Genetic characterization of upper respiratory tract virome from nonvaccinated Egyptian cow-calf operations

Abdou Nagy1, Fatma Abdallah1, Hend M. El Damaty2, Ahmed Tariq3, Abdallah M. A. Merwad4, Bader Y. Alhatlani5, Ibrahim Elsohaby2,6,7*

1 Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig City, Sharkia, Egypt, 2 Infectious Diseases, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig City, Sharkia, Egypt, 3 Veterinary, Zagazig City, Sharkia, Egypt, 4 Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig City, Sharkia, Egypt, 5 Department of Medical Sciences, Applied College, Qassim University, Unayzah, Saudi Arabia, 6 Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada, 7 Department of Infectious Diseases and Public Health, Jockey Club of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

* balhatlani@qu.edu.sa (BYA); ielsohaby@upei.ca (IE)

Abstract

Bovine respiratory disease (BRD) is the costliest complex disease affecting the cattle industry worldwide, with significant economic losses. BRD pathogenesis involves several interactions between microorganisms, such as bacteria and viruses, and management factors. The present study aimed to characterize the nasal virome from 43 pooled nasal swab samples collected from Egyptian nonvaccinated cow-calf operations with acute BRD from January to February 2020 using metagenomic sequencing. Bovine herpesvirus-1 (BHV-1), first detection of bovine herpesvirus-5 (BHV-5), and first detection of bovine parvovirus-3 (BPV-3) were the most commonly identified in Egyptian cattle. Moreover, phylogenetic analysis of glycoprotein B revealed that the BHV-1 isolate is closely related to the Cooper reference strain (genotype 1.1), whereas the BHV-5 isolate is closely related to the reference virus GenBank NP_954920.1. In addition, the whole-genome sequence of BPV-3 showed 93.02% nucleotide identity with the reference virus GenBank AF406967.1. In this study, several DNA viruses, such as BHV-1 and first detection BHV-5, and BPV-3, were detected and may have an association with the BRD in cattle in Egypt. Therefore, further research, including investigating more samples from different locations to determine the prevalence of detected viruses and their contributions to BRD in cattle in Egypt, is needed.

Introduction

Bovine respiratory disease (BRD) is one of the most economically important disease affecting the cattle industry worldwide [1]. The cause of BRD is multifactorial, including infectious agents and environmental, management, and host factors. The infectious agents include different viruses, such as bovine herpesvirus-1 (BHV-1), bovine viral diarrhea virus (BVDV), bovine
coronavirus, bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus, bovine adenovirus, influenza D virus (IDV) and bovine rhinitis virus, and bacteria, such as *Mycoplasma bovis*, *Pasteurella multocida*, and *Mannheimia haemolytica* [2]. Despite the widespread use of BRD vaccines and different effective antibiotics, mortalities caused by BRD have steadily increased since the mid-1990s [3, 4]. This indicates that the etiology and pathogenesis of BRD are not fully understood.

Recently, viral metagenomics has been used to characterize the virome associated with complex diseases [5–8]. Viral metagenomics has been used to identify large numbers of known and novel viruses associated with enteric diseases [9, 10] and respiratory diseases [11, 12]. Previous studies on the bovine respiratory tract using metagenomic sequencing showed a significant association between bovine adenovirus-3 (BAdV-3), bovine rhinitis A and B virus, BRSV, BPIV3, BHV-1, BVDV, bovine parvovirus (BPV), IDV, and BRD [7, 11, 13]. BHV-1 and bovine herpesvirus-5 (BHV-5) are DNA viruses that belong to the family *Herpesviridae*. Although BHV-1 causes low mortality, it is responsible for severe economic loss to the cattle industry due to its impact on growth and milk production [14]. BHV-5 causes nonsuppurative meningoencephalitis in young cattle [15].

There are six bovine parvovirus sub-species, and the most significant three sub-species are BPV-1 to BPV-3, which cause diarrhea in neonatal calves and respiratory and reproductive diseases in adult cattle [16]. In addition to these viruses, recent metagenomic studies showed a significant association between IDV and BRD in dairy calves [11] and beef cattle [13].

