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Surveillance, isolation and complete genome sequence of bovine parainfluenza virus type 3 in Egyptian cattle

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ABSTRACT

Parainfluenza virus type 3 (PIV-3) can infect a wide variety of mammals including humans, domestic animals, and wild animals. In the present study, bovine parainfluenza virus type 3 (BPIV-3) was isolated from nasal swabs of Egyptian cattle presenting with clinical signs of mild pneumonia. The virus was isolated in Madin-Darby bovine kidney (MDBK) cells and confirmed by reverse transcription-polymerase chain reaction (RT-PCR). The complete genome of Egyptian BPIV-3 strain was sequenced by using next generation (Illumina) sequencing. The new isolate classified with genotype A of BPIV-3 and was closely related to the Chinese NM09 strain (JQ063064). Subsequently in 2015–16, a molecular surveillance study was undertaken by collecting and testing samples from cattle and buffaloes with respiratory tract infections. The survey revealed a higher rate of BPIV-3 infection in cattle than in buffaloes. The infection was inversely proportional to the age of the animals and to warm weather. This report should form a basis for further molecular studies on animal viruses in Egypt.

1. Introduction

Bovine parainfluenza virus type 3 (BPIV-3) is a member of *respirovirus* genus in the family *Paramyxoviridae* [1]. The virus is pleomorphic and enveloped and has a diameter of 150–300 nm [2]. The non-segmented negative-stranded RNA genome contains approximately 15,000 nucleotides (nt) organized to encode six structural proteins including nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large polymerase protein [3]. The HN (hemagglutinin-neuraminidase) and F (fusion) proteins are surface glycoproteins found in all parainfluenza viruses (PIVs). There are significant differences in the number of HN glycosylation sites among PIVs, and even among strains within a single type, which may be part of a strategy to escape immune detection [4]. The F protein is integral to virus-host interactions and helps in fusion of membranes, which allows the viral nucleocapsid to enter and infect a host cell [5]. The M protein interacts with the surface glycoproteins (HN and F) and directs their insertion and aggregation at specific cell membrane sites [6]. The L, P and N proteins are closely associated with the viral RNA [7].

Parainfluenza virus type 3 (PIV-3) can infect a wide variety of mammals including humans, domestic animals, and wild animals [8,9]. Cross-species infections have also been reported e.g., BPIV-3 in humans and sheep and ovine PIV-3 in cattle [10,11]. The three genotypes of BPIV-3 are named as A (BPIV-3a), B (BPIV-3b) and C (BPIV-3c) [12] of which genotype A, which was first recognized in the USA, is the most common. Genotypes B and C were detected for the first time in Australia and China, respectively [12,13]. It was previously thought that genotype C was circulating only in China but recently it has been reported from both Korea and Argentina [13,14].

The BPIV-3 is one of the causes of bovine respiratory disease complex (BRDC) [15]. This virus may cause tissue damage and immunosuppression resulting in severe bronchopneumonia due to secondary bacterial infections, especially when animals are under stressful conditions [16]. The virus was first isolated in the United States in 1959 from nasal discharge of cattle with shipping

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fever and was initially named as myxovirus SF-41 [1]. In Egypt, the virus was first isolated in 1963 from lung tissues of imported Somali cattle with respiratory illness at the Suez quarantine station [17]. Since then, the virus has been involved in several outbreaks in Egypt and neighboring countries either alone or with other viruses e.g., infectious bovine rhinotracheitis virus (IBRV), bovine respiratory syncytial virus (BRSV) and/or bovine viral diarrhea virus (BVDV) [18,19].

In 2014, we observed sporadic cases of pneumonia in bovine calves in Sharkia province in Egypt. Inoculation of nasal swab suspensions in MDBK (Madin-Darby bovine kidney) cells followed by RT-PCR revealed the presence of BPIV-3. No other virus e.g., IBRV, BVDV, BRSV or bovine enterovirus (BEV) was identified. In this study, we report on the complete genome sequence of BPIV-3 using Illumina sequencing. The Egyptian BPIV-3 strain was classified as a member of genotype A based on its nucleotide and amino acid (aa) sequences. The study was extended to investigate the presence of BPIV-3 in cattle and buffaloes in 2015–16. The investigation included examination of nasal swabs of animals suffering from respiratory manifestation and pneumonia and those admitted to the clinic at Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.

2. Materials and methods

2.1. Samples and virus isolation

In 2014, nasal swabs were collected from 12 sporadic cases of calf pneumonia from the Abo-Kebeer area of Sharkia province in Egypt and were tested for the presence of BRDC viruses (IBRV, BVDV, BRSV and BPIV-3) and BEV. Briefly, the swabs were squeezed in phosphate buffered saline (PBS, pH 7.4) solution followed by centrifugation at 2500*g* for 10 min. The supernatants were filtrated through 0.22 μm filters and then inoculated in MDBK cells for virus isolation. The inoculated cells were incubated at 37 °C for 2 h before adding modified Eagle's minimum essential medium (MEM) supplemented with 1.5 mg/mL trypsin (Fisher Scientific, Loughborough, UK), 4% fetal bovine serum (Fisher Scientific), 100 μg/mL streptomycin (Sigma-Aldrich, Dorset, UK), 100 U/mL penicillin (Sigma-Aldrich, Dorset, UK), and 2.5 μg/mL fungizone (Fisher Scientific, Loughborough, UK). The inoculated cells were observed daily under a light microscope for the appearance of virus-induced cytopathic effects (CPE). When CPE appeared in 80% of the cell monolayer, the virus was harvested by three cycles of freezing and thawing, followed by centrifugation at 1200*g* for 15 min to remove cellular debris.

