are regularly checked for PVs using specific detection and quantification methods targeting the L1 gene (Forslund et al., 1999; Gravitt et al., 2000; Iftner and Villa, 2003).

To date, 15 species of PVs have been reported to infect equines including horses and donkeys. They are *Bos taurus* papillomavirus 1 (BPV1), 2 (BPV2), and 13 (BPV13), *Equus asinus* PVs 1-2 (EaP1 and EaPV2), and *Equus caballus* PVs 1-10 (EcPV1-10), that have been completely sequenced and classified (Bromberger et al., 2023; Li et al., 2019; Munday et al., 2023; Turco et al., 2023).

Likewise occurring in human, PV infections in horses can result in the development or progression of various lesions, including verrucous lesions, genital masses, and aural plaques (Bromberger et al., 2023; Li et al., 2019; Torres and Koch, 2013; Turco et al., 2023).

Equus caballus PVs 1 and 8 have been associated to cutaneous papillomas and generalized papillomatosis, characterized by thousands of plaque types throughout the body of the horse (Dong et al., 2017; Linder et al., 2018). Additionally, EcPV2, EcPV3 and EcPV4 have been related with genital papillomatosis, squamous cell carcinoma of the prepuce and penis (SCC), and less frequently, SCC of the vulva and oropharynx in horses (Cappelli et al., 2022; Hibi et al., 2019; Lange et al., 2011; Lange et al., 2013; Sykora et al., 2017; Van Den Top et al., 2010).

Furthermore, EcPV1, 3, 4, 5, 6, and 7 have been associated with aural and genital plaques, with a significant proportion of co-infection (Lange et al., 2011; Lange et al., 2013; Mira et al., 2018; Taniwaki et al., 2013). *Equus caballus* PVs 9 was detected in semen samples obtained from a horse with a penile lesion (De Paolis et al., 2022; Li et al., 2019)**.** Whereas EcPV 10 was detected in the penile and vulvar brushes of asymptomatic horses (Turco et al., 2023).

Aural plaques are skin lesions that commonly affect, unilaterally or bilaterally, the inner surface of the ears of horses, but may also occur in other regions (Jones, 2022; Munday et al., 2022; Sousa et al., 2008). The lesions are circumscribed and depigmented hyperkeratotic plaques, occurring in isolation, as multiple lesions or as coalesced lesions (Torres and Koch, 2013). Aural plaques in horses are raised, flat surface, nonpigmented lesions that have a scaly or encrusted surface (Fairley and Haines, 1992; Torres and Koch, 2013). Despite being a benign neoplasm, aural plaques seldom regress spontaneously; this results in distress for the animals and a decrease in the commercial value of the afflicted horses as a consequence of their unsightly appearance (Sousa et al., 2008).

Epidermal hyperplasia, hyperkeratosis, regions of hypomelanosis, and mitosis of basal cells of the epithelium are observed on histopathology (Obayes, 2016).

Aural plaques have been reported in horses worldwide, but their prevalence may vary depending on geographic location. Certain environmental factors, such as temperature, humidity, and exposure to sunlight, may influence the development of aural plaques. Some horse breeds, including appaloosas, ponies, and draft breeds appear to be more predisposed to developing aural plaques. However, aural plaques can occur in horses of any horses' breed. Aural plaques can affect horses of all ages, but they are most observed in adult horses. There doesn't appear to be a significant difference in prevalence between males and females (Mira et al., 2018). Exposure to sunlight, ultraviolet (UV) radiation, insect bites, and mechanical trauma may contribute to the development of aural plaques. Horses kept outdoors or in environments with high sun exposure may be at an increased risk. Management practices, such as inadequate fly control or improper ear care, may increase the risk of aural plaque development. Regular cleaning and inspection of the ears may help prevent or reduce the severity of lesions. Overall, while aural plaques are a relatively common condition in horses, the exact epidemiology and risk factors are not fully understood (Mira et al., 2016, 2018).

Few epidemiological data are available regarding the distribution of aural plaque associated with infection of EcPVs. The objective of this study was monitoring the circulation of EcPV causes of aural plaques in Italian horses.

2. Materials and methods

2.1 Included horses and sample collection.

To assess the presence of viral DNA from EcPVs, we selected 22 horses displaying unilateral (n=19) and bilateral (n=3) lesions.

These horses were of the Italian trotter horse breed and from different regions in Italy as follows: Apulia (n=16) and Campania (n=5). The horses were selected, regardless of sex or age, for the presence of aural plaques. The lesions were evaluated according to the number and distribution in the pinna (Mira et al., 2016). Data regarding the region where the animals were housed, age, sex and breed were collected.

A total of 25 aural plaques were collected from the internal pinnae of the horses using a 6 mm disposable punch, without prior cleaning of the pinna. Prior to the biopsy procedures, the horses were sedated with 0.01 mg/kg detomidine. Then, the samples were stored at −80 °C.

All sampling procedures were conducted in accordance with the journal's ethics policies as outlined on the journal's author guidelines page, and the appropriate ethical review committee approval has been received. The study was approved by the Ethics Committee of the Department of Veterinary Medicine, University of Bari (Approval Number 26/23). All experiments were performed in

accordance with relevant guidelines and regulations.

2.2 DNA extraction and screening for pan papillomavirus

Aural plaques samples were homogenized in 10% Dulbecco's modified Eagle's medium (DMEM), homogenized at 10% by Qiagen TissueLyser (QiagenTM, Hilden, Germany) and then centrifuged at 10,000×g for 3min. Nucleic acids were extracted from 200µL of the supernatants using the IndiSpin® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany), according to the manufacturer's instructions and stored at – 80°C until use.

Screening for PV was conducted using PCR protocol, with three different broadly reactive set (pan-PV) of primers targeting L1 and E1 regions that is suitable to amplify the majority of known human and animal PV, as described elsewhere (Figure 1 and Table 1) (Forslund et al., 1999; Gravitt et al., 2000; Iftner and Villa, 2003). The 20-mL PCR mixture was arranged using Platinum II Hot-Start Green PCR Master Mix (2X) (Invitrogen, Thermo Fisher Scientific). Briefly, the thermal protocol included a first step at 94°C for 2 min followed by 35 cycles of 94°C for 15s, 42°C for 15s, and 68°C for 15s, for the first pair of primers. An annealing temperature of 50°C and 47°C was used for the second and third pair of primers, respectively. To avoid cross-contamination, a strict separation between pre-amplification for reaction mix preparation, sample preparation and addition to mix, and amplification/post-amplification was adopted. DNA extracts were added to the mixtures in separate laminar flow cabinets. Samples homogenized from bull with BPV1 infections was used as positive controls, while distilled water were used as negative control.

All PCR amplicons were purified and sent for direct sequencing to Eurofins Genomics laboratories (Germany). Sequences of approximately 450-500 nucleotides (nt) were obtained and analyzed using the web-based tools BLAST (Basic Logic Alignment Search Tool, https://blast.ncbi.nl m.nih.gov/Blast.cgi, accessed January 2024) and Papilloma Virus Episteme (PaVE, NIH) (https://pave.niaid.nih.gov/), using the default values to find homologous hits.

2.3 Full genome sequencing

Based on the direct sequencing results, the full-length genome (Figure 2), of a selection of PV-positive samples was generated. The circular DNA in selected samples was enriched by multiply primed rolling cycle amplification (RCA), using TempliPhi 100 amplification kit (GE Healthcare) and primers reverse designed specifically for each strain identified (Table 2), according to the manufacturer's instructions (Johne et al., 2009; Wu et al., 2006).

The increase in circular DNA specific for EcPV was evaluated by testing the 1:10, 1:100, 1:1000 dilution of RCA product, using Quantitative PCR for specific EcPVs.

For samples that did not give satisfactory results in RCA, i.e. there was no considerable increase in the quantity of EcPV-specific circular DNA, a set of inverse nested PCR primers was designed to

recover the viral circular genome, amplifying a fragment of about 7.5 kb. Inverse nPCR primers were designed in a diagnostic universal genome fragment. The reverse and forward primers were designed with the 5' ends of the reverse facing the 5' end of the forward primer. The design of primer pairs was based on sequencing results of the short diagnostic region obtained with degenerate primers. The primers were designed using the software Primer 3 implemented in Geneious version 10.2.4 (Biomatters Ltd., Auckland, New Zealand). 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 for 2 min, followed by 35 cycles of 94 ◦C for 30 s, 60 ◦C for 30 s, and 68 ◦C for 3 min, with a final extension of 68 ◦C for 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. One microliter of a 1:100 dilution of the inverse PCR product was used in the second-round PCR using the same mix. The thermal protocol was the same as for the first-round PCR.

Subsequently, a total of 1g of a dilution 1:100 of the purified RCA or BTB product was used as a template for a library prepared with the Ligation sequencing kit 1D SQK-LSK110 (Oxford Nanopore Technology, ONT™, Oxford, UK) for MinION sequencing. The genome was assembled using Geneious Prime version version 10.2.4. software (Biomatters Ltd., Auckland, New Zealand). The EcPvs sequence was aligned with other EcPvs sequences selected from the GenBank (NCBI) database using the Geneious Alignment plugin. The substitution model parameters for phylogenetic inference and assessment of selection pressure were selected using MEGA X version 10.0.5 (Kumar et al., 2018).

2.4 Quantitative PCR for specific EcPV screening

A quantitative real-time PCR (qPCR) was designed for each EcPV genotypes identified based on the sequence data obtained with the genomic amplification and sequencing. 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 assays was assessed with a panel of DNA viruses including Equine herpesvirus 1 and 4 (EHV-1 and EHV-4). Also, a panel of papillomavirus-positive samples identified and characterized upon sequence analysis in this study was tested to rule out cross-reactivity of the primers/probe with other EcPVs.

2.5 Sequence and Phylogenetic analysis

The purified PCR products with sufficient DNA concentration (0.10 ng/mL) were directly

sequenced in both directions by Eurofins Genomics (Ebersberg, Germany).
The obtained nucleotide sequences of the novel equine PVs EcPV11a/b, EcPV12a/b, EcPV13, EcPV14, and EcPV15 and 145 other PVs representative of animal PV disversity were imported in DAMBE (Xia and Xie, 2001) and aligned at the amino acid level. Next, the nucleotide alignments were generated using the aligned amino acid sequences as a reference guide.
This approach was independently applied to the E1, E2, and L1 ORFs. Following Gottschling et al., a compiled alignment was created by concatenating the three ORFs (Gottschling et al., 2011). The E4 region nested within the E2 gene was excluded from the molecular analyses. The evolutionary history of the novel equine PVs and the 145 PVs included in this study was reconstructed using the Maximum Likelihood method based on the General Time Reversible (GTR) model (Le and Gascuel, 2008). This method was chosen from among 24 different nucleotide substitution models as the most suitable for our data. The selection was made using the "find best DNA/protein Models" option in MEGA6 (Tamura et al., 2013) and was based on the model with the lowest BIC (Bayesian Information Criterion) score, which best described the substitution pattern. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.4341)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 3.59% sites). This analysis involved a total of 152 nucleotide sequences, and 3227 positions were considred in the final dataset. Positions containing missing data or gaps were eliminated. Bootstrapping (Felsenstein, 1985) (1000 iterations) was used to evaluate statistical support of internal branches of the trees. All evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Data analysis

The chi-squared test and Fisher's exact test were performed to determine the frequency distribution of the viral types and the associations linking the type of EcPV detected and the copresence of the viral DNA to the type and distribution of the lesion. Descriptive statistics were applied to determine the frequency of viral detection in the inter- nal pinna unilaterally or bilaterally. Statistical analyses were performed using the software R version 4.0.2 (https://www.R-project.org/) setting a statistical significance of P-value ≤ 0.05.