Egypt has a big cattle industry, with open markets for live-animal trade with different countries, mainly from Europe and Africa. Although the control of certain viruses that may contribute to the complex pathogenesis of BRD, such as BVDV, and infectious bovine rhinotracheitis has been successfully achieved with strict vaccination programs, particularly in large farms, mortality due to BRD has increased [17]. To the authors’ knowledge, no data are available on viruses that contribute to the BRD incidence in cattle in Egypt. Therefore, the purpose of this study was to characterize the virome of cattle with the BRD to identify possible viruses of interest for future investigation in a case-control design.

### Materials and methods

**Ethical approval**

This study was approved by the Zagazig University’s Animal Care and Use Committee (Ref. No. ZU-IACUC/2/F/121/2019). The farm owners provided informed verbal/written consent for the use of clinical samples collected from their animals in the present study.

**Study population**

A total of 43 nasal swabs were collected from eight cow-calf operations in Sharkia (n = 27) and Cairo (n = 16) Governorates. The average number of cattle per herd was 264. The age of cattle at the time of sampling ranged from 1 to 18 months, and the weight ranged from 40 to 450 kg. There were 81.4% (35/43) males and 18.6% (8/43) females. All samples were collected from nonvaccinated cattle. Nasal swab samples were collected only from diseased animals with severe clinical respiratory signs, such as cough, difficulty breathing, and rhinorrhea.

**Sample collection**

Nasal swabs were collected (one per animal) from cattle herds from January to February 2020. All samples were collected from nonvaccinated cattle of various ages, raised in either large intensive and/or small backyard farms. All swabs were collected from naturally infected individuals.
animals exhibiting severe clinical signs of acute respiratory disease (i.e., cough, difficulty breathing, rhinorrhea, and ocular discharge). Swabs were collected by certified veterinarians using sterile cotton swabs followed by insertion into Falcon tube containing 1 ml viral transport medium composed of sterile phosphate-buffered saline, supplemented with 1% penicillin/streptomycin (10,000 U/ml; Gibco, USA). The collected swabs were labelled with herd and animal ID and the collection date and then shipped on ice to the virology laboratory for processing and testing.

**Sample preparation and DNA extraction**

Nasal swabs were vortexed separately and then centrifuged at 2000 rpm for 10 min at 4°C for clarification. The clear supernatant was collected from each processed sample and stored at −20°C until used. Viral nucleic acid was extracted from each clear supernatant using the GeneJET Viral DNA extraction/purification kit (Thermo Fisher, MA, USA) according to the manufacturer’s instructions. The extracted viral DNA was eluted in water-nuclease free. The extracted viral DNAs from all samples (n = 43) were pooled and loaded to the FTA card and sent to the Admera’s Health LLC (South Plainfield, NJ, USA) for library preparation and next-generation sequencing.

**Library preparation and sequencing**

Isolated nucleic acid was quantified with Qubit 2.0 DNA HS Assay (Thermo Fisher) and the quality was assessed by Tapestation Genomic DNA Assay (Agilent Technologies, CA, USA). Library preparation was performed using the NexteraXT library kit (Illumina, CA, USA) according to the manufacturer’s recommendations. The final library quantity was measured by KAPA SYBR® FAST qPCR with QuantStudio® 5 System (Applied Biosystems, CA, USA), and library quality was evaluated by TapeStation HSD1000 ScreenTape (Agilent Technologies). Illumina® 8-nnt dual indices were used. Equimolar pooling of libraries in the same run was performed based on QC values and sequenced on an Illumina® NovaSeq with a read length configuration of 150 paired-end.

**Bioinformatic analysis**

Data were analyzed according to a customized pipeline. In brief, raw FASTQ files were initially checked for quality control using FastQC software version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). In brief, quality of reads were assessed for; per base sequence quality, per tile sequence quality, per base sequence content, per base N content, per sequence quality scores, per sequence GC content, levels of sequence duplications, overrepresented sequences, sequence length distribution and adapter content. High-quality files were imported into Geneious Prime 2020 (Biomatters Ltd., New Zealand), and all reads were first trimmed using default settings. In brief, BBDuk was used with the options checked to “Trim Adapters” (all Trueseq, Nextera, and PhiX adapters), trim low quality (minimum quality of 25), discard short reads (minimum length of 40 bp), and keep original order. Duplicates were removed after trimming using sequence - > Remove duplicate reads.