2.2. Polymerase chain reaction (PCR) for the detection of IBRV

Viral DNA was extracted from cell culture supernatants using DNeasy blood and tissue kit (Qiagen, Valencia, CA). Extracted DNA was subjected to PCR using HotStarTaq master mix kit (Qiagen, Valencia, CA) and IBR gB primer set (Table 1). Briefly, the amplification reaction was performed in a 25 μL reaction mixture under the following conditions: an initial denaturation step of 15 min at 95 °C, followed by 35 cycles of 1 min at 94 °C (denaturation); 1 min for annealing at 51 °C; 1 min at 72 °C (extension) and one final extension step of 10 min at 72 °C [20].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from cell culture supernatants using QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). The extracted RNA was subjected to RT-PCR using one-step RT-PCR kit (Qiagen, Valencia, CA). The primer sets used for the detection of BVDV, BRSV, BPIV-3 and BEV are shown in Table 1. The amplification protocol used 25 μL reaction mixture incubated in a thermocycler at 50 °C for 30 min in RT step and initial denaturation at 95 °C for 15 min. This was followed by 35 cycles consisting of 94 °C for 1 min for denaturation followed by annealing for 1 min at appropriate temperature for each primer set (Table 1) and then elongation at 72 °C for 1 min followed by final extension cycle at 72 °C for 10 min. The products were analyzed in ethidium bromide-stained 1.2% agarose gel. A single band of expected product size confirmed the presence of the target virus.

Table 1
Primers used in PCR and RT- PCR reactions.

Virus[a]	Primer sequence	Annealing temperature	Amplicon size
BHV-1 GB	F GTA CAC GTT CAA GGC CTA CA R TCG TCT CGC AGC ATT TC	51 °C	668 bp
BVD	F CAT GCC CTT AGT AGG AC R CTC CAT GTG CCA TGT ACA G	48 °C	400 bp
BRSV	F CAT CAA TCC AAA GCA CCA CAC TGT C R GCT AGT TCT GTG GTG GAT TGT TGT C	55 °C	381 bp
BPI-3	F AGT GAT CTA GAT GATGAT CCA R GTT ATT GAT CCA ATT GCT GT	47 °C	328 bp
BEV	F ATG GAC AAG AGG TAY GTC GTC GT R GGG CAC ACT CCG GAT TTT CTC C	55 °C	450 bp

[a] BHV-1, bovine herpesvirus type 1 or infectious bovine rhinotracheitis virus; BVDV, bovine viral diarrhea virus; BRSV, bovine respiratory syncytial virus; BPIV-3, bovine parainfluenza 3 virus; BEV, bovine enterovirus.

2.4. Next generation sequencing (Illumina sequencing)

For molecular characterization, cell culture supernatants were loaded on FTA cards and shipped to the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL), Saint Paul, Minnesota, USA. Viral RNA was extracted from loaded FTA cards using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). Equal amounts of RNA from all samples were combined to make a single pool for Illumina sequencing. The pooled RNA sample was sent to the University of Minnesota Genetic Center (UMGC) for Illumina MiSeq paired end 250 cycles. The sample passed sequencing quality assessment in which total RNA was quantified using a fluorimetric RiboGreen assay. The library was created using Illumina's Truseq RNA sample preparation kit (Cat. # RS-122-2001) and good quality libraries were sequenced on MiSeq. The obtained sequence reads were analyzed by CLC genomic workbench 6.0.[1] Trimming and sequence quality were tested followed by preparation of contigs by *de novo* assembly. Extracted contigs were analyzed by tBlastx analysis on NCBI.[2] The ORF finder tool[3] was used to find ORFs in the obtained sequences. The obtained nt sequences were translated through translator.[4] Prediction of coding and non-coding regions was done based on nt and aa alignments with reference BPIV-3 (NM09; JQ063064) sequence in GenBank.

2.5. Phylogenetic analysis and genome characterization

The alignment of compatible nt sequences, calculation of P distance and tree construction were done by using MEGA 6.0 (Molecular Evolutionary Genetic Analysis). Based on nt blast analysis on NCBI (blastn), sequences representing different geographical areas, years, and species were selected for further comparison. A phylogenetic tree of aligned sequences was constructed by selecting

[1] See: http://www.clcbio.com/products/clc-genomics-workbench/
[2] See: https://blast.ncbi.nlm.nih.gov/Blast.cgi
[3] See: http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi
[4] See: http://www.fr33.net/translator.php