3. Results

3.1 Screening pan papillomavirus

Of the 22 horses sampled, aural plaque were depigmented lesions found unilaterally in 19 horses and bilaterally in 3 horses. The aural plaques affected three or more quadrants in 16% (4/25) of the ears' internal pinna, while ears with coalescing aural plaque lesion were more frequent (60%, 15/25) followed by 24% (6/25) of the ears with limited punctate lesions (Mira et al., 2016).
All lesions (n=25 aureal plaque samples) were screened using a panel of three different pan-papillomavirus degenerate PCR protocols (PCR) (Forslund et al., 1999; Gravitt et al., 2000; Iftner and Villa, 2003). Overall EcPV was detected with a prevalence of 72% (18/25). Six (33.3%; 6/18) of these samples were positive for all three PCRs; seven (38.8%; 7/18) resulted positive for the second and third pair of oligonucleotides; three (16.6%; 3/18) were positive for the first and second PCR, and two (11.1%; 2/18) were positive only for the third PCR. Sequences obtained from PCR products were analyzed by nucleotide Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and PapillomaVirus Episteme (PaVE, NIH) (https://pave.niaid.nih.gov/) thus several papillomavirus sequences were identified. EcPV -1, -2, -5, -7, -8 and -10 were not detected in any of the 25 samples screened in this investigation. EcPV -6, -4 and -3 were the most frequently detected viral types, followed by EaPV-1, EaPV-2, and EcPV-9. The nucleotide (nt) identity with reference strains ranged from 60.8% to 100%. When evaluating whether DNA from the same EcPV was detected in both ears of the same horse, it was observed that EcPV-6 was the most common EcPV type detected bilaterally (50%, 2/4), whereas for the other two horses sampled bilaterally one of the two samples resulted negative.
Co-infection by different EcPV genotypes was detected using the sequence of the partial genome fragments amplified by endpoint PCR, which was confirmed by specific real-time PCR. Interestingly, in 9 samples, we observed the co-presence of two different EcPVs. In 4 samples, we identified three distinct EcPVs, in 3 samples four EcPVs, in 1 sample five different EcPVs, and in one sample, we identified as many as seven distinct EcPVs. Only in 4 samples were we able to identify a single EcPV (Figure 2)

3.2 Genome characterization and phylogenetic analysis

The circular DNA of selected samples was enriched by rolling cycle amplification (RCA) protocols. Subsequentially, an inverse two-round PCR strategy with primers designed to amplify the result of the circular genome was performed on the RCA products. The complete genome was generated successfully for the EcPV-6 (ITA/2023/horse/235; ITA/2023/horse/469; ITA/2023/horse/492-1; ITA/2023/horse/139), EcPV-4 (ITA/2021/horse/322-1; ITA/2022/horse/405; ITA/2023/horse/47) and EcPV-3 (ITA/2023/horse/47; ITA/2023/horse/492-1) with a 99.9% nt similarity to NC_020500, NC_017862, and NC_020085, respectively. Additionally, the entire genomes (~7.5 kb) of 7 unlabeled PVs were assembled. The novel PVs in the L1 gene showed the most similarity to EcPV-

1 (64% nucleotide identity), EaPV-2 (59.1-61.5% nucleotide identity), and EcPV-6 (57.8-66.9% nucleotide identity), respectively on BLAST investigation. Different PV species were assigned to the viruses based on the ICTV categorization criteria and PapillomaVirus Episteme (PaVE, NIH) database. The new EcPV genotypes identified in this investigation were tentatively proposed as EcPV-11, EcPV-12, EcPV-13, EcPV-14, EcPV-15. Aside from the late structural genes L1 and L2, the early genes E1, E2, E4, E6, and E7 were also identified.

3.3 Screening for specific new EcPV types by qPCR

The qPCR assays for each new EcPV genotypes were specific since it did not recognize other equine DNA viruses, and none of the other samples was positive for the pan-PV PCR that was sequenced and characterized in our collection. Based on the specific qPCR results, the infection rate was 92% (23/25) of the EcPV-positive samples. EcPV-15 (60.8%;14/23 with a Ct average of 29.95), EcPV-6 (56.5%; 13/18, with a ct average of 21.53), EcPV-3 (52.2%; 12/23, with a ct average of 25.94) and EcPV-4 (43.5%; 10/23, with a ct average of 20.64) were the most frequently detected viral genotypes, followed by EcPV12 (30.4%;7/23, with a Ct average of 29.2), EcPV-11 (26%; 6/23, with a Ct average of 2.38), EcPV-13 (4.3%;1/23, with a Ct of 25.2) and EcPV-14 (4.3%;1/23 with a Ct of 19). The compresence of the viral DNA was observed in 91.3% (21/23) of the samples positive to PV. No significant association was found when assessing lesion characteristics (type and distribution), EcPV genotypes, or copresence of viral DNA.

3.4 Phylogenetic analyses

Phylogeny was inferred based on a concatenated E1/E2/L1 nucleotide sequence alignment of the novel equine PVs with 145 PV-types belonging to different PV genera (Figure 4). According to the most recent PVs classification (Dolz et al., 2020), maximum likelihood phylogenetic trees generated in this study correctly clustered the PVs in their respective genera. Further, main genera associations were consistent with what observed by Gottschling et al. (Gottschling et al., 2011), and later studies (Lecis et al., 2014; Tore et al., 2017), and were updated with novel PV species recently discovered.

Beside the single amphibian PV RmarPV1 and the fish PVs, which groups in three main lineages of unclassified viruses, several genera associations were identified (Figure 4): 1) The Eta, Teta, Dyoepsylon, and Dyozeta genera, which include a diverse group of birds, turtles, and bats PVs, forming a distinctly separate branch; 2) The Artiodactyla-infecting Delta and Epsilon viruses; 3) Mixed groups of mammalian PVs. Equine viruses emerge in a well-defined monophyletic group. However, our approach failed to define a statistically supported phylogeny for EcPV8.

4. Discussion

Through the examination of a collection of aural plaque samples taken from 22 horses, EcPVs were detected in around 72% (18/25) of the samples that yielded positive results when subjected to three distinct pan-papillomavirus degenerate PCR procedures.

Utilizing a panel of three distinct PCR tests, this diagnostic method has greatly improved the accuracy and precision of diagnosing PV despite the possibility of higher costs. This has facilitated the detection of novel viral variants and several co-infections. Undoubtedly, the diagnostic test exhibits high sensitivity and flawless specificity within this limited sample. Nevertheless, the restricted number of disease-negative cases impacts the accuracy of specificity and other associated measures. The high positive predictive value demonstrates the test's effectiveness in accurately establishing the presence of the illness when the test result is positive. However, the negative predictive value and associated probability ratios emphasize the need to interpret negative findings carefully. These findings emphasize the need for further validation in broader and more varied groups to enhance the test's prediction accuracy and dependability.

The qPCR assays developed for each newly discovered EcPV genotype exhibited specificity since they did not exhibit cross-reactivity with other horse DNA viruses. Furthermore, none of the other samples yielded positive results when subjected to the pan-PV PCR assay, which was subsequently sequenced and described in our collection (Table X).

Based on the specific qPCR results, it was established that the infection rate among the samples that tested positive for EcPV was 92%, with 23 out of 25 samples showing infection. EcPV-15 was the most found out of the several genotypes examined, being present in 60.8% of the samples (14 out of 23). The average cycle threshold (ct) for EcPV-15 was 29.95. EcPV-6 was found in 56.5% (13 out of 18 samples) with an average ct value of 21.53. EcPV-3 was discovered in 52.2% (12 out of 23 samples) with an average ct value of 25.94. EcPV-4 was present in 43.5% (10 out of 23 samples) with an average ct value of 20.64. EcPV12 was found in 30.4% (7 out of 23 samples) with an average ct value of 29.2. EcPV-11 was discovered in 26% (6 out of 23 samples) with an average ct value of 2.38. EcPV-13 was present in 4.3% (1 out of 23 samples) with a ct value of 25.2. EcPV-14 was found in 4.3% (1 out of 23 samples) with a ct value of 19.

The co-presence of different EcPV DNA was observed in 91.3% (21 out of 23) of the samples that tested positive for PV (Figure 3) using endpoint PCR confirmed by specific real-time PCR drawn on the sequence obtained using Napore Technologies sequencing. Interestingly, in ten samples, the co-presence of two EcPVs was found. In five samples, three different EcPVs were identified; in three samples, four different EcPVs; in two samples, five different EcPVs; and in a single sample, as many as seven different types of EcPVs were identified. We were able to identify just one EcPV in two samples.

The circular DNA of selected samples underwent enrichment through rolling circle amplification (RCA) protocols. Subsequently, an inverse two-round PCR strategy, employing primers designed to amplify the circular genome, was executed on the RCA products. Successfully, complete genomes were generated for EcPV-6 (ITA/2023/horse/235; ITA/2023/horse/469; ITA/2023/horse/492-1; ITA/2023/horse/139), EcPV-4 (ITA/2021/horse/322-1; ITA/2022/horse/405; ITA/2023/horse/47), and EcPV-3 (ITA/2023/horse/47; ITA/2023/horse/492-1), exhibiting a 99.9% nucleotide similarity to NC_020500, NC_017862, and NC_020085, respectively. Additionally, entire genomes (~7.5 kb) of 7 uncharacterized PVs were assembled. BLAST investigation revealed that the novel PVs in the L1 gene exhibited the highest similarity to EcPV-1 (64% nucleotide identity), EaPV-2 (59.1-61.5% nucleotide identity), and EcPV-6 (57.8-66.9% nucleotide identity). Classification of different PV species was carried out based on ICTV categorization criteria and the PapillomaVirus Episteme (PaVE, NIH) database. The newly identified EcPV genotypes from this study were tentatively designated as EcPV-11, EcPV-12, EcPV-13, EcPV-14, and EcPV-15. Additionally, besides the late structural genes L1 and L2, the early genes E1, E2, E4, E6, and E7 were also identified.
EcPV-1, -2, -5, -7, -8, -9 and -10 were not identified in our investigation.
The examination of nucleotide sequence identities within the domain of papillomavirus (PV) phylogenetics provides a valuable comprehension of the evolutionary connections and categorization of novel PV species. EcPV13 exhibits a high sequence similarity of 69.92% to EcPV7. This close resemblance in EcPV13 suggests that it may also be a member of the *Dyorhopapillomavirus* genus or a closely related genus within the same clade, as EcPV7 is classified under the *Dyorhopapillomavirus* genus. The high identity between these PVs suggests a potential recent common ancestor or significant evolutionary conservation.
Compared to EcPV5, EcPV15 demonstrates a 63.11% identity. EcPV15's classification within the *Dyoiotapapillomavirus* genus is substantiated by this similarity. Despite the fact that the percentage of identity between EcPV13 and EcPV7 is higher than that of EcPV15, EcPV15 is still classified within a specific genus. The identity value is instrumental in verifying the genus placement and implies that EcPV15 is evolutionarily related to other members of the *Dyoiotapapillomavirus* genus.
The sequences for EcPV12, EcPV11, and EcPV14 are suggested as novel PV species within new PV genera. The unique genetic composition of these sequences, which does not closely align with any known PV sequences, is the basis for their classification as new species. The emergence of new genera for these species suggests that they belong to novel evolutionary lineages that have not been previously classified.
Over the last ten years, there has been a substantial increase in the identification of equine-infecting

PVs. Previously, the only types of papillomaviruses that had been thoroughly examined and comprehended were EcPV1 (Shin-Je et al., 2004) as well as BPV-1 and BPV-2 (Chambers et al., 2003). Papillomaviruses (PVs) were previously postulated to be involved in the pathogenesis of vaginal papillomas, carcinomas, and auditory plaques. Nevertheless, the precise characteristics of PVs, namely EcPV-2 and EcPV3, were not fully examined in samples of these disorders until 2011 (Lange et al., 2011). EcPVs-4 to -7 were first recorded in 2013 (Lange et al., 2013), EcPV8 in 2018 (Linder et al., 2018), EcPV-9 in 2019 (Li et al., 2019), and EcPV-10 in 2023 (Turco et al 2023). In 2013, researchers discovered the existence of BPV-13 in samples collected from Brazil. This strain of virus was shown to be linked to the development of sarcoids, as reported by Lunardi et al. in 2013. In 2014, researchers discovered Equus asinus papillomavirus 1 (EaPV1) in Asinara white donkeys. These donkeys did not exhibit any abnormal growths and the virus seems to have a limited capacity to cause illness (Lecis et al., 2014). However, EcPV-8 was found in horses who had extensive and severe papillomatosis (Linder et al., 2018). Furthermore, EcPV-2 has been associated with an increasing incidence of squamous cell carcinomas (SCCs). The DNA of this organism has been detected in a specific subset of stomach SCCs (Alloway et al., 2020), head SCCs and genital SCCs (Ilaria et al., 2020; Sykora et al., 2017; Tuomisto et al., 2024).