The processed/trimmed reads were then mapped to the host reference genome (Bos taurus, PRJNA33843) and the unmapped reads were subjected to de novo assembly into contigs using Velvet with default settings. Consequences sequences (150–200 bp) were extracted from contigs and analyzed against the nucleotide database of NCBI using BLASTN tool. Viral genomes with very low expectation value (E-value ~ 10^-4) were used as reference viruses for reads mapping using Geneious Prime mapper default settings with the options Medium Sensitivity/Fast Sensitivity setting that were modified by selecting the Custom Sensitivity, and Fine Tuning
was set to iterate up to five times. A consensus sequence was constructed based on the highest quality threshold and extracted using “Tools” -> “Generate consensus sequence,” calling “?” (base or gap) in the absence of coverage. BLAST tool was used to confirm the specificity of the reads aligned to each reference virus using the Megablast algorithm against the nonredundant nucleotide database of GenBank, EMBL, DDBJ, PDB, and RefSeq. The matching region of the best hit per read was retrieved. All original fastaQC files were submitted to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI). Open Reading Frame (ORF) finder algorithm embedded in Geneious Prime was used to detect ORFs from the identified viruses.

**Phylogenetic analysis**
Nucleotide sequences from genetically related viruses for BHV-1, BHV-5 and BPV-3 were downloaded from the NCBI database and aligned separately using MUSCLE [18] embedded in MEGA X with the default settings [19]. The best-fitted model of evolution was identified for each alignment using the Bayesian Information Criterion in MEGA X, and neighbor-joining trees with model/method “Maximum Composite Likelihood” were reconstructed using 1000 bootstrap replicates to evaluate the strength of branching [19].

**Results**

**Samples collection and processing**
Samples were collected from different cattle herds located at Cairo and Sharkia Governorates. Details of the locations of the herds where samples were collected are shown in Fig 1. Additionally, details about diseased animals, such as age, breed, type of the ration provided, the antimicrobials used, type of the farm rearing system are presented in (S1 Table). All samples were processed and the viral DNA was extracted (30 μl/sample), pooled (10 μl/sample). A volume of 100 μl from the final pooled viral DNAs was uploaded to one FTA card that was shipped for library preparation and next-generation sequencing.

**Virome of bovine nasal swabs**
A total of 56 million raw reads generated by the sequencing machine were submitted to the SRA under the Bioproject number PRJNA702539. Following de novo assembly, six DNA viruses were identified (Table 1). For the best blast hits, reference genomes of the identified viruses were downloaded from GenBank database and the final processed/trimmed reads (n = 8 millions) were mapped against them. All alignments were inspected manually with a threshold of minimum 20 processed/trimmed reads mapped to different regions of the reference viral genome was the virus considered detected.

Additionally, other viruses such as bovine adeno-associated virus, bovine torovirus, bovine nidovirus, and bovine hokovirus 2 were identified. However, due to the small number of reads (<20) mapped against these viruses and short length of contigs, they were considered as false-positive and so, they were excluded from further analysis.

**Bovine herpesviruses**
A total of 5958 and 4523 processed/trimmed reads were mapped to the reference viruses; BHV-1 (GenBank AJ004801.1) and BHV-5 (GenBank KY59403.1), respectively. For BHV-1, analysis of the viral glycoproteins, such as glycoprotein B (gB), showed 99.57% nucleotide identity with other BHV-1 viruses, such as the Cooper reference strain, which was isolated from USA. In addition, the study isolate (called BHV-1/Cattle/Egypt/2020) was clustered with
other BHV-1 viruses, such as the Cooper reference strain, BHV-1 isolate C18, C26, C29, C33 and C36 that have been collected from the USA (Fig 2). Moreover, 20 different genes from BHV-1/Cattle/Egypt/2020isolate showed high coverage (98–99%) when mapped to the reference BHV-1 viruses. The 20 characterized genes from BHV-1/Cattle/Egypt/2020 isolate were deposited to the NCBI database (GenBank MW805254-MW805273). On the other hand,

Fig 1. A map of herd locations in Egypt from which animal’s samples were collected.

https://doi.org/10.1371/journal.pone.0267036.g001

Table 1. Summary of the identified DNA viruses.

| Reference virus | Family         | GenBank accession number | Genome size (bp) | No. positive reads | Largest contig size (bp) | Further analysis |
|-----------------|----------------|-------------------------|------------------|---------------------|-------------------------|-----------------|
| BHV-1           | Herpesviridae  | AJ004801.1              | 135,301          | 5958                | 2523                    | Yes             |
| BHV-5           | Herpesviridae  | KY559403.1              | 137,740          | 4523                | 2661                    | Yes             |
| MdSGHV          | Hytrosaviridae | EU522111.1              | 124,279          | 1686                | 71                      | No              |
| BPV-3           | Paroviridae    | AF406967.1              | 5276             | 593                 | 1283                    | Yes             |
| BAdV-3          | Adenoviridae   | AF030154.1              | 34,446           | 26                  | 46                      | No              |

1 BHV-1: Bovine herpesvirus 1; BHV-5: Bovine herpesvirus 5; MdSGHV: Musca domestica salivary gland hypertrophy virus; BPV-3: Bovine parvovirus 3; BAdV-3: Bovine adenovirus

2 GenBank numbers are for reference virus genomes used for processed/trimmed reads mapping.

3 Samples with high coverage (~98%) and long contigs against the reference virus genomes were further investigated for annotation and phylogenetic analysis.

https://doi.org/10.1371/journal.pone.0267036.t001
analysis of gB from the BHV-5 isolate (called BHV-5/Cattle/Egypt/2020; GenBank MW805274) showed 98.28% nucleotide identity with reference BHV-5 viruses (GenBank KU KY559403.1 and NC_005261.3) and was clustered with the BHV-5 isolate 166–84 and BHV-5 isolate A663 that have been isolated from Argentina (Fig 3).

BPV-3

A total of 593 processed/trimmed reads were mapped against the reference BPV-3 virus (GenBank AF406967.1). The alignment of the whole-genome sequence of the study isolate (called BPV-3/Cattle/Egypt/2020) showed 93.02% nucleotide identity with the BPV-3 reference strain (GenBank AF406967.1), which was isolated from the USA and 91.75% nucleotide identity with the Ronda Alta isolate (GenBank MG745680.1), which was isolated from Brazil. Additionally, two main open reading frames (ORFs) were detected: (1) ORF1 is the (NS) protein starting from the nucleotide position 264 to 2222 with 97.24% amino acid identity with the reference BPV-3 virus (GenBank AAL09673.1) and (2) ORF2 is the capsid protein starting from the nucleotide position 2171 to 5068 with 97.89% amino acid identity with the reference BPV-
3 virus (GenBank AAL09674.1). Moreover, phylogenetic analysis of the partial non-structural (NS) protein gene showed that BPV-3/Cattle/Egypt/2020 clustered with BPV-3 viruses (GenBank MG745680.1, isolated from Brazil), MG026727, and MG026728 (both have been isolated from China); (Fig 4). The whole-genome sequence of the study isolate (called BPV-3/Cattle/Egypt/2020) was deposited to the NCBI database (GenBank MW805276).

Discussion

BRD is one of the most economically important complex disease affecting the cattle industry worldwide. Despite the use of several vaccines, BRD still has a worldwide distribution, with severe economic losses every year. Egypt has a big cattle industry and live-animal trade with different countries in Europe and Africa, which may facilitate the incidental introduction of new agents. The cattle industry in Egypt is based on two systems: (1) large farms where the average number of animals/herd is 100 to 1000 and (2) small farms where the average number of animals/herd is 5 to 20. Unfortunately, a high percentage of small farms do not apply annual BRD vaccination programs. Moreover, large farms are also not vaccinating young calves (average age of 6–12 months) against BRD.