However, no significant association was found when analyzing lesion characteristics (type and distribution), EcPV genotypes, or the co-presence of viral DNA.

5. Conclusions

While aural plaques are a relatively common condition in horses, the exact epidemiology and risk factors are not fully understood. Our investigation identified several novel equine papillomaviruses (EcPV) as a potential cause of these lesions. These novel viruses were phylogenetically or not related to well-known EcPVs.

EcPV13 was closely related to EcPV7, potentially indicating a shared or closely related genus within the *Dyorhopapillomavirus*. EcPV15, based on its similarity to EcPV5, belongs to the *Dyoiotapapillomavirus* genus. EcPV12, EcPV11, and EcPV14 represent novel species within newly identified genera, contributing to the expanding diversity of PV classifications.

These findings highlight the ongoing need for taxonomic refinement in the papillomavirus family and provide a basis for further research into their evolutionary relationships and functional characteristics.

Further research is needed to elucidate the underlying mechanisms and develop effective prevention and treatment strategies. If aural plaques are suspected or if the horse shows signs of discomfort or irritation associated with the lesions, veterinary attention should be sought.

References

Alloway E, Linder K, May S, Rose T, DeLay J, Bender S, Tucker A, Luff J. A Subset of Equine Gastric Squamous Cell Carcinomas Is Associated With Equus Caballus Papillomavirus-2 Infection. Vet Pathol. 2020 May;57(3):427-431. doi: 10.1177/0300985820908797. Epub 2020 Mar 17. PMID: 32180540; PMCID: PMC7335262.

Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010 May 25;401(1):70-9. doi: 10.1016/j.virol.2010.02.002. Epub 2010 Mar 5. PMID: 20206957; PMCID: PMC3400342.

Bromberger CR, Costa JR, Herman M, Hernandez JM, Albertino LG, Alves CEF, Borges AS, Oliveira-Filho JP. Detection of Equus caballus Papillomavirus in Equine Aural Plaque Samples. J Equine Vet Sci. 2023 Sep;128:104877. doi: 10.1016/j.jevs.2023.104877. Epub 2023 Jul 1. PMID: 37399911.

Cappelli K, Ciucis CG, Mecocci S, Nervo T, Crescio MI, Pepe M, Gialletti R, Pietrucci D, Migone LF, Turco S, Mechelli L, Passamonti F, Drago C, Donato GG, Varello K, Modesto P, Chillemi G, Ghelardi A, Razzuoli E. Detection of Equus Caballus Papillomavirus Type-2 in Asymptomatic Italian Horses. Viruses. 2022 Jul 31;14(8):1696. doi: 10.3390/v14081696. PMID: 36016317; PMCID: PMC9412442.

Chambers G, Ellsmore VA, O'Brien PM, Reid SWJ, Love S, Campo MS, Nasir L. Association of bovine papillomavirus with the equine sarcoid. J Gen Virol. 2003 May;84(Pt 5):1055-1062. doi: 10.1099/vir.0.18947-0. PMID: 12692268.

De Paolis L, De Ciucis CG, Peletto S, Cappelli K, Mecocci S, Nervo T, Guardone L, Crescio MI, Pietrucci D, Fruscione F, Gabbianelli F, Turco S, Varello K, Donato GG, Maurella C, Modesto P, Maniaci MG, Chillemi G, Ghelardi A, Razzuoli E. Equus caballus Papillomavirus Type-9 (EcPV9): First Detection in Asymptomatic Italian Horses. Viruses. 2022 Sep 15;14(9):2050. doi: 10.3390/v14092050. PMID: 36146856; PMCID: PMC9504741.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology. 2004 Jun 20;324(1):17-27. doi: 10.1016/j.virol.2004.03.033. PMID: 15183049.

Dolz G, Lecis R, Solórzano-Morales A, Aguilar-Vargas F, Solórzano-Scott T, Peña R, Zobba R, Tore G, Pittau M, Alberti A. Leopardus wiedii Papillomavirus type 1, a novel papillomavirus species in the tree ocelot, suggests Felidae Lambdapapillomavirus polyphyletic origin and host-independent evolution. Infect Genet Evol. 2020 Jul;81:104239. doi: 10.1016/j.meegid.2020.104239. Epub 2020 Feb 11. PMID: 32058075.

Dong J, Zhu W, Yamashita N, Chambers JK, Uchida K, Kuwano A, Haga T. Isolation of equine papillomavirus type 1 from racing horse in Japan. J Vet Med Sci. 2017 Dec 6;79(12):1957-1959. doi: 10.1292/jvms.17-0322. Epub 2017 Oct 8. PMID: 28993549; PMCID: PMC5745171.

Doorbar J. The E4 protein; structure, function and patterns of expression. Virology. 2013 Oct;445(1-2):80-98. doi: 10.1016/j.virol.2013.07.008. Epub 2013 Sep 7. PMID: 24016539.

Fairley RA, Haines DM. The electron microscopic and immunohistochemical demonstration of a papillomavirus in equine aural plaques. Vet Pathol. 1992 Jan;29(1):79-81. doi: 10.1177/030098589202900110. PMID: 1313614.

Felsenstein J. CONFIDENCE LIMITS ON PHYLOGENIES: AN APPROACH USING THE BOOTSTRAP. Evolution. 1985 Jul;39(4):783-791. doi: 10.1111/j.1558-5646.1985.tb00420.x. PMID: 28561359.

Forslund O, Antonsson A, Nordin P, Stenquist B, Göran Hansson B. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol. 1999 Sep;80 (Pt 9):2437-2443. doi: 10.1099/0022-1317-80-9-2437. PMID: 10501499.

García-Vallvé S, Alonso A, Bravo IG. Papillomaviruses: different genes have different histories. Trends Microbiol. 2005 Nov;13(11):514-21. doi: 10.1016/j.tim.2005.09.003. Epub 2005 Sep 21. PMID: 16181783.

Gottschling M, Göker M, Stamatakis A, Bininda-Emonds OR, Nindl I, Bravo IG. Quantifying the phylodynamic forces driving papillomavirus evolution. Mol Biol Evol. 2011 Jul;28(7):2101-13. doi: 10.1093/molbev/msr030. Epub 2011 Jan 31. PMID: 21285031.

Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ. Improved amplification of genital human papillomaviruses. J Clin Microbiol. 2000 Jan;38(1):357-61. doi: 10.1128/JCM.38.1.357-361.2000. PMID: 10618116; PMCID: PMC88724.

Hainisch EK, Jindra C, Kirnbauer R, Brandt S. Papillomavirus-like Particles in Equine Medicine. Viruses. 2023 Jan 25;15(2):345. doi: 10.3390/v15020345. PMID: 36851559; PMCID: PMC9966523.

Hibi H, Hatama S, Obata A, Shibahara T, Kadota K. Laryngeal squamous cell carcinoma and papilloma associated with Equus caballus papillomavirus 2 in a horse. J Vet Med Sci. 2019 Jul 19;81(7):1029-1033. doi: 10.1292/jvms.18-0461. Epub 2019 Jun 4. PMID: 31167980; PMCID: PMC6656819.

Iftner T, Villa LL. Chapter 12: Human papillomavirus technologies. J Natl Cancer Inst Monogr. 2003;(31):80-8. doi: 10.1093/oxfordjournals.jncimonographs.a003487. PMID: 12807950.

Porcellato I, Modesto P, Cappelli K, Varello K, Peletto S, Brachelente C, Martini I, Mechelli L, Ferrari A, Ghelardi A, Razzuoli E. Equus caballus papillomavirus type 2 (EcPV2) in co-occurring vulvar and gastric lesions of a pony. Res Vet Sci. 2020 Oct;132:167-171. doi: 10.1016/j.rvsc.2020.06.003. Epub 2020 Jun 5. PMID: 32590224.

Johne R, Müller H, Rector A, van Ranst M, Stevens H. Rolling-circle amplification of viral DNA genomes using phi29 polymerase. Trends Microbiol. 2009 May;17(5):205-11. doi: 10.1016/j.tim.2009.02.004. Epub 2009 Apr 15. PMID: 19375325.

Jones, S. E. (2022). Papillomaviruses in equids: A decade of discovery and more to come? Equine Veterinary Education, 34(5), 236-240. https://doi.org/https://doi.org/10.1111/eve.13506

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis

across Computing Platforms. Mol Biol Evol. 2018 Jun 1;35(6):1547-1549. doi: 10.1093/molbev/msy096. PMID: 29722887; PMCID: PMC5967553.

Lange CE, Tobler K, Ackermann M, Favrot C. Identification of two novel equine papillomavirus sequences suggests three genera in one cluster. Vet Microbiol. 2011 Apr 21;149(1-2):85-90. doi: 10.1016/j.vetmic.2010.10.019. Epub 2010 Nov 4. PMID: 21109367.

Lange CE, Vetsch E, Ackermann M, Favrot C, Tobler K. Four novel papillomavirus sequences support a broad diversity among equine papillomaviruses. J Gen Virol. 2013 Jun;94(Pt 6):1365-1372. doi: 10.1099/vir.0.052092-0. Epub 2013 Mar 13. PMID: 23486670.

Le SQ, Gascuel O. An improved general amino acid replacement matrix. Mol Biol Evol. 2008 Jul;25(7):1307-20. doi: 10.1093/molbev/msn067. Epub 2008 Mar 26. PMID: 18367465.

Lecis R, Tore G, Scagliarini A, Antuofermo E, Dedola C, Cacciotto C, Dore GM, Coradduzza E, Gallina L, Battilani M, Anfossi AG, Muzzeddu M, Chessa B, Pittau M, Alberti A. Equus asinus papillomavirus (EaPV1) provides new insights into equine papillomavirus diversity. Vet Microbiol. 2014 Jun 4;170(3-4):213-23. doi: 10.1016/j.vetmic.2014.02.016. Epub 2014 Feb 16. PMID: 24636161.

Li CX, Chang WS, Mitsakos K, Rodger J, Holmes EC, Hudson BJ. Identification of a Novel Equine Papillomavirus in Semen from a Thoroughbred Stallion with a Penile Lesion. Viruses. 2019 Aug 4;11(8):713. doi: 10.3390/v11080713. PMID: 31382657; PMCID: PMC6723834.

Linder KE, Bizikova P, Luff J, Zhou D, Yuan H, Breuhaus B, Nelson E, Mackay R. Generalized papillomatosis in three horses associated with a novel equine papillomavirus (EcPV8). Vet Dermatol. 2018 Feb;29(1):72-e30. doi: 10.1111/vde.12481. Epub 2017 Aug 22. PMID: 28833761.

Mira J, Herman M, Zakia LS, Olivo G, Araujo JP Jr, Borges AS, Oliveira-Filho JP. Factors associated with equine aural plaque in Brazil. Vet Dermatol. 2016 Oct;27(5):408-e104. doi: 10.1111/vde.12360. Epub 2016 Jul 14. PMID: 27417263.

Mira J, Herman M, Zakia LS, Olivo G, Araújo JP Jr, Borges AS, Oliveira-Filho JP. Frequency of Equus caballus papillomavirus in equine aural plaques. J Vet Diagn Invest. 2018 Jul;30(4):565-568. doi: 10.1177/1040638717753495. Epub 2018 Mar 30. PMID: 29601778; PMCID: PMC6505913.

Munday JS, Grant K, Orbell G, Vaatstra BL. Cutaneous plaques associated with a putative novel papillomavirus type in a horse. N Z Vet J. 2023 Mar;71(2):100-105. doi: 10.1080/00480169.2022.2157347. Epub 2023 Jan 12. PMID: 36484093.

Munday JS, Knight CG, Luff JA. Papillomaviral skin diseases of humans, dogs, cats and horses: A comparative review. Part 1: Papillomavirus biology and hyperplastic lesions. Vet J. 2022 Oct;288:105897. doi: 10.1016/j.tvjl.2022.105897. Epub 2022 Sep 20. PMID: 36150643; PMCID: PMC11494463.