In the present study, we investigated the nasal virome from naturally infected nonvaccinated cattle using metagenomic sequencing. We aimed to investigate contribution of viruses, particularly DNA viruses to the BRD in Egyptian cattle. Interestingly, several DNA viruses were identified in this study. Deep analysis of the BHV-1/Cattle/Egypt/2020 isolate revealed it belongs to genotype BHV-1.1, which is in agreement with previous studies [23, 24] that reported BHV-1.1 is the major circulating genotype in Egypt. Additionally, analysis of other
genes from BHV-1/Cattle/Egypt/2020, such as glycoproteins C and L and the major capsid protein, did not show a significant number of mutations when aligned with reference viruses (data not shown).

Alignment of the processed reads also showed high coverage against BHV-5 reference virus. BHV-5 is a viral cattle disease responsible for causing sporadic epizootics of fatal meningoencephalitis [25, 26]. BHV-5 is similar to BHV-1 in virion morphology, induced cytopathic effect on infected cell cultures, and antigenic properties [25, 27]. BHV-5 is formerly considered a neuropathogenic variant of BHV-1. However, many subsequent studies indicated that both viruses have different antigenic and genomic characters. BHV-1 and BHV-5 have neurotropic forms, but only BHV-5 can replicate well in the central nervous system causing neurological diseases [28, 29]. Meningoencephalitis outbreaks caused by BHV-5 have been reported in many countries, such as Australia [25], North and South America [30–32], and Europe [33, 34]. In addition, the natural transmission of BHV-5 via contaminated semen has been reported in Australia [35]. The virus has also been isolated from cryopreserved semen collected from a healthy bull [36]. Moreover, another study reported that using two species-specific nested PCR that differentiated BHV-1 and BHV-5, BHV-5 DNA was detected in all semen samples analyzed [37], while BHV-1 was detected only in 44.7% of tested samples. Surprisingly, BHV-5 DNA has been identified before in the central nervous system of the aborted fetus that gave an indication of the association of BHV-5 with bovine abortion [38]. It was surprising to detect BHV-5 sequence from nasal swab sample as this virus has high tropism for nervous and genital systems. However, BHV-5 is usually establish latent infection in nervous ganglion, and it has been shown that under stress conditions and/or excessive glucocorticoids treatments, the virus

**Fig 4. Phylogenetic analysis of the partial NS protein gene of BPV-3.** The evolutionary history was inferred using the neighbor-joining method [20]. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [21]. The evolutionary distances were computed using the maximum composite likelihood method [22] and are shown as the number of base substitutions per site. The codon positions included were 1st + 2nd + 3rd + Noncoding. Evolutionary analyses were conducted in MEGA X [19].

https://doi.org/10.1371/journal.pone.0267036.g004
reactivates. During this reactivation stage, BHV-5 can be excreted in nasal, ocular and genital discharges [39].

To our knowledge, this is the first detection of BHV-5 in Egypt. In the present study, it is unclear the source of BHV-5 introduction to Egypt; however, it is critical to carefully examine the imported frozen semen batches at artificial insemination centers using molecular biology techniques to avoid widespread of BHV-5 and to minimize its drawbacks on the cattle industry in Egypt.

The third virus detected was BPV-3/Cattle/Egypt/2020 with a whole-genome sequence of ~5286 nt long. Bovine parvovirus (BPV) belongs to genus Bocaparvovirus genus in the family Parvoviridae and was first discovered in 1961 from the gastrointestinal tract of diarrheal calves [40]. BPV has also been associated with reproductive disorders, such as spontaneous abortions and stillbirths [41]. To our knowledge, this is also the first detection of BPV type 3 in Egyptian cattle herds.

This work demonstrates the utility of metagenomic sequencing for the effective detection of viruses in cattle. There are some limitations in the present study, particularly samples pooling and the use of FTA cards. It has been reported that pooling of DNA generated challenges for accurate variant call and allele frequency. For example, sequencing errors confound with the alleles present at low frequency in the pools probably give rise to false-positive variants [42]. In addition, it has been reported that next-generation sequencing of nucleic acid samples derived from FTA cards exhibited lower proportions of poliovirus specific reads with a lower percentage of genome mapped than those obtained directly from viral isolates [43].

Conclusions
The current study shows the detection of BHV-1.1 genotype and the first detection of BHV-5 and BPV-3 from the investigated cattle herds. The detected viruses may have an association with the BRD; however, further research, including collecting and investigating more animal samples from different locations, is needed to determine the prevalence of the detected viruses and their contributions to BRD in cattle in Egypt.

Supporting information
S1 Table. Details about investigated cattle herds.
(DOCX)

Acknowledgments
We thank the veterinarians, particularly Dr. Ibrahim Yousuf, and the employees at the cattle farms where samples were collected for their technical help in animal securing and sample collection.

Author Contributions
Conceptualization: Abdou Nagy, Fatma Abdallah, Ibrahim Elsohaby.

Data curation: Fatma Abdallah, Hend M. El Damaty, Ahmed Tariq, Abdallah M. A. Merwad, Bader Y. Alhatlani.

Formal analysis: Abdou Nagy, Ibrahim Elsohaby.

Investigation: Fatma Abdallah, Hend M. El Damaty, Ahmed Tariq, Abdallah M. A. Merwad, Bader Y. Alhatlani.
Methodology: Fatma Abdallah, Hend M. El Damaty, Ahmed Tariq, Abdallah M. A. Merwad, Bader Y. Alhatlani.

Project administration: Abdou Nagy, Bader Y. Alhatlani.

Resources: Fatma Abdallah, Hend M. El Damaty, Ahmed Tariq, Abdallah M. A. Merwad, Bader Y. Alhatlani.

Software: Abdou Nagy, Ibrahim Elsohaby.

Validation: Abdou Nagy, Ibrahim Elsohaby.

Visualization: Abdou Nagy, Ibrahim Elsohaby.

Writing – original draft: Abdou Nagy, Ibrahim Elsohaby.

Writing – review & editing: Abdou Nagy, Fatma Abdallah, Hend M. El Damaty, Ahmed Tariq, Abdallah M. A. Merwad, Bader Y. Alhatlani, Ibrahim Elsohaby.

References

1. Hilton WM. BRD in 2014: where have we been, where are we now, and where do we want to go? Animal health research reviews. 2014; 15(2):120. https://doi.org/10.1017/S1466252314000115 PMID: 25358813

2. Fulton RW. Bovine respiratory disease research (1983–2009). Animal health research reviews. 2009; 10(2):131. https://doi.org/10.1017/S146625230999017X PMID: 20003649

3. Loneragan GH, Dargatz DA, Morley PS, Smith MA. Trends in mortality ratios among cattle in US feedlots. Journal of the American Veterinary Medical Association. 2001; 219(8):1122–7. https://doi.org/10.2460/javma.2001.219.1122 PMID: 11700712

4. Snowder G, Van Vleck LD, Cundiff L, Bennett G, Koohmaraie M, Dikeman M. Bovine respiratory disease in feedlot cattle: phenotypic, environmental, and genetic correlations with growth, carcass, and longissimus muscle palatability traits. Journal of animal science. 2007; 85(8):1885–92. https://doi.org/10.2527/jas.2007-0008 PMID: 17504959

5. Sadeghi M, Kapusinszky B, Yugo DM, Phan TG, Deng X, Kanevsky I, et al. Virome of US bovine calf serum. Biologicals. 2017; 46:64–7. https://doi.org/10.1016/j.biologicals.2016.12.009 PMID: 28100412

6. Altan E, Seguin MA, Leutenegger CM, Phan TG, Deng X, Delwart E. Nasal virome of dogs with respiratory infection signs include novel taupapillomaviruses. Virus genes. 2019; 55(2):191–7. https://doi.org/10.1007/s11262-019-01634-6 PMID: 30632017

7. Zhang M, Hill JE, Fernando C, Alexander TW, Timsit E, van der Meer F, et al. Respiratory viruses identified in western Canadian beef cattle by metagenomic sequencing and their association with bovine respiratory disease. Transboundary and emerging diseases. 2019; 66(3):1379–86. https://doi.org/10.1111/tbed.13172 PMID: 30873724