Obayes, A. (2016). Histological Study for Skin of Horse. Tikrit Journal of Pure Science, 21, 2016. https://doi.org/10.25130/tjps.v21i1.944

Ghim SJ, Rector A, Delius H, Sundberg JP, Jenson AB, Van Ranst M. Equine papillomavirus type 1: complete nucleotide sequence and characterization of recombinant virus-like particles composed of the EcPV-1 L1 major capsid protein. Biochem Biophys Res Commun. 2004 Nov 19;324(3):1108-15. doi:

10.1016/j.bbrc.2004.09.154. PMID: 15485669.

Sousa, N., Adorno, V., Marcondes, J., Oliveira-Filho, J., Conceição, L. G., Amorim, R., & Borges, A. (2008). Clinical and histopathological characteristics of the aural plaque in Mangalarga and Quarter Horses. Pesquisa Veterinaria Brasileira, 28, 279-284.

Sykora S, Jindra C, Hofer M, Steinborn R, Brandt S. Equine papillomavirus type 2: An equine equivalent to human papillomavirus 16? Vet J. 2017 Jul;225:3-8. doi: 10.1016/j.tvjl.2017.04.014. Epub 2017 May 3. PMID: 28720295.

Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021 Jun 25;38(7):3022-3027. doi: 10.1093/molbev/msab120. PMID: 33892491; PMCID: PMC8233496.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013 Dec;30(12):2725-9. doi: 10.1093/molbev/mst197. Epub 2013 Oct 16. PMID: 24132122; PMCID: PMC3840312.

Taniwaki SA, Magro AJ, Gorino AC, Oliveira-Filho JP, Fontes MR, Borges AS, Araujo JP Jr. Phylogenetic and structural studies of a novel equine papillomavirus identified from aural plaques. Vet Microbiol. 2013 Feb 22;162(1):85-93. doi: 10.1016/j.vetmic.2012.08.025. Epub 2012 Sep 1. PMID: 22995874.

Tore G, Cacciotto C, Anfossi AG, Dore GM, Antuofermo E, Scagliarini A, Burrai GP, Pau S, Zedda MT, Masala G, Pittau M, Alberti A. Host cell tropism, genome characterization, and evolutionary features of OaPV4, a novel Deltapapillomavirus identified in sheep fibropapilloma. Vet Microbiol. 2017 May;204:151-158. doi: 10.1016/j.vetmic.2017.04.024. Epub 2017 Apr 27. PMID: 28532795.

Torres SM, Koch SN. Papillomavirus-associated diseases. Vet Clin North Am Equine Pract. 2013 Dec;29(3):643-55. doi: 10.1016/j.cveq.2013.08.003. Epub 2013 Oct 1. PMID: 24267681.

Tuomisto L, Virtanen J, Kegler K, Levanov L, Sukura A, Sironen T, Kareskoski M. Equus caballus papillomavirus type 2 (EcPV2)-associated benign penile lesions and squamous cell carcinomas. Vet Med Sci. 2024 Jan;10(1):e1342. doi: 10.1002/vms3.1342. PMID: 38227707; PMCID: PMC10790322.

Turco S, Gabbianelli F, Mavian CN, Pietrucci D, De Paolis L, Gialletti R, Mechelli L, De Ciucis CG, Cappelli K, Dell'Anno F, Mecocci S, Donato GG, Nervo T, Fruscione F, Crescio MI, Ghelardi A, Chillemi G, Razzuoli E. Genetic Characterization of a Novel Equus caballus Papillomavirus Isolated from a Thoroughbred Mare. Viruses. 2023 Feb 28;15(3):650. doi: 10.3390/v15030650. PMID: 36992359; PMCID: PMC10059215.

Van Den Top JG, Ensink JM, Gröne A, Klein WR, Barneveld A, Van Weeren PR. Penile and preputial tumours in the horse: literature review and proposal of a standardised approach. Equine Vet J. 2010 Nov;42(8):746-57. doi: 10.1111/j.2042-3306.2010.00290.x. PMID: 21039806.

Van Doorslaer K. Evolution of the papillomaviridae. Virology. 2013 Oct;445(1-2):11-20. doi: 10.1016/j.virol.2013.05.012. Epub 2013 Jun 14. PMID: 23769415.

Van Doorslaer K, Chen Z, Bernard HU, Chan PKS, DeSalle R, Dillner J, Forslund O, Haga T,

McBride AA, Villa LL, Burk RD, Ictv Report Consortium. ICTV Virus Taxonomy Profile: Papillomaviridae. J Gen Virol. 2018 Aug;99(8):989-990. doi: 10.1099/jgv.0.001105. Epub 2018 Jun 21. PMID: 29927370; PMCID: PMC6171710.

Van Doorslaer K, Tan Q, Xirasagar S, Bandaru S, Gopalan V, Mohamoud Y, Huyen Y, McBride AA. The Papillomavirus Episteme: a central resource for papillomavirus sequence data and analysis. Nucleic Acids Res. 2013 Jan;41(Database issue):D571-8. doi: 10.1093/nar/gks984. Epub 2012 Oct 23. PMID: 23093593; PMCID: PMC3531071.

Wu L, Liu X, Schadt CW, Zhou J. Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. Appl Environ Microbiol. 2006 Jul;72(7):4931-41. doi: 10.1128/AEM.02738-05. PMID: 16820490; PMCID: PMC1489353.

Xia X, Xie Z. DAMBE: software package for data analysis in molecular biology and evolution. J Hered. 2001 Jul-Aug;92(4):371-3. doi: 10.1093/jhered/92.4.371. PMID: 11535656.

Table 1: List of oligonucleotides used in the screening for pan papillomavirus

Primer	Primer Sequnece(5'-3')	Product (bp)	Gene target	Reference
FAP59 (For)	TGACGGTDGGMMAYCCHTWYT	500	L1	(Forslund et al., 1999)
FAP64 (ReV)	CCTATVTCMMRCATRTSMCCRTC			
MY09 (For)	CGTCCMARRGGAWACTGATC	450	L1	(Forslund et al., 1999)
MY11 (ReV)	GCMCAGGGWCATAAYAATGG			
CP4 (For)	ATGGTACARTGGGCATWTGA	458	E1	(Gravitt et al., 2000)
CP5 (ReV)	GAGGYTGCAACCAAAAMTGRCT			

Table 2: List of oligonucleotides used in *quantitative PCR for specific EcPV screening*

Primers and probes	Primer Sequence (5'-3')	Product (bp)	Gene target
EcPV-3 7246 F	CCTTTCACACCTTAACACCA		
EcPV-3 7362 R	GTGGCCTGGGAACTTATATAGCG	117	L1
EcPV-3 7295 Pb	FAM-GATATCGGCCTTGGACAAAGCA-BHQ1		
EcPV-4 6312 F	CCCTGATAGAAACATTCATAATCCGGA		
EcPV-4 6457 R	TTCGGGGGTTTTCTACGTCG	146	L1
EcPV-4 6389 Pb	FAM-AGCCTATTGGAGTGTCAGTCAC-BHQ1		
EcPV-6 7213 F	AATCTAGCACATTTGACCGCTCT		
EcPV-6 7334 R	CACTTGGTGGCCAAAGAGGA	122	L1
EcPV-6 7279 Pb	FAM-GGCACCACCGGACTAGAAAGC-BHQ1		
EcPV-11 738 F	GTCAGGGGATCGGTGCTTC		
EcPV-11 851 R	CCCTTAATGAACAGTTCTGCGG	117	L1
EcPV-11 780Pb	FAM-CTACCTTCGGCATTCATTCACCAG-BHQ1		
EcPV-12 596 F	TAGAGGACGGAGACATGGTGG	117	L1
EcPV-12 699 R	GCTAATAGAATTCACCAGCTCCAGT		

EcPV-12 640 Pb	FAM-TTTGTCAACCTGCAGGAAAGCC-BHQ1		
EcPV-13 7212 F	ACACCAGAAACAGTGACCATTCT		
EcPV-13 7371 R	CTTTTTCAGGAGGTGGGGGTT	117	L1
EcPV-13 7286 Pb	FAM-AGCATCAGGGGGATTAGAAAGTAA-BHQ1		
EcPV-14 6711 F	TAGGACCACCCCCATTGAGG		
EcPV-14 6816 R	CAAAATGTCTAGAGGGGCGCT	117	L1
EcPV-14 6754 Pb	FAM-CTGGGGCAGATGGATTTTGGTAC-BHQ1		
EcPV-15 1010 F	TTAGCATGGACAATCAAGGGCA		
EcPV-15 1114 R	TGCACAGCTGGAAAATAAAACTAAGC	117	L1
EcPV-15 1045 Pb	FAM-CGTAGTAAATTTAATGTCTTCCTGCGC-BHQ1		

Figure 1: Phases of the investigation

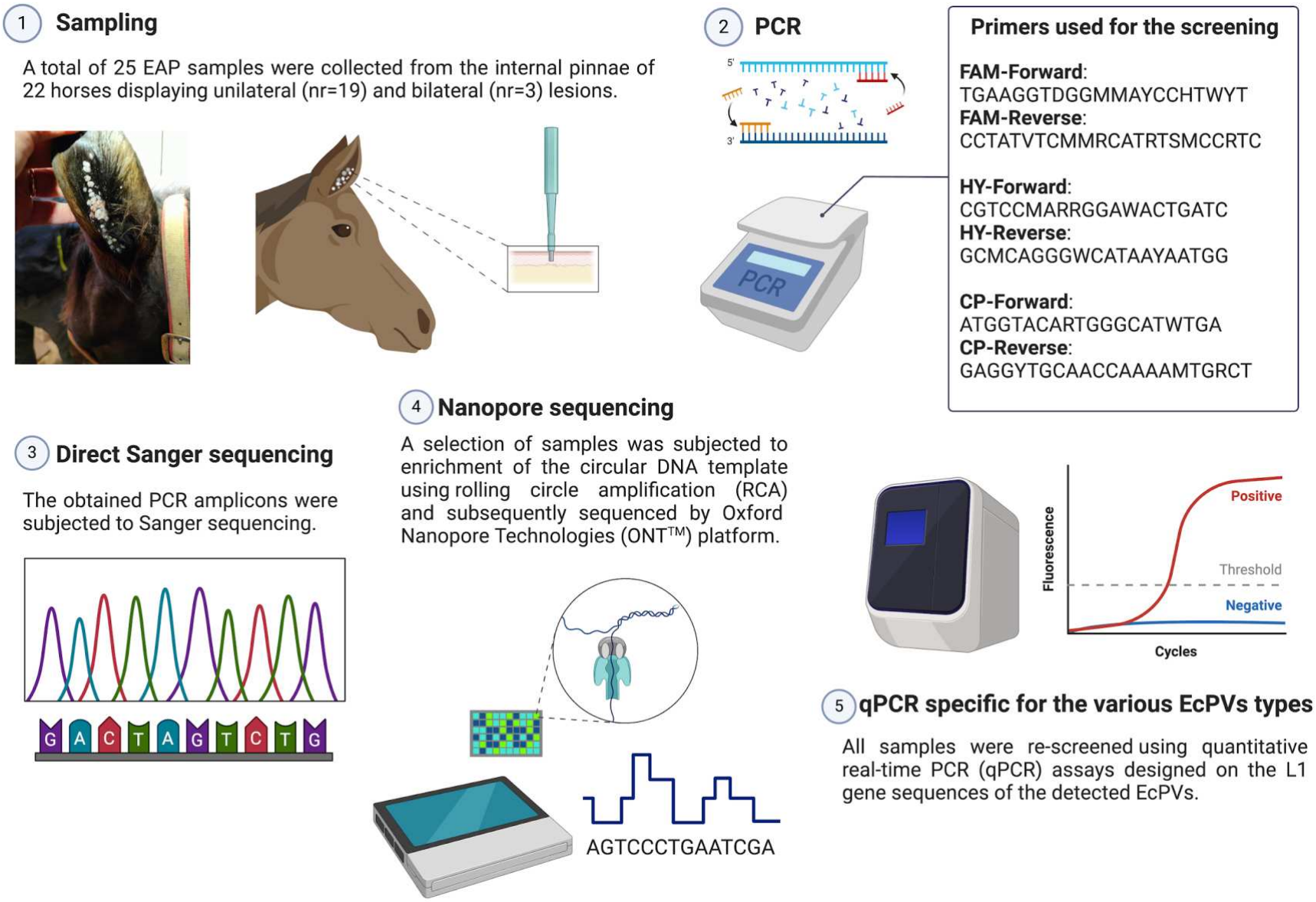


Figure 2: Distribution of EcPV types infection and coinfection of the different samples