8. Zhang M, Huang Y, Godson DL, Fernando C, Alexander TW, Hill JE. Assessment of metagenomic sequencing and qPCR for detection of influenza D virus in bovine respiratory tract samples. Viruses. 2020; 12(8):814. https://doi.org/10.3390/v12080814 PMID: 32731471

9. Shan T, Li L, Simmonds P, Wang C, Moeser A, Delwart E. The fecal virome of pigs on a high-density farm. Journal of virology. 2011; 85(22):11697–708. https://doi.org/10.1128/JVI.05217-11 PMID: 21900163

10. Zhang B, Tang C, Yue H, Ren Y, Song Z. Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China. Journal of General Virology. 2014; 95(7):1603–11. https://doi.org/10.1099/vir.0.063743-0 PMID: 24718833

11. Ng TFF, Kondov NO, Deng X, Van Eijndhoven A, Neibergs HL, Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. Journal of virology. 2015; 89 (10):5340–9. https://doi.org/10.1128/JVI.00064-15 PMID: 25740998

12. Zhang M, Hill JE, Godson DL, Ngeleka M, Fernando C, Huang Y. The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada. Transboundary and emerging diseases. 2020; 67(2):924–34. https://doi.org/10.1111/tbed.13419 PMID: 31715071

13. Mitra N, Cericchiari N, Torres S, Li F, Hause BM. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. The Journal of general virology. 2016; 97(8):1771. https://doi.org/10.1099/jgv.0.000492 PMID: 27154756
14. Biswas S, Bandypadhyay S, Dimri U, Patra PH. Bovine herpesvirus-1 (BHV-1)—a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis. Veterinary Quarterly. 2013; 33(2):68–81.

15. Chowdhury S, Lee B, Mosier D, Sur J, Osorio F, Kennedy G, et al. Neuropathology of bovine herpesvirus type 5 (BHV-5) meningo-encephalitis in a rabbit seizure model. Journal of comparative pathology. 1997; 117(4):295–310. https://doi.org/10.1016/s0021-9975(97)80078-3 PMID: 9502267

16. 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. Proceedings of the National Academy of Sciences. 2001; 98(20):11609–14.

17. El-Seedy F, Hassan H, Nabih A, Salem S, Khalifa E, Menshawy A, et al. Respiratory affections in calves in upper and middle Egypt: Bacteriologic, immunologic and epidemiologic studies. Adv Anim Vet Sci. 2020; 8(5):558–69.

18. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research. 2004; 32(5):1792–7. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

19. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution. 2018; 35(6):1547–9. https://doi.org/10.1093/molbev/msy096 PMID: 3447015

20. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution. 1987; 4(4):406–25. https://doi.org/10.1093/oxfordjournals.molbev.a040454 PMID: 17361772

21. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. evolution. 1985; 39(4):783–91. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x PMID: 28561359

22. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences. 2004; 101(30):11030–5. https://doi.org/10.1073/pnas.0404206101 PMID: 15258291

23. Hafez S, Thanaa IB, Mohsen A, Monira H. Infectious Bovine Rhinotracheitis in Egypt: isolation and serologic al identification of the virus. 1976; 36(1):129–39.

24. El-Kholy A, Abdelrahman K. Genetic character isation of the Egyptian vaccinal strain Abu-Hammad of bovine herpesvirus-1. Revue Scientifique et Technique-Office International des Epizooties. 2006; 25(3):1081–96. PMID: 17361772

25. French E. A specific virus encephalitis in calves: isolation and characterization of the causal agent. 1962; 38(4):216–21.

26. Bagust T, Clark L. Pathogenes is of meningo -encephalitis produced in calves by infectious bovine rhinotrachei tis herpesvirus . 1972; 82(4):375–83. https://doi.org/10.1016/0021-9975(72)90036-9 PMID: 4346156

27. French E. Relationship between infectious bovine rhinotracheitis (IBR) virus and a virus isolated from calves with encephalitis. 1962; 38(11):555–6.