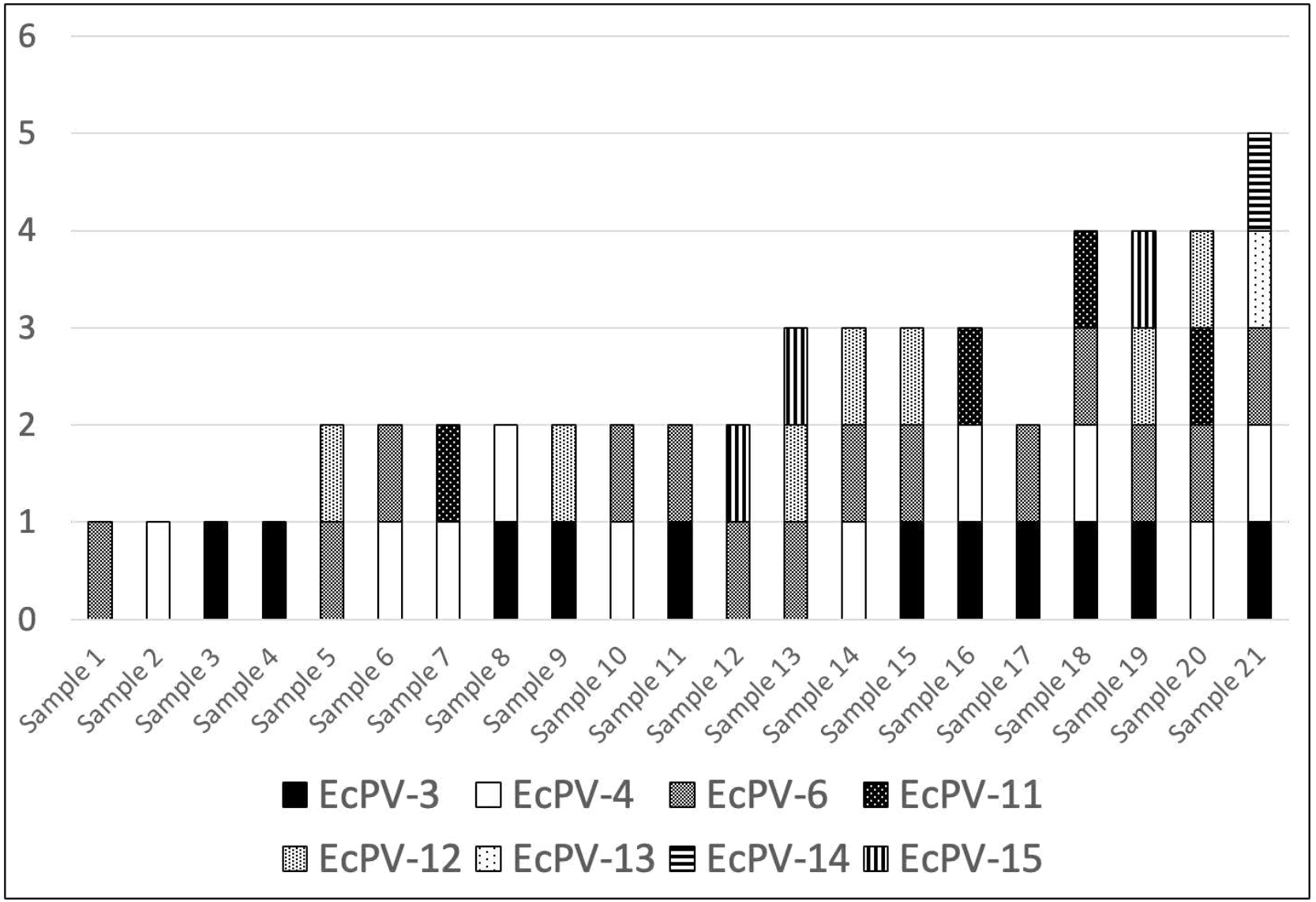


Figure 3: Genome schematic organizations of the novel types of Equus caballus papillomavirus (EcPV) viruses sequenced in this study. According to the International Committee on Taxonomy of Viruses (ICTV) and PapillomaVirus Episteme (PaVE) classification criteria of the sixteen genomes obtained, seven strains were classified into five novels putative EcPV types.

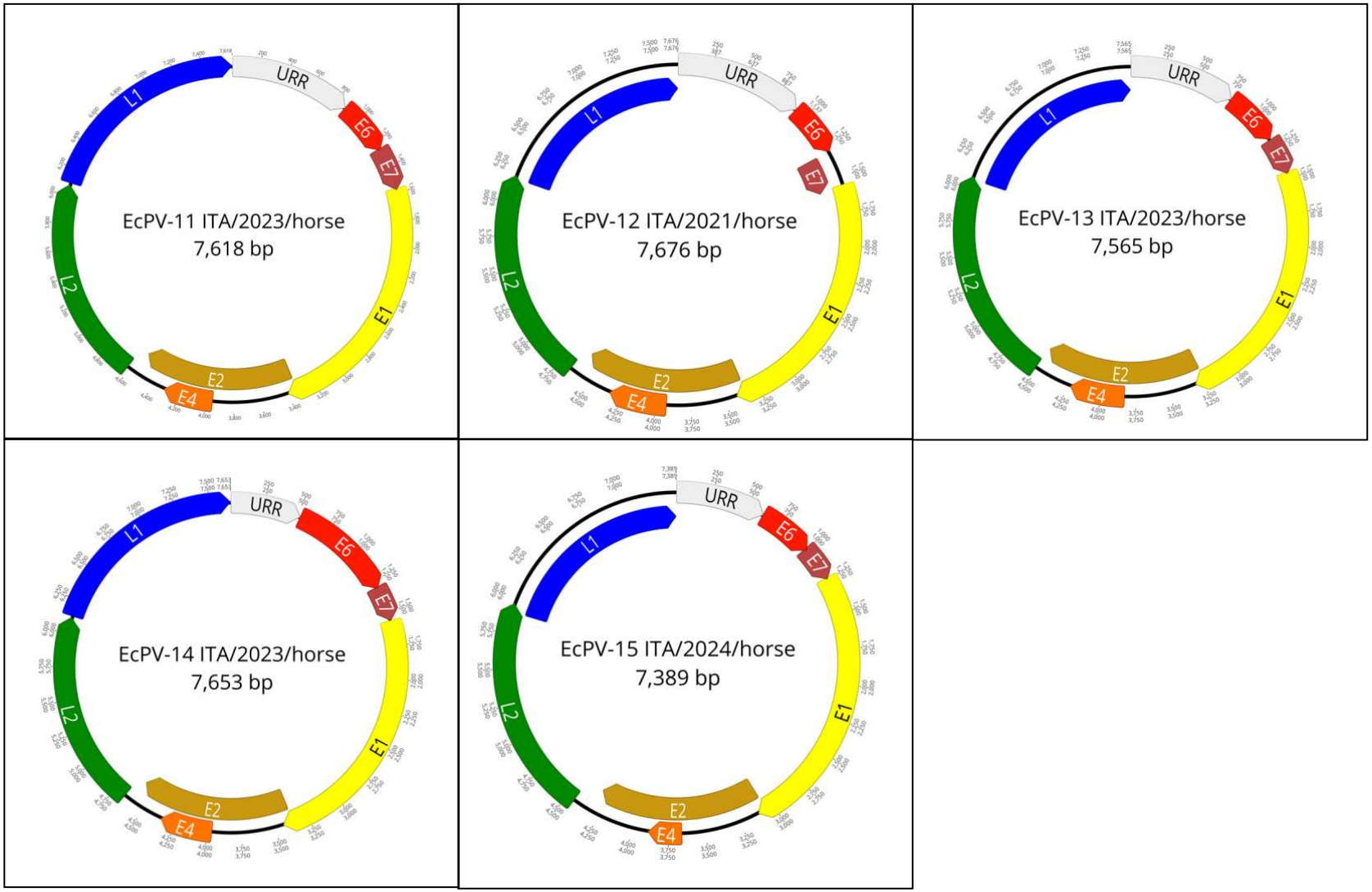
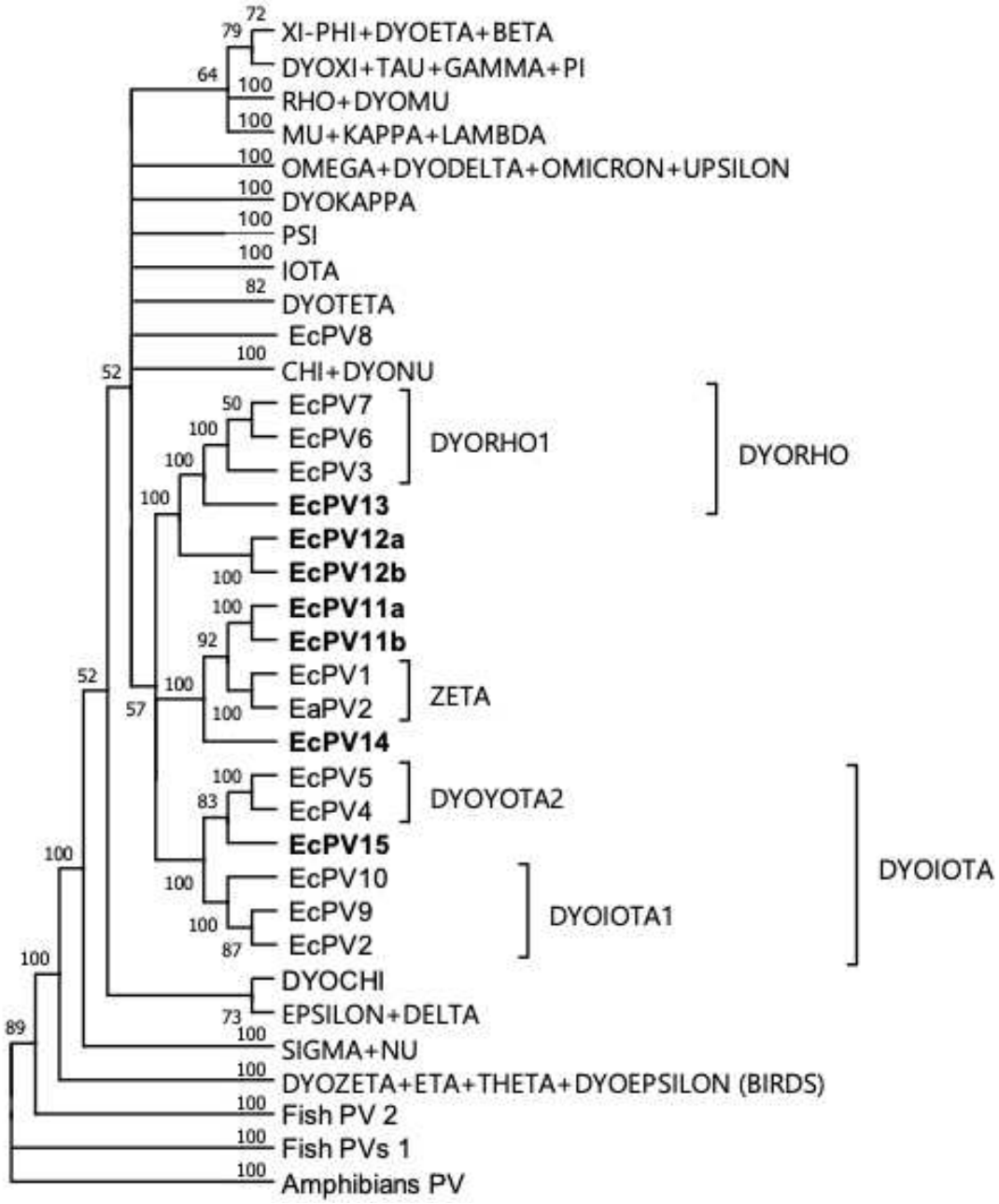


Figure 4: Maximum likelihood tree showing horses PVs diversity and evolutionary history. Equine PVs trees topology is consistent with what observed by using the L1 taxonomy tool in PapillomaVirus Episteme (PaVE).