28. Belknap E, Collins J, Ayers V, Schultheiss P. Experimental infection of neonatal calves with neuro-virulent bovine herpesvirus type 1.3. 1994; 31(3):358–65. https://doi.org/10.1177/030098589403100309 PMID: 8053131

29. Ashbaugh SE, Thompson KE, Belknap EB, Schultheiss PC, Chowdhury S, Collins JK. Specific detection of shedding and latency of bovine herpesvirus 1 and 5 using a nested polymerase chain reaction. 1997; 9(4):387–94. https://doi.org/10.1177/104063879700904040 PMID: 9376428

30. CA D, RW M, RJ S. Isolation of infectious bovine rhinotracheitis virus from calves with meningoencephalitis. 1963; 143:725–8.

31. Gough A, James D. Isolation of IBR virus from a heifer with meningoencephalitis. 1975; 16(1):313. PMID: 172213

32. Carrillo BJ, Ambrogi A, Schudel AA, Vazquez M, Dahme E, Pospischil A. Meningoencephalitis caused by IBR virus in calves in Argentina. 1983; 30(1–10):327–32. https://doi.org/10.1111/j.1439-0450.1983.tb01852.x PMID: 6310913

33. Moretti B, Orfei Z, Mondino G, Persechino A. Infectious bovine rhinotracheitis, clinical observations and isolation of virus. 1964; 15:676.

34. Bartha A, Hajdu G, Aldasy P, Paczolay G. Occurrence of encephalitis caused by infectious bovine rhinotracheitis virus in calves in hungary. 1969; 19(2):145–51. PMID: 4308193

35. Kirkland P, Gu X, Davis R, Poynting A. Infertility and venereal disease in cattle inseminated with semen containing bovine herpesvirus type 5. Veterinary Record. 2009; 165(4):111–3. https://doi.org/10.1136/ vetrec.165.4.111 PMID: 19633324
36. Diallo I, Hewitson G, Hoad J, Turner S, Corney B, Rodwell B. Isolation of bovine herpesvirus type 5 from the semen of a healthy bull in Australia. Australian veterinary journal. 2010; 88(3):93–5. https://doi.org/10.1111/j.1751-0813.2009.00545.x PMID: 20402691

37. Oliveira M, Campos F, Dias M, Velho F, Freneau G, Brito W, et al. Detection of bovine herpesvirus 1 and 5 in semen from Brazilian bulls. Theriogenology. 2011; 75(6):1139–45. https://doi.org/10.1016/j.theriogenology.2010.11.025 PMID: 21247624

38. Marin M, Morrill E, Moore D, Leunda M, Campero C, Odeón A, editors. Identificación de infecciones duales de Neospora caninum y Herpesvirus bovino tipo 5 mediante PCR en casos de aborto bovino espontáneo. the Proceedings of the 2011 Latin American Buiatric Congress, Paysandú, Uruguay; 2011.

39. Rock D, Lokensgard J, Lewis T, Kutish G. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. Journal of virology. 1992; 66(4):2484–90. https://doi.org/10.1128/JVI.66.4.2484-2490.1992 PMID: 1312639

40. FR A, MS W. Recovery of a hemadsorbing virus (HADEN) from the gastrointestinal tract of calves. Virol-ogy. 1961; 14:288–9. https://doi.org/10.1016/0042-6822(61)90206-9 PMID: 13681037

41. Barnes M, Wright R, Bodine A, Alberty C. Frequency of bluetongue and bovine parvovirus infection in cattle in South Carolina dairy herds. American journal of veterinary research. 1982; 43(6):1078–80. PMID: 6285770

42. Anand S, Mangano E, Barizzone N, Bordoni R, Sorosina M, Clarelli F, et al. Next Generation Sequencing of Pooled Samples: Guideline for Variants' Filtering (vol 6, 33735, 2016). SCIENTIFIC REPORTS. 2020; 10(1).

43. Montmayeur A, Ng T, Schmidt A, Zhao K, Magana L, Iber J, et al. Chen Q, Henderson E, Ramos E, Shaw J, Tatusov RL, Dybdahl-Sissoko N, Endegue-Za nga MC, Adeniji JA, Oberste MS, Burns CC. 2017. High-throughput next-505 generation sequencing of polioviruses. J Clin Microbiol. 55:606–15. https://doi.org/10.1128/JCM.02121-16 PMID: 27927929