General discussion and conclusions

A major aim of the present thesis was the description of molecular, epidemiological and phylogenetic patterns of important emerging viruses, such as influenza viruses, rotaviruses, hepadnaviruses and papillomaviruses. In all the above-mentioned studies, we applied the third-generation sequencing platform of Nanopore Oxford Technologies (ONT^{TM}) available in our laboratory, coupled with different strategies for efficient and selective enrichment of viral nucleic acids, based on either sequence-dependent or sequence-independent strategies.

In Chapter 1 there is the description of the reconstruction and characterization of the whole genome sequence (WGS) of an equine influenza A virus (EIAV) strain. The WGS protocol was based on PCR enrichment of the 8 RNA segments of IAV genome (Wüthrich et al., 2019), and the DNA library was subjected to deep sequencing on ONT^{TM} platform. The enrichment protocol takes advantage of conserved motives at the very 5' and 3' end of each RNA segment, used to design a primer with an anchored oligonucleotide used for a single primer amplification.
Before the advent of massive parallel sequencing techniques, genetic and phylogenetic analyses of influenza viruses have long relied on the molecular characterization of the (hemagglutinin) HA and neuraminidase (NA) segments. In the thesis work, we describe the complete genome characterization of the EIAV strain H3N8 ITA/2005/horse/Bari. This virus was identified in 2005 from an outbreak of influenza in horses in Apulia region, Italy, but the origin had remained epidemiologically unexplained. The EIAV strain was related in the H and N genes to the American virus Kentucky/5/02, whose circulation in Europe was first described in 2003 in the United Kingdom (Martella et al., 2007). Characterization of the genetic constellation of the Italian strain revealed a multi-reassortment pattern between the Eurasian lineage and the Florida 2 (FC2) sublineage. Also, the Italian strain seemed related to EIAVs circulating almost in the same years in Poland.

WGS is increasingly used in the study of IAV (Wüthrich et al., 2019), and this is helping understand the evolutionary routes of H3N8 EIAV strains on a global scale (Murcia et al., 2011). Noteworthy, H3N8 EIAVs have also been reported in domestic carnivores, swine, and camels (Daly et al., 2007; Su et al., 2014; Tu et al., 2009; Yondon et al., 2014), likely via inter-species transmission events. Also, WGS has revealed reassortment events between H3N8

EIAV strains and IAVs of non-equine origin (Khan et al., 2017; Langat et al., 2017).
The interplay of antigenic shift (reassortment) and drift (accumulation of punctate mutations) among IAVs results in the onset of novel strains (Wu and Wilson, 2020). Reassortment events are facilitated by natural reservoirs such as swine, birds, and horses (Wu and Wilson, 2020). Generating a large database of IAV genome sequences will surely improve the resolution of epidemiological investigations.

Chapter 2 describes the genome sequencing of a rotavirus A (RVA) strain retrieved from rabbits with enteritis housed in a rabbit breeding farm. The genome of RVAs is prone to evolution, leading to frequent mutations and the emergence of new strains that can efficiently spread among both humans and animals. The mechanisms driving the evolution of RVAs include the accumulation of single nucleotide mutations and reassortment (Cook, 2004; Jain et al., 2014). Insights into the evolution of RVA have been obtained in recent years, due to the increased amount of genome sequence data generated with massive sequencing technologies. This has also required efforts by researchers to improve and maintain a coherent classification system for RVAs.

The main antigenic determinants of RVAs are the G (VP7) and P (VP4) proteins, which are employed to classify rotaviruses into G (glycoprotein) and P (protease-sensitive) genotypes (Omatola and Olaniran, 2022; Caddy et al., 2021; Esona and Gautam, 2015). Currently, a total of 42 G and 58 P genotypes have been reported in both humans and animals worldwide, with over 60 G/P combinations observed in humans, among which the most common ones are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] (Omatola and Olaniran, 2022; Esona and Gautam, 2015; Donato and Bines, 2021). In 2008, the VP7-VP4 binary classification system was integrated into a genetic classification based on all 11 genome segments (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5) (Matthijnssens et al., 2011). Genotype diversity is identified by the notation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x is replaced with an Arabic number starting from 1) (Matthijnssens et al., 2008, 2011). Based on the whole genome sequencing of the 11 segments, RVA is further classified into three genogroups. The Wa-like genogroup of porcine origin, including chiefly G1P[8], G3P[8], G4P[8], G9P[8], and G12P[8] genotype combinations, with genotype-1 backbone constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1) (Heiman et al., 2008; Matthijnssens et al., 2008; Uprety et al., 2021); the DS-1-like genogroup, of bovine origin and found worldwide in human circulation, comprising genotype-2 backbone constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2)

(Matthijnssens et al., 2008; Uprety et al., 2021). Noteworthy, atypical combinations of G1P[8], G3P[8], and G9P[8] bearing a genotype-2 backbone constellation have been documented (Fukuda et al., 2020; Mwangi et al., 2020; Mhango et al., 2022). Finally, the third and less common genogroup is the AU-1-like genogroup, which comprises G3P[9] RVAs combination with a genotype-3 backbone constellation (I3-R3-C3-M3-A3-N3-T3-E3-H3) (Nakagomi et al., 1990; Matthijnssens and Van Ranst, 2012). As of December 2024, the rotavirus classification working group has identified 42G, 58P, 32I, 28R, 24C, 23M, 39A, 28N, 28T, 32E and 28H genotypes, based on the characterization of all the 11 gene segment of rotavirus (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg), demonstrating the huge genetic diversity of RVAs.

In the study described in this thesis, we used a PCR-based enrichment protocol coupled with Nanopore sequencing for reconstructing the complete genome of a rabbit RVA strain, Rabbit-wt/ITA/36-9/2022/G3[P14]. Yet, the PCR-based enrichment protocol takes advantage of conserved motives at the very 5' and 3' ends of RVA genome segments (Faizuloev et al., 2021). The primers are tagged with an oligonucleotide that can be used for a second step of selective PCR enrichment.

Based on the few studies available in the literature on the molecular features of RVA strains from rabbits, lapine RVA strains share the G3-genotype in combination with either P[14] or P[22]. The rabbit RVA strain identified in this study displayed genotype constellation G3-P[14]-I2-R2-C2-M3-A9-N2-T6-E5-H3 conserved among some human and lapine RVA G3P[14] strains. Noteworthy, in all genome segments but the NSP2, strain Rabbit-wt/ITA/36-9/2022/G3[P14] resembled G3P14] RVAs detected in human patients in Belgium (Matthijnssens et al., 2006; Bonica et al., 2015). Interestingly, the NSP2 gene (8^{th} dsRNA segment) of Rabbit-wt/ITA/36-9/2022/G3[P14] was more similar to the RVA strain Human-wt/RUS/Omsk08-442/2008/G3P[9] isolated from a human patient in Russia in 2008 (Zhirakovskaia *et al.,* 2012). On NSP2-based phylogenetic analysis, rabbit RVAs were basal to a group of human G3P[9] RVA strains, suggesting that the NSP2 gene of these human G3P[9] viruses was likely derived by reassortment with an animal strain. For some of these G3P[9] human RVAs, genome data have been generated, revealing an unusual pattern of reassortment in the genome, shared with RVAs of ruminants and cats (De Grazia *et al*., 2010, Grant *et al.,* 2011). These findings remark on the common evolutionary pathways followed by human and animal RVAs (Martella et al., 2010).

In Chapter 3 a multi-disciplinary diagnostic approach, combining clinical, pathological, virological, and serological information was used for the diagnosis of chronic infection by Domestic Cat Hepadnavirus (DCH). This combination of several techniques mirrored the diagnostic approach used for hepatitis B virus (HBV) in human patients. For the detection of HBV and predicting the stage of HBV infection in human patients, antigens, antibodies, and viral genome are profiled/quantified and this information is coupled with haematological tests (Kao, 2008; Huang et al., 2020; Lee & Kim, 2021). Since chronic HBV infection in human patients can evolve across different stages with marked fluctuations in virus activity and even with virus-undetectable phases, combining clinical, bio-chemical, virological, and serological data must be considered as an elective approach for DCH diagnosis.
We were able to obtain WGS of the DCH strain even if the viral load in the liver tissues was negligible. For efficient enrichment of DCH DNA, in this case, we used a rolling cycle amplification (RCA) protocol. This protocol can be primed using either random primers or specific oligonucleotides and can be applied to all viruses with circular DNA and also to small plasmids.

Chapter 4 reported the detection and WGS of novel papillomaviruses (PVs) in horses with aural (ears) plaques. Clinical presentation of PV infection in horses comprises dermal, aural, and genital localization. Among the 10 *Equus caballus* PVs (EcPVs) described to date, EcPV1, 3, 4, 5, 6, and 7 have been associated with aural and genital plaques, with a significant proportion of co-infection (Lange et al., 2011, 2013; Mira et al., 2018; Taniwaki et al., 2013). In the thesis work, aural plaques from 22 horses were screened for the presence of EcPVs using consensus (panviral) PCR protocols. Sequence analysis of the PCR amplicons obtained with the consensus PCR evidenced the presence of EcPV-3, -4 and -6 in several plaques. Moreover, five novel EcPV strains, likely representative of novel species/genus, were identified. To reconstruct the WGS of the novel EcPVs strains, we used enrichment of circular DNA by RCA. The enriched DNA was subsequently ligated and sequenced on ONT™ platform. Also, we used an inverted long PCR with back-to-back primers for re-sequencing of the novel EcPVs. Quantitative PCR assays based on the L1 gene confirmed the presence of novel EcPVs in the samples either alone or in co-infections with other EcPVs, suggesting that these EcPVs are common components of horse skin virome.

As mentioned, even in this case, for efficient enrichment of papillomavirus DNA, we adopted an RCA protocol. To improve sequencing on the ONT™ platform, RCA-enriched DNA was disentangled by random DNA fragmentation using an endonuclease to limit the imbalance of ONT™ membrane pores. This unbiased sequence-independent enrichment strategy was successfully applied to reconstruct the circular DNA genome of five novel EcPVs, showing a remarkable genetic diversity. These findings surely challenge the diagnostics of PVs in horses. For instance, we had to develop at least eight different quantitative assays, covering 8 of the 15 established EcPVs, for specific and sensitive diagnostics in horses. Also, the same sequence-independent strategy could be used for discovery of novel PVs in other animal species.

Virus discovery has been propelled in recent years by the development of novel diagnostic tools, including panviral PCRs and sequence-independent protocols. To improve the sensitivity of PCRs using consensus/degenerated primers, panviral PCR protocols have often been designed using a nested or heminested strategy (Reuter et al., 2018; Koukou et al., 2019, Schibler; et al., 2019, Quan et al., 2010, Drexler et al. 2012; Zinz et al., 2005). The development of pan-viral primers at the family, sub-family or genus level is a powerful tool to generate information on viruses, since the screening of large sample collections can be carried out with a cost-effective strategy. However, this approach comes with several compromises, such as the lower sensitivity of consensus/degenerated primers and the possibility to miss some highly divergent viruses. Moreover, this approach cannot be applied to all virus families, due to their massive genetic diversification hindering primers design.

Sequence-independent Amplification (SIA) PCR-based methods such as Degenerate Oligonucleotide Primed PCR (DOP-PCR) and PCR with consensus primers, have emerged in the last decades (Kricka, 1999, Rose, 2005, Haagmans et al., 2009). Moreover, sequence-independent single-primer amplification (SISPA), Virus Discovery cDNA Amplified Fragment Length Polymorphism Analysis (VIDISCA), random PCR and rolling circle amplification (RCA), were also developed to amplify viral genetic materials for cloning and sequencing (Reyes and Kim, 1991, Delwart, 2007, Pyrc et al., 2008, Thurber et al., 2009, Bexfield and Kellam, 2011). Extensive use of these viral metagenomic techniques, has led to the discovery of different viruses (Barzon et al., 2011,Chiu, 2013, Schelhorn, 2013, Boheemen, 2014).

Although, PCR-based methods were tolerant to considerable sequence variation, they lacked sensitivity and were still critically dependent on pre-existing sequence information of the virus

genera/family being investigated. This method could only amplify small fractions of viral genome, which were sometimes not enough for further analysis (Datta et al., 2015). Moreover, although these techniques allow the generation of enough nucleic acid material for sequencing, their main disadvantages remain that they can be used on samples containing only genetically uniform microbial populations, and they can distort quantitative analyses by introducing amplification bias in viral diversity studies. As a consequence, quantitative analyses of the composition of resulting viromes may not reflect the true proportions (Datta et al., 2015).

In conclusion, the results of the studies covered by this thesis demonstrate the importance of integration between novel molecular technologies, bioinformatics, and interdisciplinary collaborations. The combinations of these innovations are contributing to the evolution and improvements of virological research thus helping in anticipating pandemics, addressing global health challenges, and safeguarding the "One Health" medicine.

References

Aghazadeh M, Shi M, Barrs VR, McLuckie AJ, Lindsay SA, Jameson B, Hampson B, Holmes EC, Beatty JA. A Novel Hepadnavirus Identified in an Immunocompromised Domestic Cat in Australia. Viruses. 2018 May 17;10(5):269. doi: 10.3390/v10050269. PMID: 29772771; PMCID: PMC5977262.

Anpuanandam K, Selvarajah GT, Choy MMK, Ng SW, Kumar K, Ali RM, Rajendran SK, Ho KL, Tan WS. Molecular detection and characterisation of Domestic Cat Hepadnavirus (DCH) from blood and liver tissues of cats in Malaysia. BMC Vet Res. 2021 Jan 6;17(1):9. doi: 10.1186/s12917-020-02700-0. PMID: 33407487; PMCID: PMC7788742.

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, 7861–7884.

Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, Webster RG. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J Virol. 1992 Feb;66(2):1129-38. doi: 10.1128/JVI.66.2.1129-1138.1992. PMID: 1731092; PMCID: PMC240817.

Bexfield, N., Kellam, P., 2011. Metagenomics and the molecular identification of novel viruses. The Veterinary Journal 190, 191–198.

Boheemen, S.V.,2014. Virus Discovery and Characterization using Next Generation Sequencing. PhD Dissertation Erasmus Medical Center, Rotterdam.

Bonica, M. B., Zeller, M., Van Ranst, M., Matthijnssens, J., & Heylen, E. (2015). Complete genome analysis of a rabbit rotavirus causing gastroenteritis in a human infant. Viruses, 7(2), 844–856.

Caddy S., Papa G., Borodavka A., Desselberger U. Rotavirus research: 2014–2020. Virus Res. 2021; 304:198499. doi: 10.1016/j.virusres.2021.198499.

Capozza P, Carrai M, Choi YR, Tu T, Nekouei O, Lanave G, Martella V, Beatty JA, Barrs VR. Domestic Cat Hepadnavirus: Molecular Epidemiology and Phylogeny in Cats in Hong Kong. Viruses. 2023 Jan 3;15(1):150. doi: 10.3390/v15010150. PMID: 36680190; PMCID: PMC9865086.

Capozza P, Decaro N, Beikpour F, Buonavoglia C, Martella V. Emerging Hepatotropic Viruses in Cats: A Brief Review. Viruses. 2021 Jun 17;13(6):1162. doi: 10.3390/v13061162. PMID: 34204394; PMCID: PMC8233973.

Capozza P, Lanave G, Diakoudi G, Stasi F, Ghergo P, Ricci D, Santo G, Arena G, Grillo I, Delle Donne E, Di Lisio F, Zini E, Callegari C, Valente L, Camero M, Di Martino B, Beatty J, Barrs VR, Buonavoglia C, Martella V. A longitudinal observational study in two cats naturally-infected with hepadnavirus. Vet Microbiol. 2021 Mar;254:108999. doi: 10.1016/j.vetmic.2021.108999. Epub 2021 Jan 22. PMID: 33524809.

Chiu, C.Y.,2013. Viral pathogen discovery. Current Opinion in Microbiology 16, 468-478.

Cook, N. (2004). The zoonotic potential of rotavirus. J Infec 48, 289–302. doi: 10.1016/j.jinf.2004.01.018

Daly JM, Sindle T, Tearle J, Barquero N, Newton JR, Corning S. Equine influenza vaccine containing older H3N8 strains offers protection against A/eq/South Africa/4/03 (H3N8) strain in a short-term vaccine efficacy study. Equine Vet J. 2007 Sep;39(5):446-50. doi: 10.2746/042516407x180327. PMID: 17910270.

Datta, S., Budhauliya, R., Das, B., Chatterjee, S., Vanlalhmuaka, Veer V., 2015. Next-generation sequencing in clinical virology: Discovery of new viruses. World journal of virology 4, 265–276.

De Grazia S, Giammanco GM, Potgieter CA, Matthijnssens J, Banyai K, Platia MA, Colomba C, Martella V. Unusual assortment of segments in 2 rare human rotavirus genomes. Emerg Infect Dis. 2010 May;16(5):859-62. doi: 10.3201/eid1605.091826. PMID: 20409385; PMCID: PMC2954010.

Delwart, E.L., 2007. Viral metagenomics. Reviews in Medical Virology 17, 115-131.

Donato C.M., Bines J.E. Rotaviruses and Rotavirus Vaccines. Pathogens. 2021;10:959. doi: 10.3390/pathogens10080959.

Drexler, J.F.; Seelen, A.; Corman, V.M.; Fumie Tateno, A.; Cottontail, V.; Melim Zerbinati, R.; Gloza-Rausch, F.; Klose, S.M.; Adu-Sarkodie, Y.; Oppong, S.K.; et al. Bats Worldwide Carry Hepatitis E Virus-Related Viruses That Form a Putative Novel Genus within the Family Hepeviridae. J Virol 2012, 86, 9134–9147, doi:10.1128/JVI.00800-12.

Esona M.D., Gautam R. Rotavirus. Clin. Lab. Med. 2015;35:363–391. doi: 10.1016/j.cll.2015.02.012.

Faizuloev E, Mintaev R, Petrusha O, Marova A, Smirnova D, Ammour Y, Meskina E, Sergeev O, Zhavoronok S, Karaulov A, Svitich O, Zverev V. New approach of genetic characterization of group A rotaviruses by the nanopore sequencing method. J Virol Methods. 2021 Jun; 292:114114. doi: 10.1016/j.jviromet.2021.114114. Epub 2021 Mar 1. PMID: 33662411.

Fattovich, G. (2003). Natural history and prognosis of hepatitis B. Seminars in Liver Disease, 23(1), 47–58. https://doi.org/10.1055/s-2003-37590

Fukuda, S., Tacharoenmuang, R., Guntapong, R., Upachai, S., Singchai, P., Ide, T., et al. (2020). Full genome characterization of novel DS-1-like G9P[8] rotavirus strains that have emerged in Thailand. PLoS One 15:e0231099. doi: 10.1371/journal.pone.0231099

Grant L, Esona M, Gentsch J, Watt J, Reid R, Weatherholtz R, Santosham M, Parashar U, O'Brien K. Detection of G3P[3] and G3P[9] rotavirus strains in American Indian children with evidence of gene reassortment between human and animal rotaviruses. J Med Virol. 2011 Jul;83(7):1288-99. doi: 10.1002/jmv.22076. PMID: 21567432.

Heiman, E. M., McDonald, S. M., Barro, M., Taraporewala, Z. F., Bar-Magen, T., and Patton, J. T.

(2008). Group a human rotavirus genomics: evidence that gene constellations are influenced by viral protein interactions. J. Virol. 82, 11106–11116. doi: 10.1128/JVI.01402-08

Houghton R, Ellis J, Galiano M, Clark TW, Wyllie S. Haemagglutinin and neuraminidase sequencing delineate nosocomial influenza outbreaks with accuracy equivalent to whole genome sequencing. J Infect. 2017 Apr;74(4):377-384. doi: 10.1016/j.jinf.2016.12.015. Epub 2017 Jan 16. PMID: 28104386.

Huang, S. C., Yang, H. C., & Kao, J. H. (2020). Hepatitis B reactivation: Diagnosis and management. Expert Review of Gastroenterology & Hepatology, 14(7), 565–578. https://doi.org/10.1080/17474124.2020

Jain, S., Vashistt, J., and Changotra, H. (2014). Rotaviruses: is their surveillance needed? Vaccine 32, 3367–3378. doi: 10.1016/j.vaccine.2014.04.037

Jeanes EC, Wegg ML, Mitchell JA, Priestnall SL, Fleming L, Dawson C. Comparison of the prevalence of Domestic Cat Hepadnavirus in a population of cats with uveitis and in a healthy blood donor cat population in the United Kingdom. Vet Ophthalmol. 2022 Mar;25(2):165-172. doi: 10.1111/vop.12956. Epub 2021 Nov 22. PMID: 34806802.

Kao, J. H. (2008). Diagnosis of hepatitis B virus infection through serological and virological markers. Expert Review of Gastroenterology & Hepatology, 2(4), 553–562. https://doi.org/10.1586/17474124.2.4.553

Khan A, Mushtaq MH, Ahmad MUD, Nazir J, Farooqi SH, Khan A. Molecular Epidemiology of a novel re-assorted epidemic strain of equine influenza virus in Pakistan in 2015-16. Virus Res. 2017 Aug 15; 240:56-63. doi: 10.1016/j.virusres.2017.07.022. Epub 2017 Jul 27. PMID: 28757141.

Koukou, G.; Niendorf, S.; Hornei, B.; Schlump, J.-U.; Jenke, A.C.; Jacobsen, S. Human Astrovirus Infection Associated with Encephalitis in an Immunocompetent Child: A Case Report. J Med Case Rep 2019, 13, 341, doi:10.1186/s13256-019-2302-6.

Kricka, L.J., 1999. Nucleic acid detection technologies -- labels, strategies, and formats. Clinical Chemistry 45, 453-458.

Lanave G, Capozza P, Diakoudi G, Catella C, Catucci L, Ghergo P, Stasi F, Barrs V, Beatty J, Decaro N, Buonavoglia C, Martella V, Camero M. Identification of hepadnavirus in the sera of cats. Sci Rep. 2019 Jul 23;9(1):10668. doi: 10.1038/s41598-019-47175-8. PMID: 31337847; PMCID: PMC6650429.

Langat P, Raghwani J, Dudas G, Bowden TA, Edwards S, Gall A, Bedford T, Rambaut A, Daniels RS, Russell CA, Pybus OG, McCauley J, Kellam P, Watson SJ. Genome-wide evolutionary dynamics of influenza B viruses on a global scale. PLoS Pathog. 2017 Dec 28;13(12):e1006749. doi: 10.1371/journal.ppat.1006749. PMID: 29284042; PMCID: PMC5790164.

Lange CE, Tobler K, Ackermann M, Favrot C. Identification of two novel equine papillomavirus sequences suggests three genera in one cluster. Vet Microbiol. 2011 Apr 21;149(1-2):85-90. doi: 10.1016/j.vetmic.2010.10.019. Epub 2010 Nov 4. PMID: 21109367.

Lange CE, Vetsch E, Ackermann M, Favrot C, Tobler K. Four novel papillomavirus sequences support a broad diversity among equine papillomaviruses. J Gen Virol. 2013 Jun;94(Pt 6):1365-1372. doi: 10.1099/vir.0.052092-0. Epub 2013 Mar 13. PMID: 23486670.

Lee, J. H., & Kim, H. S. (2021). Current laboratory tests for diagnosis of hepatitis B virus infection. International Journal of Clinical Practice, 75, e14812. https://doi.org/10.1111/ijcp.14812

Liaw, Y. F., & Chu, C. M. (2009). Hepatitis B virus infection. Lancet, 373(9663), 582–592. https://doi.org/10.1016/S0140-6736(09)60207-5Seto et al., 2018

Martella V, Bányai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. Vet Microbiol. 2010 Jan 27;140(3-4):246-55. doi: 10.1016/j.vetmic.2009.08.028. Epub 2009 Aug 28. PMID: 19781872.

Martella V, Elia G, Decaro N, Di Trani L, Lorusso E, Campolo M, Desario C, Parisi A, Cavaliere N, Buonavoglia C. An outbreak of equine influenza virus in vaccinated horses in Italy is due to an H3N8 strain closely related to recent North American representatives of the Florida sub-lineage. Vet Microbiol. 2007 Mar 31;121(1-2):56-63. doi: 10.1016/j.vetmic.2006.11.017. Epub 2006 Nov 25. PMID: 17197135.

Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Bányai K, Estes MK, Gentsch JR, Iturriza-Gómara M, Kirkwood CD, Martella V, Mertens PP, Nakagomi O, Patton JT, Ruggeri FM, Saif LJ, Santos N, Steyer A, Taniguchi K, Desselberger U, Van Ranst M. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol. 2008;153(8):1621-9. doi: 10.1007/s00705-008-0155-1. Epub 2008 Jul 5. PMID: 18604469; PMCID: PMC2556306.

Matthijnssens J., Ciarlet M., McDonald S.M., Attoui H., Bányai K., Brister J.R., Buesa J., Esona M.D., Estes M.K., Gentsch J.R., et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG) Arch. Virol. 2011;156:1397–1413. doi: 10.1007/s00705-011-1006-z.

Matthijnssens J, Rahman M, Martella V, Xuelei Y, De Vos S, De Leener K, Ciarlet M, Buonavoglia C, Van Ranst M. Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. J Virol. 2006 Apr;80(8):3801-10. doi: 10.1128/JVI.80.8.3801-3810.2006. PMID: 16571797; PMCID: PMC1440464.

Matthijnssens, J., and Van Ranst, M. (2012). Genotype constellation and evolution of group a rotaviruses infecting humans. COVIRO 2, 426–433. doi: 10.1016/j.coviro.2012.04.007

Mhango, C., Mandolo, J. J., Chinyama, E., Wachepa, R., Kanjerwa, O., Malamba-Banda, C., et al. (2022). Rotavirus genotypes in hospitalized children with acute gastroenteritis before and after rotavirus vaccine introduction in Blantyre, Malawi, 1997–2019. J. Infect. Dis. 225, 2127–2136. doi: 10.1093/infdis/jiaa616

Mira J, Herman M, Zakia LS, Olivo G, Araújo JP Jr, Borges AS, Oliveira-Filho JP. Frequency of Equus caballus papillomavirus in equine aural plaques. J Vet Diagn Invest. 2018 Jul;30(4):565-568. doi: 10.1177/1040638717753495. Epub 2018 Mar 30. PMID: 29601778; PMCID: PMC6505913.

Murcia PR, Wood JL, Holmes EC. Genome-scale evolution and phylodynamics of equine H3N8 influenza A virus. J Virol. 2011 Jun;85(11):5312-22. doi: 10.1128/JVI.02619-10. Epub 2011 Mar 23. PMID: 21430049; PMCID: PMC3094979.

Mwangi, P. N., Mogotsi, M. T., Rasebotsa, S. P., Seheri, M. L., Mphahlele, M. J., Ndze, V. N., et al. (2020). Uncovering the first atypical DS-1-like G1P[8] rotavirus strains that circulated during pre-rotavirus vaccine introduction era in South Africa. Pathogens 9:391. doi: 10.3390/pathogens9050391

Nakagomi, O., Ohshima, A., Aboudy, Y., Shif, I., Mochizuki, M., Nakagomi, T., et al. (1990). Molecular identification by RNA-RNA hybridization of a human rotavirus that is closely related to rotaviruses of feline and canine origin. J. Clin. Microbiol. 28, 1198–1203. doi: 10.1128/jcm.28.6.1198-1203.1990

Newton JR, Daly JM, Spencer L, Mumford JA. Description of the outbreak of equine influenza (H3N8) in the United Kingdom in 2003, during which recently vaccinated horses in Newmarket developed respiratory disease. Vet Rec. 2006 Feb 11;158(6):185-92. doi: 10.1136/vr.158.6.185. PMID: 16474051.

Omatola C.A., Olaniran A.O. Rotaviruses: From Pathogenesis to Disease Control—A Critical Review.

Viruses. 2022; 14:875. doi: 10.3390/v14050875.

Pagani L, Thomas Y, Huttner B, Sauvan V, Notaridis G, Kaiser L, Iten A, Pittet D, Harbarth S. Transmission and effect of multiple clusters of seasonal influenza in a Swiss geriatric hospital. J Am Geriatr Soc. 2015 Apr;63(4):739-44. doi: 10.1111/jgs.13339. PMID: 25900487; PMCID: PMC7166655.

Piewbang C, Dankaona W, Poonsin P, Yostawonkul J, Lacharoje S, Sirivisoot S, Kasantikul T, Tummaruk P, Techangamsuwan S. Domestic cat hepadnavirus associated with hepatopathy in cats: A retrospective study. J Vet Intern Med. 2022 Sep;36(5):1648-1659. doi: 10.1111/jvim.16525. Epub 2022 Aug 24. PMID: 36054642; PMCID: PMC9511090.

Piewbang C, Wardhani SW, Chaiyasak S, Yostawonkul J, Chai-In P, Boonrungsiman S, Kasantikul T, Techangamsuwan S. Insights into the genetic diversity, recombination, and systemic infections with evidence of intracellular maturation of hepadnavirus in cats. PLoS One. 2020 Oct 23;15(10):e0241212. doi: 10.1371/journal.pone.0241212. PMID: 33095800; PMCID: PMC7584178.

Pyrc, K., Jebbink, M.F., Berkhout, B., van der Hoek, L., 2008. Detection of new viruses by VIDISCA. Virus discovery based on cDNA-amplified fragment length polymorphism. Methods Molecular Biology 454, 73–89.

Quan, P.-L.; Wagner, T.A.; Briese, T.; Torgerson, T.R.; Hornig, M.; Tashmukhamedova, A.; Firth, C.; Palacios, G.; Baisre-De-Leon, A.; Paddock, C.D.; et al. Astrovirus Encephalitis in Boy with XLinked Agammaglobulinemia. Emerg Infect Dis 2010, 16, 918–925, doi:10.3201/eid1606.091536.

Reuter, G.; Pankovics, P.; Boros, Á. Nonsuppurative (Aseptic) Meningoencephalomyelitis Associated with Neurovirulent Astrovirus Infections in Humans and Animals. Clin Microbiol Rev 2018, 31, doi:10.1128/CMR.00040-18.

Reyes, G.R., Kim, J.P., 1991. Sequence-independent, single-primer amplification (SISPA) of complex DNA populations. Molecular Cell Probes 5, 473–481.

Rose, T.M., 2005. CODEHOP-mediated PCR - a powerful technique for the identification and characterization of viral genomes. Virology Journal 2, 20.

Sakamoto H, Ito G, Goto-Koshino Y, Sakamoto M, Nishimura R, Momoi Y. Detection of domestic cat hepadnavirus by next-generation sequencing and epidemiological survey in Japan. J Vet Med Sci. 2023 Jun 13;85(6):642-646. doi: 10.1292/jvms.22-0439. Epub 2023 May 15. PMID: 37183016; PMCID: PMC10315552.

Scavone D, Lauzi S, Stranieri A, Tramontano G, Ratti G, Paltrinieri S. Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. Vet Rec. 2022 Aug;191(4):e1626. doi: 10.1002/vetr.1626. Epub 2022 Apr 8. PMID: 35393638.

Schelhorn, S.E., 2013. Going viral- An integrated view on virological data analysis from basic research to clinical applications. PhD Dissertation., Saarland University, Germany.

Schibler; Brito; Zanella; Zdobnov; Laubscher; L'Huillier; Ambrosioni; Wagner; Posfay-Barbe; Docquier; et al. Viral Sequences Detection by High-Throughput Sequencing in Cerebrospinal Fluid of Individuals with and without Central Nervous System Disease. Genes (Basel) 2019, 10, 625, doi:10.3390/genes10080625.

Shofa M, Kaneko Y, Takahashi K, Okabayashi T, Saito A. Global Prevalence of Domestic Cat Hepadnavirus: An Emerging Threat to Cats' Health? Front Microbiol. 2022 Jun 24; 13:938154. doi: 10.3389/fmicb.2022.938154. PMID: 35814669; PMCID: PMC9263588.

Stone C, Petch R, Gagne RB, Nehring M, Tu T, Beatty JA, VandeWoude S. Prevalence and Genomic

Sequence Analysis of Domestic Cat Hepadnavirus in the United States. Viruses. 2022 Sep 21;14(10):2091. doi: 10.3390/v14102091. PMID: 36298647; PMCID: PMC9607532.

Su S, Wang L, Fu X, He S, Hong M, Zhou P, Lai A, Gray G, Li S. Equine influenza A(H3N8) virus infection in cats. Emerg Infect Dis. 2014 Dec;20(12):2096-9. doi: 10.3201/eid2012.140867. PMID: 25417790; PMCID: PMC4257791

Taniwaki SA, Magro AJ, Gorino AC, Oliveira-Filho JP, Fontes MR, Borges AS, Araujo JP Jr. Phylogenetic and structural studies of a novel equine papillomavirus identified from aural plaques. Vet Microbiol. 2013 Feb 22;162(1):85-93. doi: 10.1016/j.vetmic.2012.08.025. Epub 2012 Sep 1. PMID: 22995874.

Thurber, R.V., Haynes, M., Breitbart, M., Wegley, L., Rohwer, F.,2009. Laboratory procedures to generate viral metagenomes. Nature protocols 4, 470-483.

Tu J, Zhou H, Jiang T, Li C, Zhang A, Guo X, Zou W, Chen H, Jin M. Isolation and molecular characterization of equine H3N8 influenza viruses from pigs in China. Arch Virol. 2009;154(5):887-90. doi: 10.1007/s00705-009-0381-1. Epub 2009 Apr 26. PMID: 19396578.

Yondon M, Zayat B, Nelson MI, Heil GL, Anderson BD, Lin X, Halpin RA, McKenzie PP, White SK, Wentworth DE, Gray GC. Equine influenza A(H3N8) virus isolated from Bactrian camel, Mongolia. Emerg Infect Dis. 2014 Dec;20(12):2144-7. doi: 10.3201/eid2012.140435. PMID: 25418532; PMCID: PMC4257804.

Uprety, T., Wang, D., and Li, F. (2021). Recent advances in rotavirus reverse genetics and its utilization in basic research and vaccine development. Arch. Virol. 166, 2369–2386. doi: 10.1007/s00705-021-05142-7

Wu NC, Wilson IA. Influenza Hemagglutinin Structures and Antibody Recognition. Cold Spring Harb Perspect Med. 2020;10(8). 10.1101/cshperspect.a038778

Wüthrich D, Lang D, Müller NF, Neher RA, Stadler T, Egli A. Evaluation of two workflows for whole genome sequencing-based typing of influenza A viruses. J Virol Methods. 2019 Apr; 266:30-33. doi: 10.1016/j.jviromet.2019.01.009. Epub 2019 Jan 21. PMID: 30677464.

Zhirakovskaia EV, Aksanova RKh, Gorbunova MG, Tikunov AIu, Kuril'shchikov AM, Sokolov SN, Netesov SV, Tikunova NV. [Genetic diversity of group A rotavirus isolates found in Western Siberia in 2007-2011]. Mol Gen Mikrobiol Virusol. 2012;(4):33-41. Russian. PMID: 23248851.

Zintz, C.; Bok, K.; Parada, E.; Barnes-Eley, M.; Berke, T.; Staat, M.A.; Azimi, P.; Jiang, X.; Matson, D.O. Prevalence and Genetic Characterization of Caliciviruses among Children Hospitalized for Acute Gastroenteritis in the United States. Infection, Genetics and Evolution 2005, 5, 281–290, doi: 10.1016/j.meegid.2004.06.010.

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